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Enzyme-substrate matching in biocatalysis: *in silico* studies to predict substrate preference of ten putative ene-reductases from *Mucor circinelloides* MUT44

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ABSTRACT

Ene-reductases are flavoproteins able to catalyse the reduction of carbon-carbon double bonds with many potential applications in biocatalysis.

The fungus *Mucor circinelloides* MUT44 has high ene-reductase activity when grown in the presence of substrates carrying different electron-withdrawing groups. Genome sequencing revealed the presence of ten putative genes coding for ene-reductases that can be potentially exploited for biocatalytic purposes. To this end, the availability of a method able to predict which isoform binds and turns over a specific substrate would help to choose the best catalyst for the desired bioconversion.

Here, homology models of the ten putative enzymes are first generated, validated and show that the proteins share the typical TIM barrel fold with a conserved β-hairpin cap on one side of the barrel and a non-conserved subdomain capping the other side, where the FMN cofactor is accommodated. The active site of the ten enzymes is different in terms of both volume and charge distribution whereas the residues responsible for substrate recognition and catalysis are generally conserved.

Docking of cyclohexenone into the active site of the ten enzymes shows a binding almost superimposable to that found in pentaerythritol tetranitrate reductase in complex with this substrate (PDB ID 1GVQ) in isoforms 1, 2, 6 and 10.

The data demonstrate that *in silico* predictions can be used for new putative fungal ene-reductases to predict the best substrate-enzyme matching for the selection of the most suitable catalyst for the desired biotransformation.

1. Introduction

Old Yellow Enzymes (OYEs) are NAD(P)H-dependent flavin-containing enzymes able to catalyse the reduction of carbon-carbon double bonds (C=C) on a wide range of α,β-unsaturated substrates [1]. OYEs were first isolated from the yeast *Saccharomyces pastorianus* [2] and then from other sources such as bacteria, plants and filamentous fungi [3] where they can participate to the metabolism of both endogenous and xenobiotic compounds [1]. Some OYEs are involved in the biosynthesis of fatty acids [4] or, in plants, 12-oxophytodienoate reductase (OPR) is involved in the biosynthesis of jasmonic acid, a compound that regulates gene expression in plant development and defense [5]. Many other OYEs are "orphans" since the physiological substrates and their role in metabolism is still unknown. However, some OYEs have attracted a lot of attention due to their ability to perform a biotechnologically important reaction, that is the stereoselective reduction of activated C=C, on a wide range of substrates of different sizes [6–8]. The resulting chiral compounds are industrially relevant and therefore OYEs are very attractive as biocatalysts [9].

Among the organisms where these enzymes have been described, fungi have been shown to possess a different number of OYEs homologs in their genomes [10]. Most of the species analyzed have from 3 to 7 genes coding for these proteins. Although some of them may be pseudogenes or expressed under control of different promoters, these data suggest a possible coexistence of different isoenzymes in fungal cells.

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Since fungi are highly versatile organisms, able to grow in different environmental conditions and using different substrates, it is also possible that there is a differential expression of the isoenzymes depending on the external stimuli such as nutrients, presence of noxious substrates, physico-chemical parameters of the surrounding environment. It can be expected that the different isoenzymes have evolved toward the recognition and reduction of specific substrates, making them even more attractive in the field of biocatalysis because of the possibility to have an enzyme portfolio catalytically optimized toward a wide range of substrates.

Recently, the fungus *Mucor circinelloides* MUT44 has been shown to be the most efficient compared to other selected fungal strains, in reducing three model substrates, cyclohexenone, α-methylcinnamaldehyde and (*E*)-α-methylnitrostyrene, all characterized by the presence of different electron-withdrawing groups (EWG) and different steric hindrance [11]. Ten putative sequences of OYE genes (McOYE1-10) were found in its genome by means of a BlastP analysis, in which the query was OYE1 sequence from *S. pastorianus*. A recent fungal OYE classification by Nizam and collaborators [10,12] clearly showed that this class of enzymes is divided in 3 distinct groups according to the structural peculiarity (e.g. core of the active site, accessory residues, loop regions): class I, class II and class III. By applying the same analysis parameters, nine out of ten OYEs from *M. circinelloides* MUT44 clustered together in class I, where OYE from bacteria, yeasts, filamentous fungi, animals and plants can be found, showing a species-specific clade, whereas only McOYE10 resulted located in class II [13]. Moreover, the expression profile of the ten enzymes is different when the fungus is grown in the presence of different substrates, suggesting that the different isoenzymes could be specialized for the conversion of different molecules [13]. In such a case, the conversion potential of the fungus can be exploited for biocatalytic purposes by using only selected isoenzymes specialized for the compound of interest.

