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Directed Evolution of Cytochrome P450 CYP102A2 from *Bacillus subtilis*

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ABSTRACT

Bacillus subtilis flavocytochrome CYP102A2 is a high activity fatty acid hydroxylase that has evolved from fusion of a eukaryotic-like NADPH-cytochrome P450 reductase (CPR) to a P450 in a single polypeptide chain. In the present work we report the directed evolution of CYP102A2 from *B. subtilis* with a focus on its substrate specificity. The highly active CYP102A2 was subjected to error-prone PCR (epPCR) to generate enzyme variants with altered substrate specificity. The library of CYP102A2 mutants was expressed in BL21(DE3) *Escherichia coli* cells and screened for their ability to oxidize several substrates (sodium dodecyl sulphate, lauric acid, 1,4-naphthaquinoline, 2-hydroxy-1,6-naphthoquinone and ε-amino-*n*-caproic acid) by means of an activity assay. After a single round of epPCR, the variant Pro15Ser/Phe160Leu was isolated which exhibited altered substrate specificity towards naphthoquinones. Molecular modeling of CYP102A2 monooxygenase domain suggests that Phe160 is located at the end of α-helix-6 and is involved in van der Waals interactions with residues positioned at the α-helix-10 which are involved partly in the formation of the substrate binding pocket. Therefore Phe160 seems to affect substrate binding and catalysis indirectly.

Keywords: biotransformation, CYP, directed evolution, enzyme immobilization, P450, substrate recognition

INTRODUCTION

Cytochrome P450 monooxygenases (CYPs) play a key role in primary and secondary metabolic pathways and in drug detoxification (Lentz *et al*. 2004; Budde *et al*. 2006; Munro *et al*. 2007). They catalyze the reductive scission of molecular oxygen, with one atom of oxygen being reduced to water and the other used to hydroxylate the substrate. Two electrons are delivered from NAD(P)H via flavoprotein and/or iron-sulfur redox partners (Munro and Lindsay 1996). The two protons required for the production of water appear to be delivered from bulk solvent via a specific channel in the P450 active site (Miles *et al*. 2000; Wade *et al*. 2004; Warman *et al*. 2005; Munro *et al*. 2007). CYPs play a pivotal role in the synthesis and metabolism of secondary metabolites, such as prostaglandins, leucotrienes and thromboxanes, steroid hormones, insect and plant hormones and some colours and odours in plants (Lentz *et al*. 2004; Budde *et al*. 2006; Munro *et al*. 2007).

Cytochrome P450 BM-3 (CYP102A1 or CYP102 or P450 BM3) from *B. megaterium*, an enzyme which is the most studied prokaryotic P450 monooxygenases, catalyzes the subterminal hydroxylation of fatty acids with a chain length of C12–C22. It is a catalytically self-sufficient monooxygenase which contains a heme domain and a flavin reductase domain on a single polypeptide chain (Gustafsson *et al*. 2004). Self-sufficient bacterial P450 monooxygenases facilitate *in vitro* applications, as they do not require any separately added electron transport partners for catalytic action (Axarli *et al*. 2005). It preferentially hydroxylates in the ω-1–3 positions with high enantioselectivity in the ω-1 and ω-2 positions (98% R, 2% S) (Truan *et al*. 1999; Wade *et al*. 2004; Huang *et al*. 2007; Branco *et al*. 2008).

Among the members of the cytochrome P450 family, the monooxygenase from *Bacillus subtilis* (CYP102A2) exhibits high turnover frequency (Budde *et al*. 2004; Gustafsson *et al*. 2004; Axarli *et al*. 2005).

