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The self-sufficient CYP102 family enzyme, Krac9955, from *Ktedonobacter racemifer* DSM44963 acts as an alkyl- and alkyloxy-benzoic acid hydroxylase

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ABSTRACT

A self-sufficient CYP102 family encoding gene (*Krac_9955*) has been identified from the bacterium *Ktedonobacter racemifer* DSM44963 which belongs to the *Chloroflexi* phylum. The characterisation of the substrate range of this enzyme was hampered by low levels of production using *E. coli*. The yield and purity of the Krac9555 enzyme was improved using a codon optimised gene, the introduction of a tag and modification of the purification protocol. The heme domain was isolated and *in vitro* analysis of substrate binding and turnover was performed. Krac9955 was found to preferentially bind alkyl- and alkyloxy-benzoic acids (≥95% high spin, K_d < 3 μM) over saturated and unsaturated fatty acids. Unusually for a self-sufficient CYP102 family member Krac9955 showed low levels of NAD(P)H oxidation activity for all the substrates tested though product formation was observed for many. For nearly all substrates the preferred site of hydroxylation of Krac9955 was eight carbons away from the carboxylate group with certain reactions proceeding at ≥ 90% selectivity. Krac9955 differs from CYP102A1 (P450Bm3), and is the first self-sufficient member of the CYP102 family of P450 enzymes which is not optimised for fast fatty acid hydroxylation close to the ω-terminus.

1. Introduction

The P450 cytochrome (CYP) superfamily of *b*-type heme-dependent monooxygenases catalyse the oxidation of organic substrates inserting a single oxygen atom from molecular dioxygen into a carbon–hydrogen bond resulting in the corresponding alcohol [1–4]. As such there is great interest in the application of CYP enzymes as biocatalysts for generating alcohols at unactivated C–H bonds, especially when these reactions are carried out with high regio- and stereoselectivity [5–10]. The isolation of new enzymes from microbes is an active area of research as these systems are often involved in the synthesis of complex natural product such as antibiotics as well as in the breakdown of available organic molecules for use as a source of energy [11–14]. In addition bacterial enzymes have been reported to have the highest activities among P450 enzymes when their electron transfer systems are available [6,10,15–20].

The self-sufficient cytochrome P450 enzymes from the CYP102 family consist of a 55 kDa P450 heme domain fused to a 65 kDa reductase domain and require only substrate, NADPH and oxygen to function [6]. These systems exhibit the highest turnover frequency (>1000 min^{-1}) of any CYP enzymes which overcomes one of the major hurdles to their use as catalysts [6,21–23]. As such they have been used as platforms for creating monooxygenase biocatalysts through protein engineering by both site-directed and random mutagenesis [5,6,23–27]. The best studied member of this family is CYP102A1 (P450Bm3) from *Bacillus megaterium* which hydroxylates saturated and unsaturated fatty acids at sub-terminal

Abbreviations: BSTFA, N,O-Bis(trimethylsilyl)trifluoroacetamide; CYP, cytochrome P450 enzyme; DEAE, diethylaminoethanol; DMSO, dimethyl sulfoxide; EMM, *E. coli* minimal media; EtOH, ethanol; GB, gblock; GC, gas chromatography; IPTG, isopropyl-β-D-1-thiogalactopyranoside; Krac0936, a second P450 enzyme from the CYP102 family found in *Ktedonobacter racemifer*; Krac9955, the holoenzyme produced by the optimised gene; Krac9955HD, the heme domain of the protein produced from the optimised gene; Krac9955NS, the holoenzyme with a histidine tag incorporated by removal of the stop codon; Krac9955HDNS, the histidine tagged heme domain; MS, mass spectrometry; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; P450Bm3, the self-sufficient P450 enzyme CYP102A1 from *Bacillus megaterium*; PCR, Polymerase chain reaction; TMSCl, Trimethylsilyl chloride; Tris, Tris(hydroxymethyl)aminomethane; 2xYT, Yeast Extract Tryptone.

