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Engineering the substrate specificity of cytochrome P450 CYP102A2 by directed evolution: production of an efficient enzyme for bioconversion of fine chemicals

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Abstract

The P450 cytochromes constitute a large family of hemoproteins that catalyze the monooxygenation of a diversity of hydrophobic substrates. CYP102A2 is a catalytically self-sufficient cytoplasmic enzyme from *Bacillus subtilis*, containing both a monooxygenase domain and a reductase domain on a single polypeptide chain. CYP102A2 was subjected to error-prone PCR to generate mutants with enhanced activity with fatty acids and other aromatic substrates. The library of CYP102A2 mutants was expressed in BL21(DE3) *Escherichia coli* cells and screened for their ability to oxidize different substrates by means of an activity assay. After a single round of error-prone PCR, the variant Pro15Ser exhibiting modified substrate specificity was generated. This variant showed approximately 6- to 9-fold increased activity with SDS, lauric acid and 1,4-naphthoquinone, and enhanced activity for other substrates such as ethacrynic acid and ε-amino-*n*-caproic acid. Molecular modeling of the CYP102A2 monooxygenase domain suggested that Pro15 is located in a short helical segment and is involved in extensive interactions between the N-terminal domain and the β2 sheet, which contribute to the formation of the substrate binding site. Thus, Pro15 appears to affect substrate binding and catalysis indirectly. These results clearly demonstrate the importance of remote residues, not readily predicted by rational design, for the determination of substrate specificity. In addition, we report here that the Pro15Ser variant of CYP102A2 can be efficiently immobilized on epoxy-activated Sepharose at pH 8.5 and 4 °C. The immobilized variant of CYP102A2 retains most of its activity (81%) and shows improved stability at 37 °C. The approach offers the possibility of designing a P450 bioreactor that can be operated over a long period of time with high efficiency and which can be used in fine chemical synthesis.

Keywords: BM3; Biotransformation; CYP; Directed evolution; Enzyme immobilization; P450; substrate recognition

1. Introduction

Cytochromes P450 (also called CYPs) constitute an extremely large family of hemoproteins that catalyze the monooxygenation of a diversity of hydrophobic substrates [1]. 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. 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. Genes coding for diverse CYPs have been found in

Abbreviations: BMP, the heme domain of P450 BM3; CPR, NADPH: cytochrome P450 reductase; CYP, cytochrome P450; CYP102 or P450 BM3, cytochrome P450 monooxygenase from *Bacillus megaterium*; CYP102A2, cytochrome P450 monooxygenase from *Bacillus subtilis*; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; $NADP^+$, β-nicotinamide-adenine dinucleotide phosphate; NADPH, β-nicotinamide-adenine dinucleotide phosphate, reduced form; pCYP102A2, the expression vector pCR®T7/CT-TOPO® which contains the gene of CYP102A2; SDS, sodium dodecyl sulphate

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virtually all living organisms [2–4]. The most common reaction catalyzed is hydroxylation (Eq. (1)), but it has also been reported to participate in other reactions, such as dimerizations, isomerizations, dehydrations and reductions [5,6]:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+ \quad (1)$$

CYPs can be classified in two classes according to the type of electron transfer system utilized to deliver electrons from NADPH or NADH to the P450 heme [7]. In class I CYPs (bacterial/mitochondrian systems) electron transfer to P450 heme is mediated by a NAD(P)H-dependent ferredoxin reductase (FDR) and a ferredoxin (FD). In contrast with class I, class II CYPs (microsomal systems) utilizes a single NADPH dependent FAD/FMN containing reductase, which is called NADPH: cytochrome P450 reductase (CPR), in order to deliver electrons to P450 heme. CYPs have molecular masses ranging from 45 to 62 kDa and many have <15% amino acid sequence identity. However, their overall 3D structure is conserved particularly in the sequences of heme-binding site [5,8].

