In-silico and In-vitro based studies of Streptomyces peucetius CYP107N3 for oleic acid epoxidation

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Certain members of the cytochromes P450 superfamily metabolize polyunsaturated long-chain fatty acids to several classes of oxygenated metabolites. An approach based on in silico analysis predicted that Streptomyces peucetius CYP107N3 might be a fatty acid-metabolizing enzyme, showing high homology with epoxide enzymes. Homology modeling and docking studies of CYP107N3 showed that oleic acid can fit directly into the active site pocket of the double bond of oleic acid within optimum distance of 4.6 Å from the Fe. In order to confirm the epoxidation activity proposed by in silico analysis, a gene coding CYP107N3 was expressed in Escherichia coli. The purified CYP107N3 was shown to catalyze Δ5-CΔ6 epoxidation of oleic acid in vitro to 9,10-epoxy stearic acid confirmed by ESI-MS, HPLC-MS and GC-MS spectral analysis.

INTRODUCTION

The cytochrome P450 (CYP) superfamily represents a highly diversified set of heme-containing enzymes found in bacteria, fungi, animals and plants (1). These enzymes are a major class of biocatalysts related to the oxidative metabolism of many drugs. Most often, this is brought about by the CYP metabolisms increasing the solubility of compounds facilitating metabolic processes within the cell.

Fatty acid-oxidizing enzymes have been the subject of an increasing number of studies in all organisms, since the products of their reactions exhibit fundamental biological activities (2-4). For instance, CYP epoxygenases convert ω3-polyunsaturated fatty acids, such as eicosapentaenoic acid or docosahexaenoic acid to epoxy-derivatives (5) which are potent dilators of coronary arteries (6-8) or the pulmonary artery (9), and inhibit platelet aggregation (10). It is also well known that CYPs play a prominent role in oxidative metabolism. CYP102A1 (Bacillus megaterium BM-3), a bacterial CYP that closely resembles eukaryotic microsomal CYPs is mainly a ω-hydroxylase for arachidonic acid and a highly specific epoxidegenase for eicosapentaenoic acid. Replacement of phenylalanine 87 (F87) with valine (V) converts CYP102A1 into a highly specific arachidonic acid epoxidegenase (11). The epoxidation of polyunsaturated fatty acid double bonds, particularly of arachidonic acid, has generated much interest because of the biological activities of the resulting metabolites (12, 13). These epoxidation reactions of arachidonic acid (C 20:4) are catalyzed by members of the CYP2C subfamily and by the CYP2J2 isoform (14, 15). Human CYP4F8 and CYP4F12 isoforms are able to epoxidize docosahexaenoic acid (C 22:6) (16).

With an objective of finding a new epoxidegenase from Streptomyces species, we carried out in silico analysis of all 23 CYPs present in the Streptomyces peucetius. A BLAST search revealed CYP107N3 has high sequence identity with well characterized epoxidegenases including OleP (S. antibioticus) and ChmPll (S. bikiniensis) (17, 18). Homology modeling and docking studies revealed oleic acid to be the best candidate for in vitro studies. Herein, we report the heterologous expression and functional characterization of S. peucetius CYP107N3 as oleic acid epoxide which was screened for its putative epoxidase activity by in silico analysis.

RESULTS

Sequence alignment and homology modeling

The putative S. peucetius CYP107N3 gene encodes for 414 amino acids with a predicted molecular mass of 46 kDa. The overall G + C content of the gene is 71%, which is characteristic of such genes. The amino acid sequence of CYP107N3 was aligned with other epoxidegenases by Clustal X. The alignment between CYP107N3 and other CYPs was performed using Align3D, followed by manual modification (Fig. 1A). This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
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Fig. 1. (A) Multiple sequence alignment of CYP107N3 with three highly homologous templates CYP158A2 (PDB ID: 2D0E, 42% identity), CYP105A1 (PDB ID: 2ZBX, 40% identity) and CYP105P1 (PDB ID: 3E5L, 40% identity). Deep green color shows conserved residues in all three templates sequence, and red boxes indicate helixes or beta-sheets. (B) Superimposition of CYP107N3 model (blue) over CYP158A2 (green).

Fig. 2. (A) Oleic Acid docking into CYP107N3 model. The distance between iron in the porphyrin and C9 atom of oleic acid is 4.6 Å as shown by the green line. (B) 2D display of amino acids within active site surrounding the ligand. B:415 denotes the heme ligand. Amino acids with green circles represent residues involving in van der Waals interactions with the ligand, whereas those with pink circles interact with the ligand via a charge or polar based interaction. Two hydrogen bonds between carboxyl oxygen of the ligand and Met328/Gly329 are shown in green dashed lines.