Because of their broad substrate specificity, there is an increasing interest to use P450s in biotechnology, for example for the production of pharmaceuticals or the optimization of lead compounds and existing drugs (Guengerich 2002; Lentz *et al*. 2004; Urlacher *et al*. 2004; Budde *et al*. 2006; Otey *et al*. 2006; Urlacher and Eiben 2006; Andreadeli *et al*. 2008; Damsten *et al*. 2008). However, practical applications of P450s are in general not economically viable because of the requirement of the expensive pyridine nucleotide cofactors such as NAD(P)H. Due to the high cost of cofactors, *in situ* cofactor regeneration is necessary to be coupled with NAD(P)H-dependent oxidation for preparative applications (Kataoka *et al*. 2003; van der Donk and Zhao 2003; Schewe *et al*. 2007; Kosjek *et al*. 2008; Andreadeli *et al*. 2009).

In the present work we report the heterologous expression, purification and directed evolution of CYP102A2 from *B. subtilis* with a focus on the factors affecting substrate specificity.

MATERIALS

The pCR®T7/CT-TOPO®TA Expression Kits were purchased from Invitrogen (UK). β-nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH, tetrasodium salt, *ca*. 95%), crystalline bovine serum albumin (BSA) (fraction V), DEAE Sepharose CL-6B, 2,5-ADP-agarose and other analytical reagents were purchased from Sigma-Aldrich (St. Louis, USA).

METHODS

Cloning and expression of the wild-type CYP102A2 from *E. coli* BL21 (DE3) cells

Cloning and expression of the wild-type CYP102A2 from *E. coli* BL21 (DE3) cells was carried out as described in Axarli *et al*. (2005).

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Original Research Paper

Purification of the wild-type and mutants of CYP102A2 from *E. coli* BL21 (DE3) cell-free extract

CYP102A2 was purified by a method similar to that described elsewhere (Gustafsson *et al*. 2004). Cell paste (0.22 g) was resuspended in potassium phosphate buffer (0.15 M, pH 6.7, 0.66 mL) containing 1 mM MeSH), sonicated, and centrifuged at 13,000 × *g* for 5 min. The supernatant was collected and dialysed overnight (4°C) against 1000-volumes of 50 mM Tris-HCI buffer containing 1 mM EDTA, pH 7.4. Dialyzed cell-free extract (0.7 mL, 7.1 U/mL, 6.5 mg protein) was applied to a column of DEAE Sepharose CL-6B (1 mL) previously equilibrated with 50 mM Tris-HCI buffer containing 1 mM EDTA, pH 7.4. Non-adsorbed protein was washed off with 10 mL equilibration buffer. Bound CYP102A2 was eluted with a step-wish gradient of 50-300 mM KCI in the equilibration buffer (total volume of 36 mL). Collected fractions were assayed for CYP102A2 activity and protein (Bradford 1976). Fractions with high enzyme activity (eluted with equilibration buffer containing 250 mM KCl), were pulled and dialysed overnight (4°C) against 1000-volumes of 50 mM Tris-HCI buffer pH 7.4, containing 1 mM EDTA. The dialysate was loaded onto a 2,5-ADP-agarose column (0.5 mL) previously equilibrated with 50 mM Tris-HCI buffer pH 7.4, containing 1 mM EDTA. Non-adsorbed protein was washed off with 4 mL equilibration buffer. Bound CYP102A2 was eluted with equilibration buffer containing 5 mM $NADP^+$ (2 mL). Collected fractions were assayed for CYP102A2 activity and protein (Bradford 1976). Protein purity was judged by SDS-PAGE. Purification of mutants were carried out as described for the wild-type enzyme.

Kinetic analysis

Enzyme assays were performed at 37°C at a Hitachi U-2000 double beam UV-Vis spectrophotometer carrying a thermostated cell holder (10 mm pathlength). Activities were measured by determining the rate of NADPH conversion to $NADP^+$ and following the decrease of absorbance at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyses the conversion of 1 μmol NADPH to $NADP^+$ per minute at 37°C.

