Enzyme and Microbial Technology 55 (2014) 121–127

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Virtual screening of mandelate racemase mutants with enhanced activity based on binding energy in the transition state

Jiali Gu, Min Liu, Fei Guo, Wenping Xie, Wenqiang Lu, Lidan Ye, Zhirong Chen, Shenfeng Yuan, Hongwei Yu *

Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, PR China

ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 22 October 2013
Accepted 23 October 2013

Keywords:
Virtual screening
Mandelate racemase
Binding energy
Transition state
Molecular dynamics simulation

ABSTRACT

Mandelate racemase (MR) is a promising candidate for the dynamic kinetic resolution of racemates. However, the poor activity of MR towards most of its non-natural substrates limits its widespread application. In this work, a virtual screening method based on the binding energy in the transition state was established to assist in the screening of MR mutants with enhanced catalytic efficiency. Using *R*-3-chloromandelic acid as a model substrate, a total of 53 mutants were constructed based on rational design in the two rounds of screening. The number of mutants for experimental validation was brought down to 17 by the virtual screening method, among which 14 variants turned out to possess improved catalytic efficiency. The variant V26I/Y54V showed 5.2-fold higher catalytic efficiency (k_{cat}/K_m) towards *R*-3-chloromandelic acid than that observed for the wild-type enzyme. Using this strategy, mutants were successfully obtained for two other substrates, *R*-mandelamide and *R*-2-naphthylglycolate (V26I and V29L, respectively), both with a 2-fold improvement in catalytic efficiency. These results demonstrated that this method could effectively predict the trend of mutational effects on catalysis. Analysis from the energetic and structural assays indicated that the enhanced interactions between the active sites and the substrate in the transition state led to improved catalytic efficiency. It was concluded that this virtual screening method based on the binding energy in the transition state was beneficial in enzyme rational redesign and helped to better understand the catalytic properties of the enzyme.

1. Introduction

Due to its remarkable stability and broad substrate spectrum, mandelate racemase (MR) is an attractive catalyst for the racemization of α-hydroxy carboxylic acids. However, except for the electron-withdrawing substituent in the *p*-position of the aryl moiety, MR shows poor activity against the non-natural substrates [1]. Hence, it is of great scientific interest to reconstruct the enzyme for enhanced catalytic performance.

Currently, two major reconstruction strategies are applied in enzymatic engineering: directed evolution and rational design. High-throughput screening methods are limited for directed evolution of racemase, while rational design is a suitable choice since the crystal structure of mandelate racemase is available and the catalytic mechanism is well studied [2,3]. However, the efficiency of rational design could not meet the requirement of enzyme engineering [4,5]. Therefore, it is necessary to improve its efficiency.

Virtual screening is a promising approach to enhance the efficiency of rational design, which is facilitated via a quick search of the large enzyme library based on computational simulation to identify potential candidates for further experimental validation. However, there are few reports about its application in enzyme redesign, probably due to the lack of generally accepted screening standards. Recently, Zheng et al. employed virtual screening to facilitate the screening of cocaine hydrolase mutants with improved activity, using the interaction energy and energy barrier as the screening criteria [6]. On one hand, they did not consider the solvation effect and intramolecular interactions during the calculation of interaction energy, which might lead to reduced accuracy. On the other hand, the energy barrier calculation performed by the sophisticated and hybrid quantum mechanical/molecular mechanical (QM/MM) simulation was time-consuming and CPU-demanding. Therefore, it is necessary to develop an alternative route with reasonable accuracy and acceptable time cost.

Binding energy is of fundamental importance for enzyme catalysis [7]. Enzymes use substrate-binding energy not only to promote

* Corresponding author at: Institute of Bioengineering, Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, PR China. Tel.: +86 571 8795 1873.
E-mail address: yuhongwei@zju.edu.cn (H. Yu).

