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Understanding substrate specificity and enantioselectivity of carbonyl reductase from *Candida parapsilosis* ATCC 7330 (CpCR): Experimental and modeling studies

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ABSTRACT

The whole cells of *Candida parapsilosis* ATCC 7330 are a well-established biocatalyst used for oxidation and reduction of various organic compounds to generate chiral synthons. Recombinantly expressed carbonyl reductase (CpCR) from the same strain reduces aryl α–ketoesters to their respective optically pure alcohols but preferentially reduces aliphatic and aryl aldehydes to primary alcohols. The prochiral substrates viz. aryl α-ketoester [Ethyl-2-oxo-4-phenylbutanoate], aryl ketone [Acetophenone] and aliphatic β-ketoester [Ethyl-4,4,4-trifloro-3-oxo-butanoate] get reduced to (*R*)-alcohols with CpCR while an aryl ketoaldehyde [2-oxo-2-phenylacetaldehyde] gives the (*S*)-alcohol. The optimal orientation required for the high conversion and desired enantioselectivity was analyzed by docking the α/β ketoesters, ketoaldehyde and a ketone with a modeled CpCR. Aryl α-ketoester, having the lowest free energy (-8.43 kcal/mol), shows the most favorable binding with CpCR (Interaction Energy = 7.9 kcal/mol). Also, the close proximity of aryl α-ketoester to the cofactor NADPH (2.82 Å) facilitates a better *Pro-R* hydride transfer as compared to other substrates.

1. Introduction

Carbonyl reductases (CRs) and alcohol dehydrogenases (ADHs) belong to the oxidoreductase class of enzymes that use molecules other than oxygen as electron donors or acceptors [1]. They catalyze the oxidation of alcohols and/or reduction of ketones and aldehydes by using $NAD(P)^+$ and NAD(P)H as the electron acceptor and donor respectively [2]. ADHs are further divided into three superfamilies *viz*; Fe-dependent ADH, short chain alcohol dehydrogenases/reductases (SDRs) where Zn is usually absent and medium-chain alcohol dehydrogenases/reductases (MDRs) containing Zn^{2+} as one of the cofactors [3]. CRs and ADHs differ in their directional preference of biological catalysis. This is expressed as the ratio of reaction rate constants, $k_R/k_O \geq 1$ for carbonyl reductases and $k_R/k_O \leq 1$ for ADHs where k_R and k_O are reaction rate constants in the direction of carbonyl reduction and alcohol oxidation respectively. However, there is no clear mechanistic distinction between them [4]. Various carbonyl reductases isolated from different strains of *Candida parapsilosis* (*Cp*) have the ability to reduce a variety of natural and unnatural conjugated polyketones, quinones, ketoesters, aldehydes, ketones, ketoacetals, keto acids and amides into important chiral molecules [5–17]. Key chiral intermediates for the synthesis of cholesterol regulation drugs are generated using the recombinantly expressed carbonyl reductases [18–20].

From our lab, we have shown that the whole cells of *Candida parapsilosis* ATCC 7330 are an efficient biocatalyst to prepare industrially important chiral building blocks [21]. One of the recombinantly expressed carbonyl reductases from this strain (CpCR) [7] is an MDR, based on the characteristic Rossmann fold in the C terminal and anti-parallel β sheets in the N terminal domain, separated by a cleft accommodating Zn^{2+} and the nicotinamide cofactor [22]. CpCR reduces Ethyl-2-oxo-4-phenylbutanoate [EOPB] to (*R*)-Ethyl-2-hydroxy-4-phenylbutanoate [EHPB] with > 99% ee (enantiomeric excess). The stereospecificity of enzyme-catalyzed reduction of ketones can be predicted using Prelog's rules, which depend on the alignment of the

Abbreviations: CPCR, carbonyl reductase from Candida parapsilosis ATCC 7330; EOPB, ethyl-2-oxo-4-phenylbutanoate; ACP, acetophenone; ETOB, ethyl-4,4,4-trifloro-3-oxo-butanoate; OPHEN, 2-oxo-2-phenylacetaldehyde; EPHEN, ethyl-2-oxo-4-phenylacetate; EHPB, (R)-Ethyl-2-hydroxy-4-phenylbutanoate

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substrate inside the active site that is structured into a larger and a smaller pocket to give the distinct faces i.e. the *Re*-face and the *Si*-face of the carbonyl compound. These rules also take into account the preferential hydride (*Pro-R* and *Pro-S*) transfer to either the *Si*- or *Re*-face of the ketone to give (*R*)- or (*S*)-alcohols, respectively [23]. Many ADHs including yeast ADH, horse liver ADH and *Thermoanaerobium brockii* ADH which catalyze asymmetric reductions follow Prelog's rules in terms of stereochemical outcomes [24]. CRs from various strains of *Cp* can reduce ketones to chiral alcohols with anti-Prelog's stereospecificity [8–12]. CpCR does not follow Prelog's rules strictly in reducing different prochiral substrates to their respective alcohols. In this study, the substrate scope and enantioselectivity of CpCR are investigated experimentally and by *in silico* studies.

2. Experimental

2.1. Materials

All the chemicals and media were purchased from Sigma Aldrich, Alpha Aesar, SRL biochemical, Merck and BD Difco. Molecular biology grade biochemicals and the enzyme Formate dehydrogenase were obtained from Sigma. AKTA protein purification system and GST affinity column were purchased from GE Healthcare Life Sciences. Homology modeling was performed using SWISS-MODEL. Docking was performed using Molecular Operating Environment (2016.08.1) [25]. The details of all other software and online resources used for the *in silico* studies are mentioned in the supporting information (Table S2).