Steady-state kinetic measurements were performed at 37°C in 0.15 M potassium phosphate buffer, pH 6.7 by varying the concentration of the substrates (NADPH, SDS). Initial velocities were determined in the presence of 0.139 mM SDS, while the NADPH concentration range was 6.6-100 μM. When NADPH was used at a fixed concentration (0.1 mM), the SDS was varied in the range of 0.013-0.12 mM. In this case the data are best fitted to the Hill function since the curves are nonhyperbolic (sigmoidal curves). The kinetic parameter K_m was calculated by non-linear regression analysis of experimental steady-state data using the computer program GraFit (Erithacus Software Ltd.).

Determination of protein concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (fraction V) as standard.

Construction of error prone PCR library

Error-prone PCR was performed as following: the PCR mixture (total volume 50 μL) contained 0.1 μM forward (5′-ATGAAGGAA ACAAGCCCGATTCCTCAGCCG-3′) and reverse (5′-TTTAGA TCTCTATATCCCTGCCCAGACATC-3′) primer, 7 mM $MgCl_2$, 5 ng of template DNA (mutant Pro15Ser), 200 μM dATP and dGTP and 600 μM dTTP and dCTP and 2.5 U of *Taq* DNA polymerase (Promega, U.K.). The reaction was carried out, in a Gene Amp 9700 PE Applied Biosystems thermcycler. The PCR procedure comprised 35 cycles of 96°C for 2 min, 55°C for 2 min and 72°C for 6 min followed by 20 min at 72°C. The resulting PCR amplicon was TOPO ligated into a T7 expression vector (pCR®T7/CT-TOPO®). The resulting library was used to transform competent BL21 (DE3) *E. coli* cells. Recombinant *E. coli* cells were grown at 37°C in 100 mL LB medium containing 100 μg/mL ampicillin. The synthesis of mutated forms of CYP102A2 was induced by the addition of 1 mM IPTG when the absorbance at 600 nm was 0.6-0.8. Four hours after induction, cells were harvested and analyzed.

Bioinformatics analysis and molecular modelling

A molecular model of the heme domain of CYP102A2 was constructed using SWISS-MODEL (http://www.expasy.org/swissmod/) (Guex and Peitsch 1997), as described by Axarli *et al*. (2005). The determined X-ray crystal structures of the heme domain of P450 BM3 [PDB codes 1JPZ, 2HPD, 1FAG, 1BU7, with which the CYP102A2 enzyme shares 63% sequence identity, was used as a template. The program iMolTalk was used to analyze interactions in the modeled structure (Diemand and Scheib 2004). Sequences homologous to CYP102A2 were sought in the NCBI using BLASTP (htt://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*. 1990). The resulting sequence set was aligned with Clustal W (Thompson *et al*. 1994). ESPript (http://espript.ibcp.fr/ESPript/ESPript/) (Gouet *et al*. 1999) was used for alignment visualization.

Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) on a slab gel containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. The protein bands were stained with Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

Bioinformatic analysis and purification of *Bacillus subtilis* CYP102A2 monooxygenase

Two P450 monooxygenases [CYP102A2 (accession number O08394) and CYP102A3 (accession number O08336)] within the *Bacillus subtilis* genome, with high similarity to the well-known cytochrome P450 BM-3 (CYP102A1) of *Bacillus megaterium* have been recently identified (Budde *et al*. 2004; Gustafsson *et al*. 2004; Axarli *et al*. 2005). The CYP102A2 is a natural fusion enzyme consisting of a heme domain and a reductase domain. **Fig. 1** shows the amino acid sequence alignments resulting from the BLAST search of CYP102A2. The heme domain of P450 BM3 (BMP domain) of CYP102A2 showed 63% sequence identity with the CYP102A1 from *Bacillus megaterium*, whereas significantly higher identity was observed with the homologues enzymes from *Bacillus cereus* (Chowdhary *et al*. 2007) and *Bacillus anthracis* (~80%).