Expression and spectral analysis of CYP107N3
The expressed His-tag fused CYP107N3 was purified using Co2+ affinity chromatography where the target protein was eluted at 500 mM imidazole. The predicted molecular weight of the protein (46 kD) was subsequently confirmed by 12% SDS-PAGE (Fig. 3A). When substrate is unbound to a cytochrome, heme iron is in ferric form coordinated by six water molecules. In this state, heme iron shows absorbance at 420 nm. Substrate bound CYP, on the other hand, a hexa-coordinated heme iron is changed to a penta-coordinated state from perturbation of the electronic shell. This change in the water coordination causes a spin state shift of the Soret band from approximately 418 nm (representing the low-spin substrate free form), to around 390 nm for the high-spin, which results in a classical type I spectrum (21). Incubation of CYP107N3 with oleic acid results in a type I spectrum indicating oleic acid confirmed that the model was reliable (data not shown). The Ramachandran plot \( \phi / \psi \) distribution of backbone conformation angles for each residue of the refined structure revealed that, 92.8% were in the favored region, 4.2% of amino acids were in allowed region, and 3.0% were in outlier region. These validation results consistently demonstrated that the CYP107N3 model was reasonable and could be employed for the further docking study. The model substrate oleic acid was docked into the CYP107N3 model using LigandFit (20), and the top binding mode for each, as adjudged by LigScore, was overlaid and displayed in the binding site (Fig. 2A). The distance between the unsaturated bond of oleic acid and the heme was 4.6 Å which is within the range for successful hydroxylation reaction to occur. The oleic acid chain was bound almost entirely via hydrophobic interactions with amino acid side chains (Pro92, Leu91, Leu101, Leu186, Ala247, Ala251, Gly302, Ile250, Ile297, Val404, Glu254, Thr255, Thr299, Met403 and Asp301) and via polar interactions (Gly298, Val300 and Ser327). Two hydrogen bonds, between carboxyl oxygen of oleic acid and Met328/Gly329, were also observed, further helping the stabilization of the ligand within the active site (Fig. 2B).
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Protein assay for epoxidation
The epoxidase activity of CYP107N3 was determined with oleic acid as a substrate. The protein extracts from E. coli mutant, harboring only expression vector (pET28a), were used as a negative control. The extracted product from the reaction sample was analyzed by ESI-MS showed a mass of 297.2, which is a possible epoxidated product (Fig. 4A). Subsequent LC/MS analysis of the peak clearly indicated an epoxidated product with a mass of 297.5 in negative mode (Fig. 4B). Furthermore, GC-MS analysis showed the major peak with m/z of 155 and 185 along with 298 which is characteristic mass fragmentation patterns of epoxidated oleic acid (Fig. 4C).

