Design of Motifs Interfacial Interactions by Co-evolved Analysis of D-amino Acid Dehydrogenase for Stability Enhancement

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Abstract

To design D-amino acid dehydrogenase (DAADH) for enhanced stability, the interactions of the subunit interfaces of DAADH were analyzed. Interaction network analysis of DAADH indicated that there are only weak interactions between the A and B subunits. Several co-evolved residue pairs were selected for mutation to enhance interfacial interactions of subunits, and 11 designed MDHs were obtained. DA06 and DA11 were selected for experimental verification for their salt bridges are 1.4 and 1.2-fold of that of DAwild, respectively. DA11 can maintain 93% activity in 80, while it was only 40% for DAwild. Thermostabiliy study indicated the half-life of DA11 was 2-fold of DAwild. Molecular dynamics simulations revealed that the extraordinary stability of the DA11 was due to the formation of extra interfacial salt bridges. The paper provided a strategy of mutations outside the active site of enzyme by co-evolutionary analysis which can reduce the effect of the activity-thermostability trade-off.

1. Introduction

Engineering of proteins to improve their thermostability is an area of intensive research. Robust enzymes are required to withstand industrial process conditions for developing biobricks for synthetic biology. Rational design approaches to engineering thermostability require structural information, but even with advanced computational methods it is challenging to predict with sufficient precision to anticipate the results of a given mutation. Directed evolution is an alternative when structures are unavailable but it requires extensive experiments for the screening of mutant libraries. Novel enzymes has been fueled by the diversification of enzymes for billions of years evolution[1]. Therefore, new design method based on the co-evolution of amino acid residues is proposed [2,3].

D-amino acids are key intermediates for pharmaceuticals and agrochemicals [4]. L-amino acid dehydrogenases dependent on NAD(P)H have been wildly applied for the effectively synthesis of L-amino acids. By contrast, wide application of D-amino acid dehydrogenase (DAADH) has not been achieved due to few DAADHs have been explored from natural resources. Furthermore, compared with L-amino acid dehydrogenases, DAADHs are unstable and low reaction rates[5]. Thermostability is the bottleneck for DAADHs. Therefore, creating a stable and high active DAADH and demonstrated that it is applicable in industry is a challenge[6]. Designed mutations based on the structures analysis of*meso* -DAPDH and DAADH to enhance enzyme activity[7]. Cheng et al.[8] studied the site-saturation mutagenesis of DAPDH from*Symbiobacterium thermophilum* (StDAPDH) and obtain a double-site mutant W121L-H227I, which showed improved activities towards various sterically bulky 2-keto acids.

The development of *in sillco* design of enzyme reduce the experimental costs and time[9].These interfacial interactions are generally regulated by the key residues on the surface of motif of enzyme. Zhu et al.[10] studied the modification of subunit interface of *Leifsonia* alcohol dehydrogenase (LnADH) and yielded the mutant T100R-S148I with thermostability with and catalytic efficiency enhancement. Boucher et al. [11] investigated the structural basis for the hyperthermostability of a FN_3-like protein domain from *Ther-*

moanaerobacter tengcongensis by molecular dynamics simulations. Three arginine residues (R23, R25, and R72), which stabilize the protein by salt bridges were identified and mutated into alanine, which reduced melting temperature from 97.5 to 22 °C.

How to quickly define the key interficial amino acid residues which greatly effect stabilitiy of enzyme is crticial for protein engineering. Co-evolution analysis can narrow down the selection range of mutated sites, and enhance the successful rate based on the natural evolved selection. Conservation and co-evolution analysis indicated the interfacial interaction of domains and subunits are key factors behind enzyme's stability [12,13]. A limited number of co-evolved residues located at the oligomerization interfaces, which are expected to highlight most key sites involved in their thermostability[14]. Wang et al.[15] redesigned the interactions of the subunit interfaces of glycerol dehydratase from *Klebsiella pneumoniae.* Based on evolutionary analysis, three co-evolved residue pairs were selected for mutation to enhance subunits interfacial interactions. The design of salt bridges are considered to be an easy and efficient ways to assembly motifs, domains and subunits. Kleiner et al.[16] found that the interdimeric interface of methionine S-adenosyltransferase from *Ureaplasma urealiticum* is subject to rapid evolutionary changes. The indel loop affects the release pathway for *meso* -DAP and pyruvic acid and *meso* -DAP[17] . Insights from co-evolution perspective can define the key residues the interaction hotspots across the interfacial of subunits[18]. Therefore, the novel strategy combined with co-evolution and interfacial interaction network analysis is developed.

D-amino acid dehydrogenase (DAADH) from *Ureibacillus thermosphaericus* was selected for thermostability enhancement study (Scheme 1). Redesign of interfacial interactions of domains and subunits by integrating interfacial interaction network analysis with co-evolution analysis (Fig. 1). Several novel NADPH-dependent DAADHs were designed by multi-point mutations for interfacial interaction enhancement. Experiments were carried out to verify the design and reveal the mechanism of enhancement of thermostability.