Bacillus subtilis CYP102A2 monooxygenase gene was cloned and expressed using the T7 expression system which appeared very useful for expressing prokaryotic CYPs (Gustafsson *et al*. 2004; Axarli *et al*. 2005, 2010). The recombinant enzyme was purified by a 2-step procedure comprising anion-exchange chromatography and affinity chromatography on 2,5-ADP-agarose Sepharose column. Anion exchange chromatography on DEAE-Sepharose proven to be a convenient technique for the preliminary purification of CYP102A2. The enzyme was adsorbed at pH 7.4 and

Table 1 Purification of CYP102A2 using a two-step procedure employing anion-exchange chromatography on DEAE-Sepharose CL 6B and affinity chromatography on 2,5-ADP-Sepharose CL 6B column. The procedure was carried out at 4°C.

Step	Volume (mL)	Units	Protein (mg)	SA [a]	Purification (fold)	Yield (%)
Crude extract	0.7	4.958	6.500	0.763	1	100
Anion-exchange chromatography on DEAE-Sepharose	1	2.930	0.975	3.005	3.938	59.1
Affinity chromatography on immobilized 2,5-ADP-Sepharose CL 6B column	1	1.610	0.125	12.880	16.880	32.5

[a]: Specific activity , Units/mg

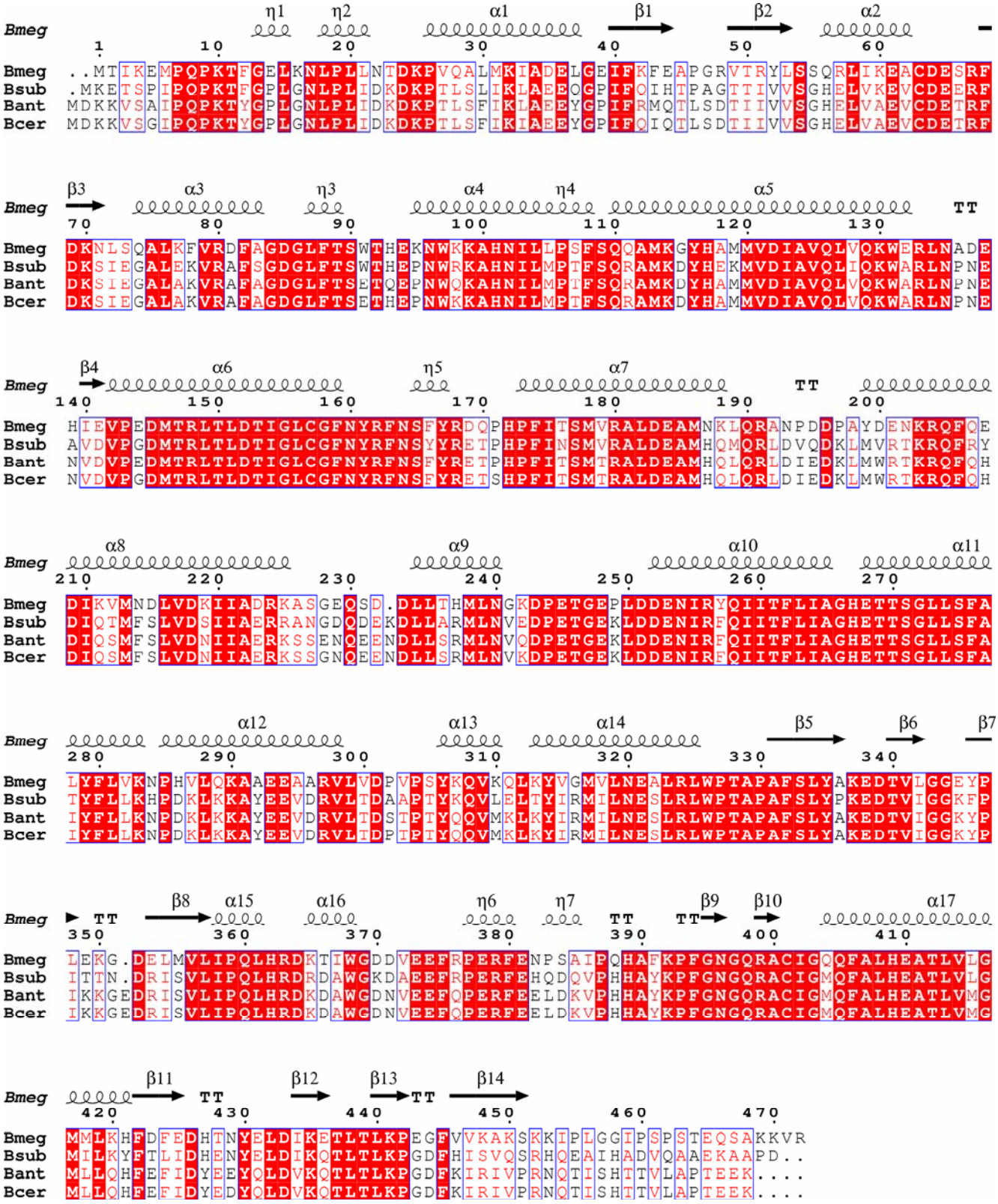

Fig. 1 Amino acid sequence alignments. Sequence alignments of heme domain of CYP102A1 (residues 1-472) of *B. megaterium* flavocytochrome P450 BM3 (A34286) with the respective domain of CYP102A2 from *B. subtilis* (O08394), *B. cereus* (NP979541), *B. anthracis* str. Sterne (YP_029250). NCBI accession number for the P450 enzymes are in brackets. The alignments were produced using Clustal W (Thompson *et al*. 1994) and visualised using ESPript (Gouet *et al*. 