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positions, close to the ω-terminus [6,28,29]. The optimal activity is for longer chain polyunsaturated fatty acids such as arachidonic acid (5,8,11,14-all-*cis*-eicosatetraenoic acid) [30,31]. Other self-supporting fatty acid oxidising P450s have been identified and investigated including CYP102A family variants from *Bacillus* species, CYP102D1 from *Streptomyces avermitilis* MA4680 and CYP505 ($P450_{foxy}$) from the fungus *Fusarium oxysporum* [6,21,32–36].

Recently we identified two self-sufficient CYP102 family member encoding genes (*Krac_0936* and *Krac_9955)* in the bacterium *Ktedonobacter racemifer* DSM44963, a species which is representative of a group of organisms which is genealogically highly isolated [37,38]. Preliminary studies indicated that both could oxidise fatty acid substrates *in vivo* but their substrate profiles were narrower than CYP102A1. *In vitro* analysis showed that Krac0936 more closely resembled CYP102A1 with a preference for pentadecanoic acid and longer monounsaturated fatty acids such as oleic acid (*cis*-9-octadecenoic acid). However, unlike CYP102A1, which hydroxylated linear saturated fatty acids at ω-1, ω-2 and ω-3 with no apparent preference, Krac0936 showed a strong preference for the ω-1 and ω-2 positions.

Initial studies on Krac9955 were hampered by low levels of protein production using *E. coli* resulting in turnover assays being performed on extracts from cell lysates rather than purified enzyme. Krac9955 showed optimal activity with the shorter tridecanoic acid and favoured oxidation at more sub-terminal positions with the preferred site of hydroxylation being eight carbons from the carboxylate group. The gene encoding Krac9955 contained more clusters of rare codons than that of CYP102A1 and here we report the improved production of the holoenzyme using codon optimised genes and the use of affinity tag purification protocols. We also generated constructs to enable the production of the heme domain of Krac9955 in order to assess substrate binding and product formation for the first time.

2. Experimental section

2.1. Cloning, expression and purification

General DNA and microbiological experiments were carried out using standard methods [39]. The KOD polymerase, used for the PCR steps, and pET26a expression vectors were from Merck Biosciences, UK. T4 DNA ligase and restriction enzymes for molecular biology were from Lucigen and New England Biolabs. Codon optimised DNA was purchased from Integrated DNA Technology (IDT, USA). The Krac9955 gene was purchased as two gene blocks (GBs) one of which (GB1) covered the P450 domain, whilst the other (GB2) encompassed the remainder of the sequence, including the reductase domain (Supporting Information). GB1 incorporated a 5′ NdeI and 3′ BamHI and HindIII restriction sites, and GB2 a 5′ BamHI and 3′ HindIII site (Fig. S1). These were cloned together into the vector pET26 using standard techniques to create the full length optimised gene (Fig. S2). The heme domain (HD) Krac9955HD was obtained via the polymerase chain reaction (PCR) using the respective GB1 as a template and the primers listed in the Supporting Information. Additionally a C-terminal 6 x histidine tag was incorporated into the Krac9955 holoprotein and heme domains by using PCR to remove the stop codon (Krac9955NS and Krac9955HDNS, Supporting Information). This method extended each protein sequence by 13 amino acids by integrating additional codons from the pET26 vector including those which encode the 6 x His tag (Supporting Information). PCR was performed using KOD Hot Start DNA polymerase and the following method: 94 °C, 30 s; 55 °C, 45 s; 68 °C, 205 s (or 100 s for the heme domains) for 30 cycles; 72 °C final extension 10 min and 10 °C final hold. The sequences of all clones were confirmed by DNA sequencing (Australian Genome Research Facility, Adelaide node) using the T7F and T7T primers of the pET26 vector and additional sequencing primers (Supporting Information).