DISCUSSION
As epoxidated and hydroxylated fatty acids have gain grounds as valuable pharmaceutical and antimicrobial agents, the search for new fatty acid hydroxylase compounds, or novel engineering of current ones, has intensified. The hydroxylated fatty acid 7S,10S-dihydroxy-8E octadecanoic acid, isolated from Pseudomonas aeruginosa, has been found to stop the growth of the pathogenic yeast Candida albicans, whereas 7S,10S,12R-trihydroxy-8E-octadecenoic acid, isolated from the same species, exhibits antimicrobial activity and curtails the rice blast fungus (Magnaporthe grisea) (22). Candida yeasts have been widely used for fatty acid oxygenation e.g. the production of α,ω-dicarboxylic acids. Apart from yeast, several bacteria such as Pseudomonadaceae, Bacilli or Rhodococci are able to hydroxylate fatty acid in a terminal or subterminal manner (23, 24). For enzymatic synthesis of hydroxyl fatty acid, E. coli transformed with a P450BM-3 has been used with a fatty acid uptake system (25, 26) but still biocatalytic hydroxylation and epoxidation of fatty acids by bacterial CYP is in its infancy. Moreover, plant and animal CYP has been studied and found to be better oleic acid epoxidase. Arabidopsis thaliana CYP77A4 was the first CYP which was determined to be able to catalyse the epoxidation of free fatty acid in plants (27). Similarly, P4502CAA and P4502C2, from the rabbit, have also been studied for the epoxidation of oleic acid (28). To find a new epoxidase from Streptomyces sp., we conducted a BLAST
search and identified CYP107N3 as having high identity with previously reported epoxidase from Streptomyces sp. Homology modeling and docking studies of CYP107N3 revealed oleic acid to be the best candidate for in vitro studies. Previously, the crystal structure of CYP102A1 in complex with palmitoleic acid has shown that the acid group binds to positively charged or hydrogen-bonding residues near the entrance of the hydrophobic substrate access channel, while the alkyl chain penetrates the channel, and reaches the catalytic center in a geometric arrangement allowing hydroylation to occur (29), consistent with the docking of oleic acid determined in this study. The carboxyl groups form hydrogen-bonds with Met328 and Gly329 residues near the entrance. The distance of 4.6 Å from heme iron to the site of epoxidation was the optimum distance for reaction to occur. To establish oleic acid as a substrate of CYP107N3, the CYP was expressed in E. coli. The substrate binding assay conducted with oleic acid showed a Type I spectrum, indicating that oleic acid binds at the catalytic site of CYP107N3 near the heme. The reaction product obtained from in vitro enzyme assay, using spinach ferredoxin and ferredoxin reductase as reduct partner, was analyzed and found to be the epoxidized product of oleic acid, i.e. 9,10-epoxyoleic acid. Further studies investigating whether CYP107N3 can also accept other substrates are ongoing, for the introduction of epoxy substituent into different fatty acid derivatives.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions
Standard methods were used for DNA cloning, plasmid isolation, and restriction enzyme digestion (30, 31). E. coli strains were grown at 37°C in Luria Bertani (LB) media in both liquid and agar plates supplemented with the appropriate amount of antibiotic (ampicillin 100 μg ml⁻¹). pGEM-T easy vector (Promega, USA) and pET-28a(+) (Novagen, Germany) were used for the cloning of polymerase chain reaction (PCR) products and for the expression of the gene, respectively. E. coli XL1-Blue (MRF) (Stratagene, USA) was used as a host cell for the preparation of recombinant plasmid and manipulation of DNA, whereas E. coli BL21 (DE3) (Stratagene, USA) was used as the host for the expression. Reagent grade chemicals were purchased from Sigma/Aldrich or Merck, UK. In some cloning experiments, 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG) and 50 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were included in the LB agar plates for screening.

Sequence analysis and homology modeling
The computer-based analyses and comparison of protein sequences were performed using the programs BLAST, FASTA, CLUSTALW and GENEDOC. The homology modeling of CYP107N3 and docking were performed using Accelrys Discovery Studio V3.1 (Accelrys Software Inc., San Diego, CA, USA). The homologue search was done from the ExPASy web site (http://swissmodel.expasy.org). The structures of three highly homologous proteins, 2DOE (CYP158A2, 2.15 Å, length 407 aa) (32), 2ZBX (CYP105A1, 1.5 Å, length 412 aa) (33) and 3ESL (CYP105P1, 2.40 Å, length 403 aa) (34) were selected as templates for modeling. The final 3D model was generated by MODELER, an original program (19) that performed automatic protein homology modeling and loop modeling for CYP107N3. The refined model was validated with ProSa2003 (35), and Ramachandran Plot (36). RSMD (Root Mean Square Deviation) analysis of the predicted model from its templates was calculated using SUPERPOSE (37). Substrate for docking was drawn using a sketch toolbar application and converted to stereo-chemically correct configuration and optimized by Dreiding-like force-field in DS V3.1. The protein-ligand interaction study was performed using LigandFit/LigandScore (20), an automated tool for protein-small molecule docking/scoring.

Construction of recombinant plasmid
Recombinant pN107N3 was constructed for E. coli hosts following. A set of primers 107N3F (5'-GGC CGG CGG GCG ACC GA-3') and 107N3R (5'-GGT TTC CAA TGC CCA CCA GGT GAT CGG CAT G-3') (the underlined letters indicate the restriction sites) was used for the amplification of CYP107N3 coding region (GenBank accession no. CAE53712). Polymerase chain reaction (PCR) was performed in a thermocycler (Takara, Japan). The amplification conditions for PCR were: 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, 66°C for 1 min, 72°C for 1 min and finally 72°C for 10 min. DNA amplification was performed in a total volume of 20 μl containing 5 μl PCR Mix (Genotech Co., Korea). The PCR products (1,245 bp) were cloned into pGEM-T (Promega, USA) and sequenced prior to cloning into the expression vector in order to verify that no mutation had been introduced during PCR amplification. The purified PCR product was again cloned into the EcoRI-HindIII sites of the pET-28a(+) vector. The recombinant expression vector pN107N3 was introduced into E. coli BL21 (DE3) by heat-pulse transformation (31).