Scheme.1 Reductive amination of phenylpyruvate catalyzed by DAADH

2. Materials and methods

2.1. Structures

The X-ray diffraction structure of D-amino acid dehydrogenase of *Ureibacillus thermosphaericus* was retrieved from the Protein Data Bank (PDB). In this work, the crystal structure of the DAADH (PDB code 5gz6) was employed for amino acid residues interaction analysis and molecule dynamic simulation.

2.2. Amino acid residues interaction analysis

The web server of Protein Contacts Atlas(http://www.mrc-lmb.cam.ac.uk/pca/) was applied for analysis of non-covalent contacts, such as hydrogen bonds, hydrophobic interactions, cation-pi interactions, salt bridges, and etc. The default threshold of the atoms' distance for interaction calculations is set as 0.5 Å. Multiple non-covalent contacts at different scales of structure, namely, amino acid residues, secondary structure, subunits, and entire complexes were analyzed[19]. Protein side-chain networks (PScN) is then constructed by setting amino acid residues as nodes. The edges are constructed between the nodes based on non-covalent interactions, side-chain interaction between them.

2.3. Co-evolution and conservation and analysis

The calculation of co-evolved residue pairs was based on Evcoupling webserver. Evcoupling can calculate co-evolution score for each residues in the corresponding protein family[20]. The co-evolution score reflect a network of interactions between residue pairs based on the evolutionary information in the family[21]. Conservation analysis was carried out by the ConSurf webserver (http://consurf.tau.ac.il/2016/) with HMMER E-value was set as 0.0001.

2.4. Thermodynamic parameters calculation

Thermodynamic parameters, such as change of enthalpy(ΔH_m), change of Gibbs free energy of stability

(ΔG_r), change of heat capacity (ΔC_p) and melting temperature (T_m) were calculated by the SCooP webserver, which is freely available at http://babylone.ulb.ac.be/SCooP.

2.5. Chemicals

Kanamycin and isopropyl-β-D-thiogalactopyranoside (IPTG) are purchased from Trans-Gen Biotech (Shanghai, China). NADPH and phenylpyruvate are purchased from Sigma Chemical Company (Tianjing, China). The ionic liquids (99% purity) are purchased from Sigma Chemical Company (Tianjing, China). All other chemicals are analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

2.6. Enzyme expression and purification

The daadh gene encoding the wild type D-amino acid dehydrogenase (DAADH) was synthesized by Sangon Biotech (Shanghai) Co. Ltd. DAADH originates from Ureibacillus thermosphaericus (PDB ID: 5gz6) was selected. An expression vector for DAADH_5gz6 with polypeptide linker (amino acid sequence (RTHRK)4) were constructed by amplifying the DAADH_5gz6 gene fragment infusion by PCR.

The amplified gene fragment was double-digested with NdeI and XhoI and ligated cloned into plasmid pET-28a(+), with the expression vector E.coli BL21(DE3)/pET28a. The plasmid extraction and gel recovery kit (Takara) was used. Cells were cultivated at 37 °C and 200 r/min in 10 mL of LB medium containing 50 μg/L kanamycin until the optical density (OD) at 600 nm reached 0.6-0.8. Then, gene expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) for an additional 12 h at 25 °C.

The harvested cells were washed twice with 20 mM Tris-HCl buffer (pH 7.0) and suspended in a buffer of 50 mM Na_2HPO_4 (pH 7.0). Suspended cells were disrupted by ultrasonication and centrifuged at 100,000×g for 1 h. The supernatant was purified using a Ni^{2+}-NTA column (Seven sea biotech, Shanghai, China) to obtain samples for activity assay.

2.7. Enzyme activity assay

The activities of DAADHs were determined in NH_4Cl-$NH_3{\cdot}H_2O$ buffer (200 mM, pH 9.5) with phenylpyruvate and NADPH. Enzyme activity was measured by detecting the NADPH concentration at 340 nm by Infinite 200 PRO spectramicroplate reader (TECAN). The initial velocities was calculated as consumption rate of NADPH when the conversion rate was below 5%. The amount of enzyme consumed for generation or consumption of 1 μmol of NADPH per minute was defined as one unit of enzyme activity. Specific activity (U/mg) of DAADH is defined as the ratio of enzyme activity (U/mL) to enzyme concentration (mg/mL). Each data was obtained by five parallel experiments.

D-phenylalanine was analyzed by HPLC. HPLC analysis with standard solution of L-phenylalanine and D-phenylalanine showed that the peak time of L-phenylalanine was 25 min, and the peak time of D-phenylalanine was in 33 min. HPLC detection condition for D/L phenylalanine was as follow: Chromatographic column: Philomon Chirex 3126 (D)-Penicillamine 250 nm*4.6 nm, 5 μm; mobile phase, 5% (v/v) acetonitrile and 95% (v/v) 2.0 mM, $CuSO_4$ aqueous solution; flow rate, 0.8 mL/min; detection wavelength, 254 nm[22].