In this work, sequence analysis and structure prediction through homology modeling are used to gain information about the degree of similarity among the ten putative isoenzymes and possible structural differences also compared to homologs from yeasts and plants. Moreover, we performed docking simulations with cyclohexenone and compare the results to the crystal structure of pentaerythritol tetranitrate reductase in complex with the same molecule to find out which isoforms are able to bind this substrate effectively.

2. Experimental

2.1. Sequence analysis and alignments

The putative OYE homologues of *M. circinelloides* and sequence IDs according to JGI database (http://genome.jgi.doe.gov/programs/fungi/index.jsf) are reported in Table 1. Multiple sequence alignments were perfomed using Clustal Omega software [14]. Primary structure analysis was performed using PROTPARAM [15] and TOPPRED [16]. Secondary structure prediction was performed using PSIPRED server [17].

2.2. Homology modeling of Mucor circinelloides MUT44 (McOYEs)

The software Modeller v9.11 [18,19] was used for homology modeling. The search of homologs of known crystal structure was carried out using PSI-Blast. Multiple templates were chosen on the basis of sequence identity, full coverage and high resolution of their crystal structure. The templates chosen for McOYE1-9 models are the crystal structures of plant 12-oxophytodienoate reductase 1 from tomato (PDB ID 1ICS, 3HGR) [20,21], the Old Yellow Enzyme from *Saccharomyces pastorianus* (PDB ID 1OYA) [22] and from *Kluyveromyces marxianus* AKU4588 (PDB ID 4TMB) [23]. For McOYE10, the templates used were the thermostable OYE from *Thermoanaerobacter pseudethanolicus* E39 (PDB ID 3KRU) [24], *Thermus scotoductus* SA-01 (PDB ID 3HF3) [25], YqjM from *Bacillus subtilis* (PDB ID 1Z41) [26], OYE from *Geobacillus kaustophilus* (PDB ID 3GR7) [27] and the xenobiotic reductase A from *Pseudomonas putida* 86 (PDB ID 2H8X) [28]. Thus, for McOYE10, the best structural homologs were all bacterial enzymes.

For each McOYE, ten models were generated and the best model was chosen on the basis of the normalized Discrete Optimized Protein Energy (DOPE) parameter, an atomic distance-dependent statistical score [29]. In order to optimize the side chain packing and interactions, all models were subjected to energy minimization through AMBER 03 force field. The models were analyzed from a structural point of view and their quality was checked by PROCHECK [30] ProSA [31] and the QMEAN scoring function [32].

2.3. Analysis of active site, surface charge and FMN binding site

The active site volume of each model was estimated through the CASTp server [33]. The surface charge of the models was also analyzed through the Coulombic surface coloring tool available on the UCSF Chimera software [34], in order to identify densely charged regions on the enzyme surface that could suggest interactions with other partners such as proteins bearing surface patches with opposite charge.

FMN binding sites were analyzed with the program LigPlot$^+$ [35].

2.4. Ligand docking

The chemical structure of cycloehexenone was subjected to molecular geometry optimization with YASARA [36]. The substrates were docked into the models of the putative McOYEs with the program AutoDock v4 [37,38] available in the YASARA package using 100 runs of flexible docking. A simulation cell (18 Å × 18 Å × 18 Å) was built around the FMN group of McOYEs. Since the crystal structure of an OYE in complex with cyclohexenone is available (PDB ID 1GVQ) [39], the substrate was removed from this PDB entry and a first docking simulation was performed on this protein with the same substrate to validate our approach and to calculate the binding energy. The same simulation cell was then used for the McOYEs models. The binding energies and dissociation constant (K_D) values were predicted using the scoring function included in the YASARA embedded AutoDock package. A set of structures having a root mean square deviation (RMSD) of less than 1 Å was included into single clusters and the clusters were ranked according to their binding energies. The best cluster in terms of binding energy for each simulation was used for predicting the final pose of the protein/ligand interaction.

3. Results and discussion

3.1. Sequence alignments and analysis of the primary structure of McOYEs

Primary sequences of the ten McOYEs considered in this work are from 364 to 396 amino acids long, and with an identity percentage ranging from 24.6% for isoforms 9 and 10 to 91.8% for isoforms 1 and 2 (Table S1).

Sequence alignments of McOYEs with representative members of different classes of OYEs of known crystal structure (Figs. S1 and S2) shows that the consensus sequence for substrate binding, that is HX_1X_2HGY or HX_1X_2NGY, where X_1 and X_2 are often small residues such as glycine, alanine or serine, is almost fully conserved with some significant exceptions. The two His or one His and one Asn of