Expression and purification of His-tag fused protein
E. coli BL21 (DE3) was transformed with the recombinant plasmid (pN107N3), and the overnight culture was diluted 1:50 in fresh medium. IPTG was added to a final concentration of 0.4 mM when the culture reached an optical density at 600 nm (OD₆₀₀) of 0.7, and the induction was carried out for 20 h in a 20°C shaker. 1 mM 5-aminolevulinic acid (ALA) and 0.5 mM FeCl₃ were added to the culture medium 10 min before IPTG induction in order to increase the yield of active CYP (38). After harvest, the cells were washed with 25 ml of 50 mM phosphate buffer (pH 7.5) and resuspended in 2 ml of storage buffer [10% glycerol along with 1 mM DTT, 1 mM PMSF and 0.1 mM EDTA in 50 mM potassium phosphate buffer (pH 7.6)]. The cell pellets were disrupted by ultrasonication, and the crude cell extract was obtained by centrifugation at 12,000g for 20 min. The molecular masses of the denatured proteins were observed in SDS-PAGE analyses.
compared with standard molecular mass protein markers (Novagen, USA). His-tag fused protein in crude cell extracts was purified by immobilized Co²⁺-affinity chromatography (TALON, USA) according to the manufacturer's instructions. The proteins were eluted with a linear gradient of imidazole (from 10 to 500 mM) in water. The pure fractions were dia lyzed with storage buffer (50 mM phosphate buffer, pH 7.5) and the purified proteins were analyzed by 12% SDS-PAGE.

**Protein concentration and spin-state shift determination**

The CYP content was determined as described previously (39). Briefly, 0.5 ml samples (crude or purified) were diluted in 4.5 ml of 50 mM potassium phosphate buffer (pH 7.6) with 1 mM EDTA and 10% (v/v) glycerol, and dithionite-reduced carbon monoxide difference spectra at 450 and 490 nm were measured. To perform this, a few crystals of sodium dithionite were added to the CYP-rich fraction obtained from the heterologously expressed strains, and were then mixed and divided into two cuvettes. The mixture was then saturated with 30 to 40 bubbles of CO at a rate of about 1 bubble per second. The absorbance difference between reduced CYP and CO bound CYP was measured using an extinction coefficient of the unreduced form (ε=91 mM⁻¹ cm⁻¹). To determine the spin-state shift upon substrate binding, 1 μM of protein in buffer (50 mM potassium phosphate buffer, pH 7.6) was incubated with 1 μM of substrate dissolved in ethanol in sample cuvette. The spectral changes between 350 and 500 nm was recorded. The reference cuvette contained the same concentration of protein and an equal volume of ethanol, but with no substrate.

**Reconstituted assay of CYP107N3 with oleic acid**

The activity of CYP107N3 was determined with oleic acid as a substrate. The reaction mixture consisted of 5 μM spinach fer redoxin, 0.1 unit of ferredoxin reductase, 4 μM CYP107N3, 1 mM NADH and 1 mM oleic acid, 10 mM of glucose 6-phosphate and 0.24 unit of glucose 6-phosphate dehydrogenase in 50 mM potassium buffer. The reaction mixtures were incubated at 37°C for 6 h, and an equal quantity of ethyl acetate was added to stop the reaction at the end of the incubation, which was followed by centrifugation to obtain the supernatant. The extract was analyzed by electron spray ionization-mass spectrometry (ESI-MS), high performance liquid chromatography-mass spectrometry (HPLC-MS) and gas chromatography-mass spectrometry (GC-MS) to determine product formation. The ESI-MS analysis was carried out with a probe temperature of 392°C and a source voltage of 31.2 V using Finnigan AQA GC-MS (Agilent 5973) inert MSD, equipped with HP-5ms column. The conditions for GC-MS analysis was as follows, the injector and detector temperatures were set at 250°C and 285°C, respectively, with helium as carrier gas. Sample (1 μl) was injected for analysis. The column temperature was maintained at 180°C for 2 min, increased to 300°C at a rate of 8°C min⁻¹, and held at 300°C for 5 min. Conversion products were identified by their characteristic mass fragmentation patterns.

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