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Engineering thermostable (*R*)-selective amine transaminase from *Aspergillus terreus* through *in silico* design employing B-factor and folding free energy calculations

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ABSTRACT

Amine transaminases have recently gained a lot of attention for the synthesis of chiral amines. Using (*R*)-selective amine transaminase from *Aspergillus terreus* (AT-ATA) as a transaminase model, *in silico* design was applied employing B-factor and folding free energy ($\Delta\Delta G^{fold}$) calculations. Mutation sites were selected by targeting flexible regions with the greatest B-factors, and were substituted with amino acids that were determined by folding free energy calculations ($\Delta\Delta G^{fold} < 0$) to be more rigid than the original ones. By site-directed mutagenesis, we obtained four stabilized mutants (T130M, T130F, E133F and D134L) with improved stability from 19 candidates. Compared to the wild type, the best single mutant (T130M) showed an increase in thermal stability with a nearly 2.2-fold improvement of half-life ($t_{1/2}$) at 40 °C and a 3.5 °C higher $T_{1/2}^{10\,min}$. The optimum catalytic temperature of T130F was increased by 10 °C. In addition, the T130M/E133F double mutant displayed the largest shift in thermostability with 3.3-fold improvement of $t_{1/2}$ at 40 °C and a 5.0 °C higher $T_{1/2}^{10\,min}$. Modeling analysis showed that new hydrophobic interactions and hydrogen bonds might contribute to the observed thermostability improvement.

1. Introduction

Chiral amines are important building blocks for the pharmaceutical and fine chemical industries. Transaminases (EC 2.6.1.x), utilizing pyridoxal-5-phosphate (PLP) as a cofactor, catalyze the reversible transfer of an amino group from an amino donor to the carbonyl carbon atom of an α-keto acid, a ketone, or an aldehyde, and are the most suitable enzymes for preparing chiral amines [1,2]. According to their substrate scope, transaminases can be divided into α-, ω-, and amine transaminases (ATAs); ATAs are used for the asymmetric synthesis of enantiomerically pure amines in the pharmaceutical and fine-chemical industries [3–6]. For example, Savile et al. [7] reported an efficient chiral amine asymmetric synthesis by an (*R*)-selective ATA to replace a rhodium-catalyzed asymmetric enamine hydrogenation to manufacture antidiabetic sitagliptin. Both (*S*)- and (*R*)-selective ATAs have been found in nature, and (*S*)-selective ATAs are more abundant than the (*R*)-selective enzymes. Höhne et al. [3] used an *in silico* approach to identify novel (*R*)-selective ATAs from the NCBI protein database, based on motif rational assignments. However, few (*R*)-selective ATA thermostability studies have been reported using the promising strategy of rational protein engineering, although it is of great importance to improve (*R*)-selective ATA thermostability.

To minimize the screening effort and time, a protein engineering strategy by rigidifying flexible sites has been proven to be highly effective in increasing protein stability [8–11]. Highly flexible residues, which have less contact with other amino acids, can trigger protein unfolding because of their large thermal fluctuations. Based on the "Wood Barrel" theory, the identification and intensification of flexible residues as the "short board" contributes more to overall stability than stabilization of rigid residues [12]. As a measure of dynamic mobility, B-factor (or B value), which reflects equilibrium fluctuations of each atom, is commonly used to represent flexible sites, and the residues with higher B-factors are more flexible [9,13].

Abbreviations used: AT-ATA, amine transaminase from *Aspergillus terreus*; DMSO, dimethylsulfoxide; ΔG^{fold}, folding free energy; MD, molecular dynamics; RMSF, root mean square fluctuations; MBA, (*R*)-α-methylbenzylamine; SD, standard deviation.

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Candidate residues having the greatest B-factors are selected for mutation and subsequent thermal stability analysis. Saturation mutagenesis is a useful approach to rigidify flexible sites based on library construction and screening, but the size of the library to be screened is still too large. If the three-dimension structure of the protein is available, these flexible sites can be substituted with more rigid amino acids through folding free energy ($\Delta\Delta G^{fold}$) calculations. There is approximately 5–20 kcal/mol free energy difference between the native and denatured states owing to several stabilizing and destabilizing interactions [14]. The thermodynamic stability effects of mutations ($\Delta\Delta G^{fold}$) are computed with the protein design software such as FoldX [15].

Here, we applied X-ray data (B-factors) to study the overall and local flexibility of (*R*)-selective amine transaminase from *Aspergillus terreus* (AT-ATA) as a model enzyme, and folding free energy ($\Delta\Delta G^{fold}$) calculations to design thermostabilizing mutations that rigidified the predicted flexible sites. The predicted stabilizing effects of these mutations were experimentally verified by thermal inactivation. Homology modeling and molecular dynamics (MD) simulations were employed to investigate the structural basis of the improved thermostability.

2. Materials and methods

2.1. Media and reagents

The AT-ATA cDNA sequence containing the *Nco*I and *Xho*I restriction sites was synthesized at General Biosystems (AnHui) Co., Ltd (Chuzhou, China), and the pET28a(+) expression vector (pET28a(+)-AT-ATA) was constructed as a template for site-directed mutagenesis. PrimeSTAR® Max DNA was purchased from Takara Biotechnology (Dalian, China). Services for primer synthesis and DNA sequencing were provided by GenScript Corp (Nanjing, China). *Dpn*I was purchased from Thermo Fisher (Waltham, MA, USA). Kanamycin sulfate, isopropyl-β-D-thiogalactoside (IPTG), tryptone, glucose, and yeast extracts were purchased from Sangon Inc. (Shanghai, China). The Ni-IDA-Sefinose™ resin kit, DNA ladder, and protein marker were purchased from Bio Basic Inc. (Toronto, Canada). PurePlasmid Mini Kit, DNA Clean-up Kit, and SDS-PAGE Gel Kit were purchased from CWBIO, Co. Ltd. (Beijing, China).