The recombinant plasmids were transformed into *Escherichia coli* strain BL21(DE3) and these cells were cultured in 2xYT medium at 37 °C with 30 μg mL^{-1} kanamycin. When the OD_{600} of the culture reached 0.6–0.8 the temperature was reduced to 20 °C and the media was supplemented with 3 mL L^{-1} of trace elements solution (0.74 g $CaCl_2 \cdot H_2O$, 0.18 g $ZnSO_4 \cdot 7H_2O$, 0.132 g $MnSO_4 \cdot 4H_2O$, 20.1 g Na_2EDTA, 16.7 g $FeCl_3 \cdot 6H_2O$, 0.10 g $CuSO_4 \cdot 5H_2O$, 0.25 g $CoCl_2 \cdot 6H_2O$), and 1 mM 5-aminolevulinic acid. Finally 0.1 mM IPTG was added in order to induce the production of the protein. After further growth for 18 h at 20 °C, cells were harvested by centrifugation, resuspended in 40 mM potassium phosphate, pH 7.4 (buffer P), 1 mM in dithiothreitol and lysed by sonication on ice. The crude extracts were then centrifuged at 37000 *g* for 25 min at 4 °C to remove the cell debris.

For Krac9955HDNS the supernatant was loaded directly onto a GE-Healthcare DEAE fast-flow Sepharose column (XK50, 200 × 50 mm). The protein was eluted using a linear gradient, 100–400 mM KCl in Tris buffer, pH 7.4. The P450 containing fractions were combined and concentrated to a final volume of ~15 mL by ultra-filtration using a Vivacell 100 (Sartorius Stedim, 10 kDa membrane) aided by centrifugation (1500 g). The concentrated protein was loaded onto a 5 mL nickel NTA column, pre-equilibrated with buffer T1 (300 mM NaCl, 20 mM imidazole, 1 mM DTT in 50 mM Tris, pH 7.4). The protein was then washed with buffer T1 and eluted with buffer T2 (300 mM NaCl, 300 mM imidazole, 1 mM DTT in 50 mM Tris, pH 7.4). The eluted protein was concentrated and buffer exchanged with 50 mM Tris, pH 7.4, before being stored. Krac9955NS (holoprotein with 6 x His tag) was extracted from *E. coli* by the same method described above. The supernatant was subjected to an ammonium sulfate precipitation. The 25–60% fraction was purified by the nickel NTA column, as described for Krac9955HDNS. All the proteins were stored at −20 °C in 50% (v/v) glycerol.

Glycerol and salts were removed from proteins immediately prior to experiments using a GE Healthcare 5 mL PD-10 desalting column pre-equilibrated with 50 mM Tris buffer, pH 7.4. The concentration of the proteins was calculated using the extinction coefficient of the CO reduced difference spectrum $\varepsilon 450 = 91\ mM^{-1}\ cm^{-1}$ [28,40]. Using this value, the extinction coefficients for the ferric resting state of the heme domain and holoprotein (Krac9955HDNS and Krac9955NS) were both estimated to be approximately $\varepsilon 419 = 105\ mM^{-1}cm^{-1}$.

2.2. Substrate binding and turnover assays

For substrate binding assays the P450 heme domains were diluted to ~1–2 μM using 50 mM Tris, pH 7.4 and aliquots of the substrate were added from a 20 mM stock solution in DMSO until the spectra stopped shifting. The high spin heme content was estimated, to approximately ±5%, by comparison with a set of nine other spectra (10%–90% in increments of 10%) generated from the sum of the weighted averages of the spectra of the substrate-free form (>95% low spin, Soret maximum at 418 nm) and camphor-bound form (>95% high spin, Soret maximum at 392 nm) of wild-type CYP101A1 (see supporting information).

For dissociation constant determination the CYP199A4 enzyme was diluted to 0.5–2.0 μM using 50 mM Tris, pH 7.4, in 2.5 mL and 0.5–2 μL aliquots of the substrate were added using a Hamilton syringe from 1, 10 or 20 mM stock solutions in ethanol or DMSO. The maximum difference in the Soret peak-to-trough absorbance (ΔA) was recorded between 700 nm and 250 nm. Further aliquots of substrate were added until the peak-to-trough difference of the