1999). The secondary structure of CYP102A1 (pdb code 1FAG) and numbering are shown above the alignment. Alpha helices and beta strands are represented as helices and arrows, respectively, and beta turns are marked with TT. Conserved areas are shown shaded. A column is framed, if more than 70% of its residues are similar according to physico-chemical properties.

subsequently eluted using KCl step-gradient. Affinity chromatography was the next and final step for CYP102A2 purification. The enzyme was adsorbed at pH 7.4 (50 mM Tris-HCI buffer, containing 1 mM EDTA). Elution was carried out biospecifically with $NADP^+$ (5 mM). The results from a typical purification run are shown in **Fig. 2** and summarized in **Table 1**.

P450 monooxygenases catalyze a broad range of reactions, with different members of the family exhibiting quite varied substrate specificity (Gustafsson *et al*. 2004; Lentz *et al*. 2004; Axarli *et al*. 2005). CYP102A2 is more active in oxidation of SDS than any other characterized P450 mono-

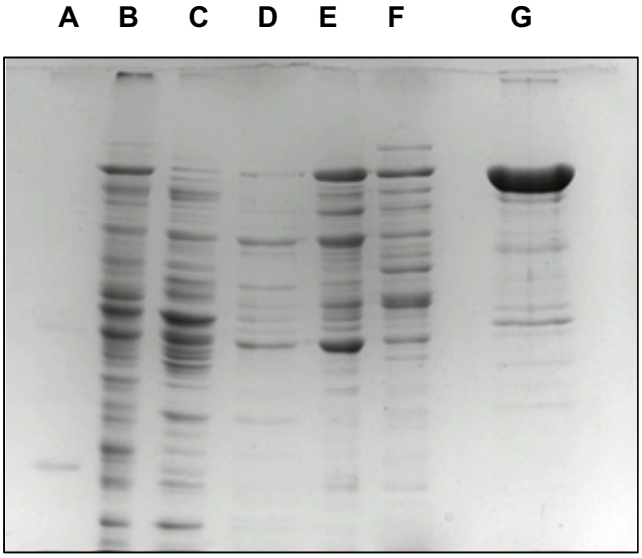

Fig. 2 SDS-polyacrylamide gel electrophoresis of CYP102A2 preparations. Protein bands were stained with Coomassie Brilliant Blue R-250. Lane A, molecular weight markers; Lane B *E. coli* crude extract after induction with 1 mM IPTG; Lane C, D, E, F, eluted fraction from DEAE Sepharose CL-6B chromatography; Lane G, CYP102A2 eluted from the 2,5-ADP-Sepharose CL 6B column.

oxygenase and catalyses its conversion to ω-3, ω-2 and ω-1 hydroxylated products (**Fig. 3**). Even and odd-chain as well as unsaturated fatty acids (e.g. myristic, pentadecanoic, oleic acids) were all exclusively hydroxylated at positions ω-3, ω-2 and ω-1 (Gustafsson *et al*. 2004).