2.2. In silico design procedure

We used the crystal structure of AT-ATA (PDB ID: 4CE5) to design a thermostable transaminase [16]. The residues having the highest B-factors, except for the *N*-terminal and *C*-terminal amino acids, were chosen as substitution targets. After analyzing the B-factor profile of the protein, the residues Gly129-Asp134 were predicted to be relatively flexible, since their B-factors were 2.5-fold higher than the average B-factor. The thermodynamic stability changes of mutations were computed using the protein design tool FoldX (version 3.0 beta5.1). $\Delta\Delta G^{fold}$ of mutations were obtained by comparing the energy values of the mutant with those of the wild type, and negative values of $\Delta\Delta G$ indicated that the mutants were more stable.

2.3. Site-directed mutagenesis of AT-ATA

Polymerase chain reaction (PCR)-based AT-ATA site-directed mutagenesis was conducted using a modified QuickChange™ method. All mutagenic primers are shown in Table S1. The reaction mixture contained DNA template (pET28a(+)-AT-ATA, 100 ng), forward and reverse primers (100 μM, 1 μL each), 2x PrimeSTAR Max Premix (25 μL), and autoclaved water to a final volume of 50 μL, and was subjected to the following PCR conditions: 98 °C for 1 min, 30 cycles of 98 °C for 15 s, 55 °C for 15 s and 72 °C for 3 min, and a final extension at 72 °C for 10 min. The PCR products were treated with *Dpn*I for 3 h at 37 °C to remove the parent plasmid, purified with a PCR purification kit, and used to transform competent *E. coli* DH5α bacteria by heat shock. The transformed bacteria were spread on a Luria-Bertani (LB) plate containing 50 μg/mL kanamycin and incubated at 37 °C overnight. Plasmid DNA was isolated with the PurePlasmid Mini Kit from several colonies, and the presence of the desired mutations was confirmed by sequencing.

2.4. Protein expression and purification

E. coli BL21 (DE3) transformed with wild-type or mutant pET28a(+)-AT-ATA was grown in LB media at 37 °C until the optical density at 600 nm (OD_{600}) reached a value of 0.6–0.8. The culture was then cooled to 25 °C and protein expression was induced by adding IPTG at a final concentration of 0.5 mM. Following 18 h culture under shaking at 25 °C, the cells were harvested by centrifugation at 6000 × *g* for 8 min at 4 °C, resuspended in buffer A (50 mM sodium phosphate buffer, 300 mM NaCl, 20 mM imidazole, 0.1 mM PLP, pH 8.0), and lysed by sonication at 4 °C in an ice-water bath (output level 300 W, duty time 3 s, interval time 6 s, total time 10 min). The cell extract was centrifuged at 12,000 × *g* at 4 °C for 30 min and the supernatant used for protein purification. Recombinant proteins with *N*-terminal hexahistidine tags were purified from the cell lysate by Ni-affinity chromatography, using the Ni-IDA-Sefinose™ resin kit according to the manufacturer's instructions. Purified proteins were eluted in buffer B (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, 0.1 mM PLP, pH 8.0), and concentrated using an Amicon Ultra-15 centrifugal filter unit with a membrane molecular weight cut-off of 10 kDa (Millipore, Billerica, MA, USA).

2.5. Enzyme activity assay

The activities of wild-type and mutant AT-ATA were determined according to the method of Schätzle et al. [17]. Kinetic spectrophotometric measurements were conducted in UV 96-well microtiter plates at 25 °C at 245 nm ($\varepsilon_{245nm} = 12\ mM^{-1}\ cm^{-1}$) for 5 min using an MD 190 photometer (Molecular Devices, Sunnyvale, CA). The enzyme reaction mixture contained 0.25% dimethylsulfoxide (DMSO), 2.5 mM (*R*)-α-methylbenzylamine (MBA) and 2.5 mM pyruvate in 180 μL phosphate buffer (50 mM, pH 8.0), and an appropriate amount of purified enzyme (20 μL). One unit of activity was defined as the amount of enzyme that produced 1 μmol acetophenone from (*R*)-α-MBA per minute. When determining the K_m for (*R*)-α-MBA ($K_m^{(R)\text{-}\alpha\text{-MBA}}$), AT-ATA substrate concentrations were varied in the range of 0–3 mM of (*R*)-α-MBA with a fixed concentration of pyruvate (2.5 mM); when determining the K_m for pyruvate ($K_m^{pyruvate}$), pyruvate was varied in the range of 0–8 mM with a fixed concentration of (*R*)-α-MBA (2.5 mM). The data were fitted to the Michaelis–Menten equation by nonlinear regression using Origin 7.5 (Northampton, MA, USA).

2.6. Thermal inactivation kinetics of wild-type and mutant AT-ATA

Wild-type and mutant AT-ATA were incubated at 40 °C for different time intervals (0–50 min), cooled on ice for 10 min, and their enzyme activities were assayed at 25 °C, as described above. The activity of the enzyme that was not incubated at 40 °C (0 min) was considered to be 100%. The data were fitted to the equation $y = \exp(-k_d \cdot t)$ and the first-order rate constants (k_d) were determined by nonlinear regression using Origin 7.5. The thermal inactivation half-life ($t_{1/2}$) was defined as the time required for the

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