0141-0229/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.enzmictec.2013.10.008

the ground-state association, but also to stabilize the reaction transition state, reducing the energy barrier of the catalytic process [8]. The virtual dissociation constant of the enzyme-substrate complex in the transition state (K_{tx}) is defined by the Eq. (1)

$$K_{tx} = \frac{k_{non}}{k_{cat}/K_m} \quad (1)$$

where k_{non} is the rate constant of the non-enzymatic reaction, k_{cat} is the turnover number, K_m is the Michaelis constant [9]. The binding energy of the enzyme-substrate complex in the transition state is calculated as follows:

$$\Delta G = -RT \ln\left(\frac{1}{K_{tx}}\right) = -RT \ln\left(\frac{k_{cat}/K_m}{k_{non}}\right)$$

$$= RT \ln(k_{non}) - RT \ln\left(\frac{k_{cat}}{K_m}\right) \quad (2)$$

For enzymes that catalyze the same reaction, the k_{non} value is fixed, the catalytic efficiency of these enzymes could thus be compared based on their difference in the binding energy ($\Delta\Delta G$):

$$\Delta\Delta G = \Delta G_2 - \Delta G_1 = RT \ln\left(\frac{k_{cat}}{K_m}\right)_1 - RT \ln\left(\frac{k_{cat}}{K_m}\right)_2$$

$$= RT \ln\left(\frac{(k_{cat}/K_m)_2}{(k_{cat}/K_m)_1}\right) \quad (3)$$

Great effort has been made to develop computational methods that allow reliable estimation of the binding energy for a given catalytic process [10–12]. Among them, molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) is widely used to provide relatively accurate binding energy values at moderate computational cost [13–15]. In addition, the MM/PBSA approach allows for the analysis of individual energy contributions by means of free energy decomposition, which gives additional energetic insights to the system of interest. Therefore, calculation of binding energy based on MM/PBSA could be suitable for the virtual screening.

Herein, we aimed to develop a virtual screening method based on the binding energy of the enzyme-substrate complex in the transition state to facilitate the screening of positive MR mutants towards non-natural substrates. Using *R*-3-chloromandelic acid as a model substrate, the binding energies of variants from the mutant library were calculated and the potential candidates were selected for further experimental investigation. The mechanisms behind the improved catalytic efficiency were studied from the perspective of energy and structure. Furthermore, the screening method was applied to screen positive variants towards two other non-natural substrates (*R*-2-naphthylglycolate and *R*-mandelamide).

2. Materials and methods

R-3-chloromandelic acid and all other reagents, unless mentioned otherwise, were purchased from Sigma–Aldrich Chemical Company. Recombinant mandelate racemase from *Pseudomonas putida* containing the MR open reading frame (ORF) and N-terminal hexahistidine tag was overproduced and purified from *Escherichia coli* strain BL21 cells transformed with a pET-30a plasmid (Novagen, Madison, WI). The enzyme was purified by metal ion affinity chromatography as described in the Novagen protocols. The purity of the enzyme was confirmed by SDS-PAGE (Fig. S1).

2.1. Site-directed mutagenesis

The pET-30a plasmid containing the recombinant MR gene was used as a template for polymerase chain reaction (PCR)-based site directed mutagenesis. Site directed mutagenesis was performed by using the QuikChange™ method (Stratagene, La Jolla, CA). The two synthetic primers used to construct the mutants were listed in Table S1 in the supplementary material. The genes of the mutants were sequenced to verify that no other alterations in the nucleotide sequence were introduced. The mutants were purified using the same procedure as described for the wild-type enzyme.

2.2. Enzyme assay

MR activity was assayed at 25 °C in HEPES-buffer (100 mM, pH 7.5, 3.3 mM $MgCl_2{\cdot}2H_2O$) based on the online measurement of the change of the optical rotation versus time using a Rudolph Research Autopol IV Automatic Polarimeter. Spontaneous racemization under the reaction conditions was checked for all the substrates in the absence of enzyme and was proven to be <3% within 72 h. For all assays, the substrate solutions were incubated at 25 °C prior to the reaction, and the concentrations of substrates ranged from 0.15 to 10 mM. Reactions were initiated by addition of the enzyme to give a final enzyme concentration of 1–100 ng ml^{-1}.