2.2. Protein expression and purification

The overexpression and purification of CpCR were performed as per reported methodology using a GST affinity chromatography [7].

2.3. Determination of the enzyme activity and substrate specificity

The enzyme assay is performed in a total volume of 1 ml by monitoring the decrease in the absorbance of NAD(P)H spectrophotometrically at 340 nm and 30°C using a UV visible spectrophotometer (Jasco V 530, Easton, USA). The assay mixture consists of 4 mM of substrate and 0.2 mM of NAD(P)H in 100 mM of Potassium phosphate buffer (pH 7.5) and an appropriate amount of the pure enzyme. One unit of the enzyme activity is defined as the amount of enzyme that oxidizes 1 µmol of NAD(P)H per minute at 30°C. Different aldehydes, ketones and ketoesters were used to determine the substrate specificity of the enzyme by monitoring their conversion to respective alcohols using the above assay. Each substrate was assayed at least thrice and the activity values are reported with standard deviation.

2.4. Enantioselectivity assay

The biotransformation with purified CpCR was performed in 1.0 ml of reaction mixture consisting of 5 mM of the substrate, 1 mM of NADH and the cofactor regeneration system (1U Formate dehydrogenase, 150 mM Formate, 2U purified carbonyl reductase) in 10 mM of Phosphate buffer (pH 7.0). The reaction was carried out at 30°C, 200 rpm for 3 h. The product formed was extracted with ethyl acetate. The samples were analyzed for the conversion of the substrate and enantiomeric excess of the product by HPLC (Jasco PU-1580 with a Jasco MD-1515-PDA detector, Easton, USA) using a C18 and chiral column respectively as reported in the literature [26–28]. The reaction time was extended from 3 to 12 h for substrates which showed low conversion. The enantioselectivity assay was repeated thrice with a fresh batch of purified CpCR.

2.5. Homology modeling of CpCR

The Fasta sequence of CpCR (PDB ID - 4OAQ) was downloaded from PDB-RCSB database [29]. Proteins with high sequence similarity to CpCR were obtained by a Smart-BLASTp search of NCBI [30]. The BLAST search results were further iterated with NCBI Conserved domain database (CDD) [31] search. The closest match to CpCR was found to be cinnamyl alcohol dehydrogenase (PDB ID: 1PS0, resolution: 3 Å) with 39% sequence identity when aligned using CLUSTAL Omega 1.2.4 [32] (Fig. S1) and closest conserved domain search. The homology model was built using the above template in the SWISS-MODEL user template interface [33,34].

2.6. Validation of homology model

The homology model was validated using SAVES [35] and RAMPAGE [36]. RMSD of the model was calculated by superimposing it on the template in PyMOL [37]. The 3D structure was validated using Verify 3D [35] and RAMPAGE [36]. The outliers were redesigned by reorienting the loops in which those residues were present.

2.7. Docking of different substrates with CpCR

Docking of substrates EOPB, OPHEN, ETOB and ACP with CpCR was performed using Molecular operating environment (MOE) 2016.0801 [38]. The modeled protein structure was energy minimized using an Amber99 force field. 2-D structure of the substrates was drawn in ChemDraw Ultra 14.0 [39] and subsequently energy minimized in MOE using MMff94x force field. The protein structure was fixed and NADPH was rendered flexible for enzyme-cofactor docking. For the docking with substrates, both protein and the substrates were considered flexible. The residues within 8 Å proximity from the catalytic Zn^{2+} were selected as the active site and were specified by creating "dummy atoms" at that site [34,40]. Docking was performed thrice independently with 100 confirmations. The docked structures were analyzed to study the ligand interactions with cofactor and Zn^{2+} and the feasible orientations with respect to these units. The docking scores obtained are estimates of the free energy of binding of a ligand for a given pose.

3. Results and discussion

3.1. Substrate specificity and enantioselectivity

CpCR shows highest activity against aldehydes in comparison to α–ketoesters, ketones, β–ketoesters, keto acids and ketoaldehydes (Table 1). The reduction of aldehydes is cofactor (NADPH) specific unlike the α–ketoesters which show dual specificity i.e. both NADH and NADPH. In comparison to EOPB, Ethyl-2-oxo-4-phenylacetate [EPHEN] (Table 1, entries 1 and 2) shows up to 40 times less specific activity which suggests that CpCR activity is strongly affected by the number of carbons separating the phenyl ring and the carbonyl carbon of the α-ketoester which is attacked by the hydride. CpCR reduces both aliphatic and aromatic aldehydes to their corresponding alcohols (Table 1, entries 3–8). Aliphatic and aryl β–ketoesters and aryl ketones show very low (Table 1, entries 11 and 12) or no activity (Table S1) with CpCR unlike the SRED [8] from the same *Candida* strain. The specific activity of CpCR is comparatively high against ketoaldehyde 2-oxo-2-phenylacetaldehyde [OPHEN] than α-ketoester EPHEN and ketoacid 2-oxo-2-phenylacetic acid (Table 1, entries 10, 2 and 9). The overall conversion and enantioselectivity of four substrates, each representing the functional groups α-ketoester, ketoaldehyde, ketone and β–ketoester were further studied and summarized in Table 2.

The enantioselectivity of CpCR was established using EOPB as a standard substrate. EOPB was reduced by CpCR to (*R*)-EHPB (Table 2, entries 1a & 1b) with an ee > 99.0%. (*R*)-EHPB is an important

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