Error prone PCR of wild type CYP102A2

We used the polymerase chain reaction (PCR) to perform an error-prone mutagenesis based on the mutant Pro15Ser gene. The Pro15Ser mutant enzyme was recently characterised (Axarli *et al*. 2005) and showed approximately 6- to 10-fold increased activity to SDS, lauric acid and 1,4-naphthoquinone and enhanced activity for other substrates such as ethacrynic acid and ε-amino-n-caproic acid. In order to identify enzyme forms with altered specificity, mutated clones from the error-prone library were screened using the NADPH-based assay and employing the several different substrates (sodium dodecyl sulphate, lauric acid, 1,4-naphthaquinoline, 2-hydroxy-1,6-naphthoquinone and ε-amino-*n*-caproic acid). After activity screening one enzyme variant, designated CYPvar8 was isolated and analysed. Sequencing of the CYPvar8 gene showed that the mutant contained two base substitutions (C→T, at codon No 16 and T→C, at codon No 160) leading to the amino acid exchanges Pro15Ser/Phe160Leu. The mutant was expressed in *Escherichia coli*, purified and its kinetic properties were analyzed. The steady-state turnover kinetic parameters were determined by monitoring the SDS dependent oxidation of NADPH. The effect of SDS and $NADP^+$ concentration on the enzyme activity was studied at 37°C and pH 6.7 and the results are shown in **Table 2**. The results showed that the mutations do not change appreciably the affinity of the enzyme for $NADP^+$ whereas contribute significantly to the affinity for SDS. In particular, the double mutant Pro15Ser/Phe160Leu showed a 9.7-fold increase in K_m values for SDS, compared to the wild type enzyme and 52.3-fold higher K_m compared to the mutant Pro15Ser. These findings suggest that Phe160 is involved in important interactions which contribute to substrate binding and catalysis. **Table 3** shows the relative specific activity the Pro15Ser/Phe160Leu enzyme variant exhibited for SDS, lauric acid, 1,4-naphtha-

Table 2 Kinetic parameters of the wild-type and mutants Pro15Ser and Pro15Ser/Phe160Leu. Steady-state kinetic measurements were performed at 37°C in 0.15 M potassium phosphate buffer, pH 6.7. All initial velocities were determined in triplicate. The kinetic parameters k_{cat} and K_m for NADPH were calculated by non-linear regression analysis of experimental steady-state data using the GraFit (Erithacus Software Ltd.) program (Leatherbarrow 1998). The $S_{0.5}$ values for SDS were determined by fitting the plotted *v versus* substrate concentration to the Hill equation using the GraFit (Erithacus Software Ltd) program (Leatherbarrow 1998).

Enzyme	K_m[a]	$S_{0.5}$[b]
Wild-type	7.81 ± 0.52	0.0330 ± 0.003
Pro15Ser	7.44 ± 0.45	0.0065 ± 0.0004
Pro15Ser/Phe160Leu	6.12 ± 0.63	0.3400 ± 0.111

[a]: μM NADPH
[b]: mM SDS

Table 3 Specific activities of the *B. subtilis* CYP102A2 wild-type enzyme and its mutant Pro15Ser/Phe160Ile, against selected substrates. Data for the wild-type enzyme and Pro15Ser were taken from Axarli *et al*. 2005 and included for comparison. As 100% was taken the specific activity of the wild type enzyme against SDS.