P450 BM3 (also called CYP102 and CYP102A1 according to Nelson's classification system [9]) from *Bacillus megaterium* is a water-soluble cytoplasmic enzyme of 119 kDa. It is the first bacterial CYP found to belong to the class II CYPs [10,11]. Unlike other CYPs it is catalytically self-sufficient, containing on a single polypeptide chain both a monooxygenase and a reductase domain. It catalyzes the hydroxylation of long-chain fatty acids, the epoxidation of the double bonds of long-chain unsaturated fatty acids [11–15], and exhibits the highest catalytic activity known for a P450 monooxygenase [16].

The enzyme contains heme, FAD and FMN in equal molar ratios. It is comprised of two distinct domains: a CPR domain (65 kDa) linked covalently via a short hydrophilic linker region to the heme domain (BMP) (55 kDa). The heme domain, also known as cytochrome P450, is located on amino-terminal domain and the NADPH: cytochrome P450 reductase (CPR) on carboxyl-terminal domain [13]. The crystal structure of BMP has been determined in the substrate-free form [17] and in complex with its natural substrate and substrate analogues such as arachidonic acid and *n*-palmitoylglycine [18]. Also the crystal structure of BMP/FMN domain is available [19].

P450 BM3 is a stable, highly active, self-sufficient CYP that shares high sequence homology with mammalian CYPs [20] and can be prepared in large amounts using recombinant expression systems. Due to these properties P450 BM3 has become an excellent model system especially for mammalian CYPs. Furthermore, protein engineering approaches have been used to modulate P450 BM3 in order to catalyze the oxygenation of substrates with emerging applications to pharmaceuticals and vaccines [21,22], and of substrates with harmful effects on humans such as PAHs [23,24].

The immobilization of enzymes has proven very useful in biotransformations as this leads to enhanced operational and storage stability and simple product separation. There are at least four main areas in which immobilized enzymes may find applications, i.e. industrial, environmental, analytical and chemotherapeutic [25,26]. In this paper, we report the use of directed evolution for improving the catalytic activity of CYP102A2 along with the demonstration that a variant of CYP102A2 can efficiently immobilized on epoxy-activated Sepharose CL6B and can be used in fine chemical synthesis.

2. Materials and methods

2.1. Materials

The pCR®T7/CT-TOPO®TA Expression Kit were purchased from Invitrogen (Holland). NADPH (tetrasodium salt, ca. 95%), ethacrynic acid, cholic acid, 1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, ε-amino-*n*-caproic acid, sodium dodecyl sulfate (SDS), lauric acid and crystalline bovine serum albumin (BSA) (fraction V) were obtained from Sigma-Aldrich Co. (St Louis, USA).

2.2. Methods

2.2.1. Bioinformatics analysis and molecular modelling

A molecular model of the heme domain of CYP102A2 was constructed using SWISS-MODEL (http://www.expasy.org/swissmod/) [27]. 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. Protein structure quality assessment, using Verify 3D [28] was used. Analysis of packing, solvent exposure and stereochemical properties suggests the final model to be of good overall quality. Sequences homologous to CYP102A2 were sought in the NCBI using BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/) [29]. The resulting sequence set was aligned with Clustal W [30]. ESPript (http://prodes.toulouse.inra.fr/ESPript/cgi-bin/ESPript.cgi) [31] was used for alignment visualization and manipulation. Residues contacts were analyzed by iMolTalk (http://i.moltalk.org/) [32] using a distance threshold set to 3.4 Å.

2.2.2. Assay of enzyme activity and protein

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 consumption to $NADP^+$ and following the decrease of absorbance at 340 nm. The final assay volume of 1 ml contained 0.1 M potassium phosphate buffer, pH 7.2 (when lauric acid is used as substrate the pH was 6.5); 0.1 mM NADPH; 0.1–0.5 mM of organic substrate, and sample containing enzyme activity. One unit of enzyme activity is