2.3. Data analysis

The values of V_{max} and K_m were determined from plots of the initial velocity (V_i) versus the substrate concentration [*S*]) by fitting the data to Eq. (4) using nonlinear regression analysis. All kinetic parameters were determined in triplicate. The protein concentration was measured by absorption spectroscopy in a UV-2800 UV/Vis Spectrophotometer using the BSA kit (Bio-Rad) and k_{cat} values were obtained by dividing the V_{max} values by the total enzyme concentration ($[E]_t$).

$$V_i = \frac{V_{max}[S]}{K_m + [S]} \quad (4)$$

2.4. Molecular dynamic (MD) simulation

The starting geometry coordinates for the calculation were taken from the 2.2 Å resolution X-ray crystal structure of the MR with the analogue of putative *aci*-carboxylate intermediate, benzohydroxamate (PDB ID: 3UXK) [16]. The *aci*-carboxylate intermediate, taken as the transition state, was located in the active site to match the maximum number of atoms with benzohydroxamate in the X-ray structure. Partial charges and force field parameters of the substrates were generated automatically by the antechamber programme using the general AMBER force field (GAFF) [17,18]. The non-polar hydrogen atoms were added to the enzyme by AMBER 11 simulation package using the ff10 force field. The protonation state of all the residues was set according to pH 7.0, except Glu317, which was protonated to reproduce the hydrogen bond with the carboxyl oxygen atom of substrate. Both the ε-N and δ-N of residue His 297 were protonated in the transition state. Residues 86–101 from the related subunit, which are located around the active site, were added to the protein model [19]. Counterions of Na^+ were added to neutralize the system and the whole system was immersed in a rectangle box of TIP3P water molecules [20], extended 10 Å from the dissolved atoms in all three dimensions. Each MD simulation included minimization, heating, density equilibration at constant temperature, equilibration under constant pressure (NPT) and production run in the NPT ensemble according to our previous work [21].

2.5. MM/PBSA analysis

The calculation and decomposition of binding energy between the ligand and the enzyme was evaluated using the MM/PBSA method as implemented in AMBER 11 with a thermodynamic cycle that combined the molecular mechanical energies with the continuum solvent approach. As many as 100 snapshots were taken after the MD trajectory of enzyme-substrate complex reached a plateau. The binding energy ΔG_{bind} between a ligand (L) and a receptor (R) required for forming a complex (C) was calculated as follows:

$$\Delta G_{bind} = \Delta H - T\Delta S \approx \Delta E_{MM} + \Delta G_{sol} - T\Delta S$$

$$\Delta E_{MM} = \Delta E_{internal} + \Delta E_{electrostatic} + \Delta E_{vdw}$$

$$\Delta G_{sol} = \Delta G_{PB} + \Delta G_{np}$$

where ΔE_{MM}, ΔG_{sol}, $T\Delta S$, stand for the changes of the gas phase MM energy, the solvation free energy, and the conformational entropy, respectively. ΔE_{MM} is the sum of $\Delta E_{internal}$ (bonds, angles and dihedrals energy), $\Delta E_{electrostatic}$ (electrostatic energy), and ΔE_{vdw} (van der Waals). G_{sol}, which accounts for the solvation energy, is divided into the electrostatic solvation energy (ΔG_{PB}) and the non-polar solvation energy (ΔG_{np}). The entropic contributions were excluded due to the little contribution of entropy to the stabilization of the transition state [22], the cancelling out of entropic contribution in the binding energy difference between the wild type and mutants [23–25], and the time-consuming nature of entropy determination [26]. The dielectric constants inside and outside the molecule were 1.0 and 80.0, respectively. "The single-trajectory method" [27,28] was used where the internal energy term ($\Delta E_{internal}$) was zero.

2.6. Statistical analysis

F-test and Student's *t* test were performed to test if the difference between the wild type and the mutants in the binding energy was significant. Based on the mean