Substrate	Wild-type enzyme (%)	Pro15Ser (%)	Pro15Ser/Phe160Ile (%)
SDS	100	571.	8.4
Lauric acid	27	234.2	7.4
Ethacrynic acid	2.9	6.4	ND[a]
1,4-naphthoquinone	92	821.9	142
2-hydroxy-1,6-naphthoquinone	15.7	25.3	38.8
ε-amino-n-caproic	0.3	1.8	1.6

[a]: No detectable activity

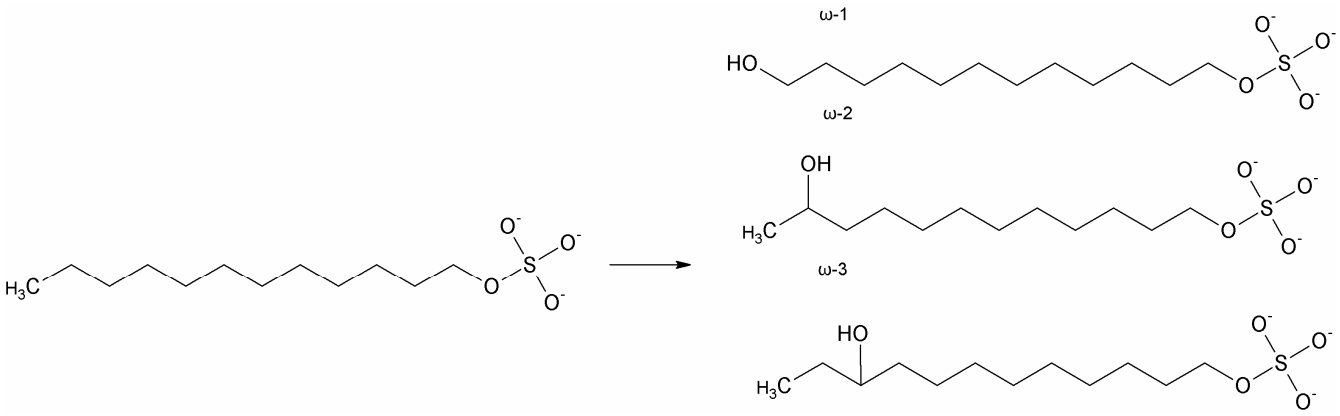

Fig. 3 Reaction scheme of fatty acid oxidation by CYP102A2. CYP102A2 hydroxylates SDS at the ω-1, ω-2 and ω-3 positions.

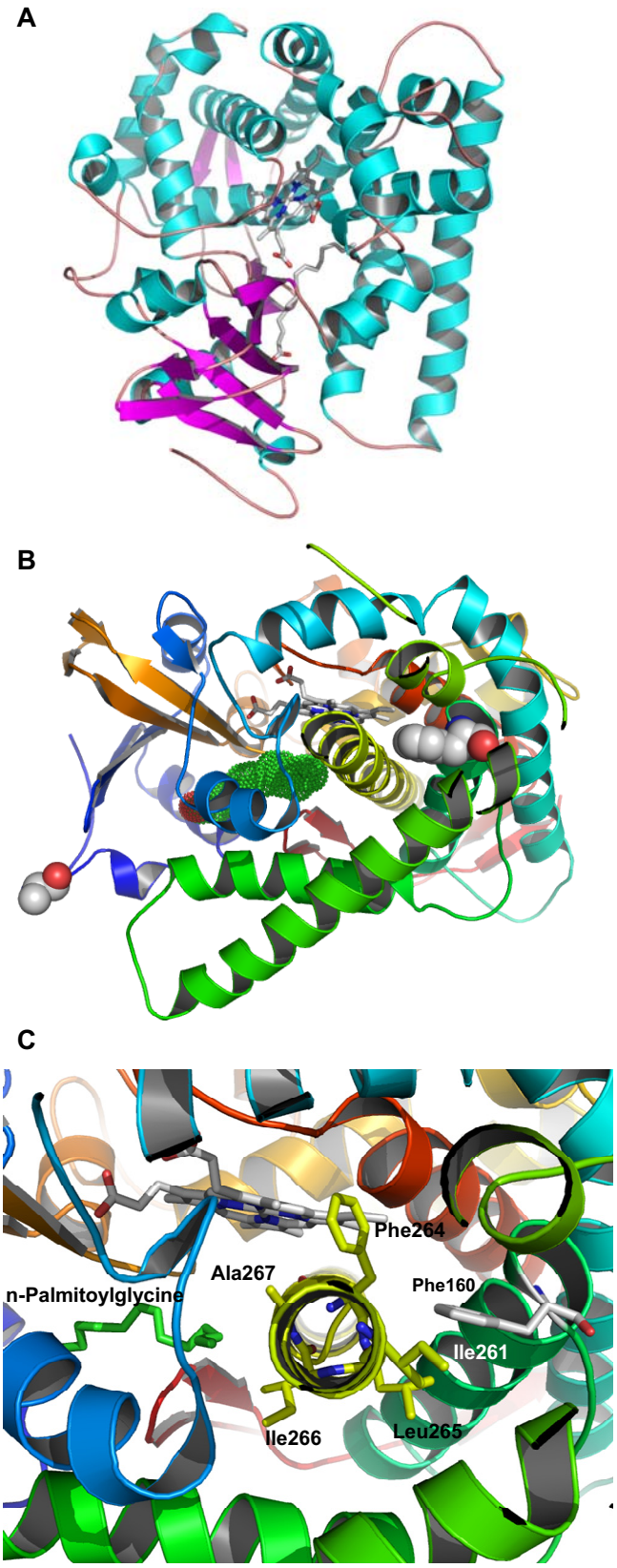

Fig. 4 Structural representations of the heme domain of CYP102A2 (residues 1-472) of *B. subtilus*. (**A**) The bound heme and the organic substrate n-palmitoylglycine are shown in a stick representation. β-Sheets are shown in magenta and α-helices in turquoise. (**B**) Structural representation of the Pro15 and Phe160. Pro15 and Phe160 are shown as spheres. The bound heme is shown in a stick representation and the organic substrate n-palmitoylglycine is shown as dotted spheres. (**C**) Representation of the putative interactions in the substrate binding site. Important residues are represented as sticks and labelled.

quinoline, 2-hydroxy-1,6-naphthoquinone and ε-amino-*n*-caproic acid. With SDS as substrates this variant showed about 12-fold lower specific activity, whilst with 1,4-naphthaquinoline, 2-hydroxy-1,6-naphthoquinone and ε-amino-*n*-caproic acid showed higher specific activity, compared to the wild-type enzyme (**Table 3**).

A molecular model of CYP102A2 was constructed to put the activity data in a structural context (**Fig. 4**). The model was constructed based on the known crystal structures of CYP102A1 from *B. megaterium*. The possible role of Pro15 was recently analysed (Axarli *et al.* 2005). Briefly, Pro15 is located on the surface of the protein onto the short helical segment formed by residues 14 to 16, and is involved in interactions with Pro46 which is located in a β-turn that connects the β1 and β2 sheets (**Fig. 1**). Part of the β2 sheet forms the entrance of the substrate access channel and is responsible for the tight binding and subsequent orientation of the substrate in the active site (Maves *et al.* 1997). Therefore, the Pro15Ser mutation may affect substrate binding, which may affect the K_m of the enzyme for SDS.

The other mutated residue (Phe160Leu) found in the double mutant is a conserved residue (**Fig. 1**). Phe160 is located at the end of α-helix-6 (**Fig. 4**), and is involved in van der Waals interactions with Ile261, Phe264 and Leu265 which are located at α-helix-10 (**Fig. 1**). Residues at α-helix-10 (Ile266 and Ala267) are involved in the formation of the substrate binding pocket (**Fig. 4B, 4C**). One way in which the Phe160Leu mutation could affect substrate binding is through the perturbation of the structure of the α-helix-10. This would lead to altered conformations for the important residues Ile266 and Ala267 that form part of the substrate binding site.

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