3. Result and discussions

3.1. Interfacial interaction analysis

In an effort to redesign DAADH for improved stability, interfacial interactions of A and B subunits of DAADH from *Ureibacillus thermosphaericus* (DAwild) were analyzed by Protein Contacts Atlas. Amino acid residues interaction analysis indicated that there are only weak interfacial interactions between the A and B subunits, which are only one salt bridges and 12 hydrogen bonds. The salt bridge was form by GLU136 of A subunit and LYS316 of B subunit with the distance of 3.177 Å (Fig. 2A). Interactions heatmap of Helix 15 and 16 of A subunit and Helix 23 of B subunit were investigated and key residues were highlighted (Fig. 2B). Therefore, the interfacial interactions of subunits and motifs can be further improved to enhance the stability of the enzyme [23].

3.2. Define key residues by conservation and coevolution analysis

Conservation scores were calculated for each residue of DAADH using ConSurf webserver (Fig. S1). Conservation analysis indicated that key residues which maintain the interfacial interactions are more conserved than others during the evolution (Table 1) [24].

Evolutionary coupling reflect the network of interactions between residue pairs based on their evolutionary information in the family[13]. Several co-evolved residues pairs are identified by co-evolution analysis of dimeric interfacial interactions (Table 2). Co-evolutionary analysis of protein residues can reveal the underlying remote interactions of amino acid residues (Fig. 3A). Therefore, several co-evolved residues pairs which are selected for mutation to enhance the subunits interaction (Fig. 3B).

3.3. Design of interfacial interactions

Double mutations of V137I-S314L, F306H-G311I, L134W-F310I were designed to enhance the hydrogen bonds and salt bridges, which aim to promote structure rigidity of enzyme. Calculation of mutation energy was carried out by the webserver Calculate Mutation Energy of Discovery Studio 2016 Client. All mutation energy of double mutated enzymes was negative, which indicated the enhanced thermostability. Therefore, positive mutations were further combined and the designed enzymes DA06 to DA11 were obtained. None of mutated sites is located within active sites, which would not arise activity change from binding substrate. Mutation energy for the combined mutations indicated the cooperative effect on the improvement of thermostability (Table 2).

Thermodynamic parameters of all designed enzymes were calculated by Scoop and were listed in Table 3 [25]. Parameters such as change in enthalpy (ΔH_m), change in heat capacity (ΔC_p), melting temperature (T_m) and difference in Gibbs free energy of stability (ΔG_r), were calculated. These thermodynamic parameters which have important implications for enzyme thermostablility[26].

3.4. Energy and interaction analysis of designed enzymes

Molecular dynamics simulations (MDS) of designed enzyme were carried out. Energy decomposition analysis of mutation was studied by data obtained from MDS. Co-evolved residues pairs were mutated in order to form new and strong salt bridges. Electrostatic interaction energy and total interaction energy was calculated for further selection of mutated pairs (Table 4), which indicated that electrostatic interaction energy contribute to the most part of interaction energy. Buried interactions network can enhance interfacial interactions of subunits and enzyme stability [27].

The benefits of considering dynamic conformation change for enzyme design can also enable engineering enzymes from the prospect of both structure and function[28]. The structure of the enzyme undergoes dynamic conformational adjustments with increasing temperature, resulting in gradual loosening of the unstable secondary structure and consequently disfolding. The introduction of interfacial hydrogen bonds contributes to the thermostability of the enzyme[29]. Energy provided by each hydrogen bonds is about 0.6 kcal/mol [30]. MDS also indicated that the average number of hydrogen bonds of wild-type DAwild is 108. Therefore, there are 8 extra hydrogen bonds of DA11 than DAwild, which provided extra energy barrier for unfolding (Table 5).

The average number of salt bridges of DAADHs were calculated by MDS result with 0.1 M NaCl. Salt bridges networks play an important role in maintain the rigidity of the three-dimensional structure of enzyme. Formation of more salt bridges inside the protein molecular is beneficial to maintain the structural stability[31]. The average number of salt bridges of DA06 and DA11 are 1.4 and 1.2-fold of that of DAwild, respectively. This result indicated that increased salt bridges and hydrogen bonds enhanced structural rigidity and improved thermostability.

Therefore, DA06 and DA11 were selected for further experiment study. The formation of a large number of non-covalent interactions within the modified enzyme is beneficial to the thermostability, which has been strengthened by multipoint mutations.

3.5. Experimental verification by mutation

Activity of mutations were tested. The activity of DAwild and DA11 at 25 °C were 2.46 and 2.47 U/mg, respectively, while the activity of DA06 was only 0.27 U/mg. This result indicated the DA11 of interfacial interactions would not affect the enzyme activity. Normally, engineering enzyme with improved the rigidity for thermostability enhancement cause the decrease of activity. Multi-site mutation for enzyme activity improvement can affect the stability, which is a compromise between enzyme activity and stability [28].

Interaction analysis indicated that the 12 mutations of DA06 (L133W-V136I-F305C-F309I-G310W-S313Q) led to the formation of salt bridges between 346LYS-408GLU, 349LYS-375GLU, 349LYS-408GLU (Table S2). New formed salt bridges elucidated the mechanism of a key amino acid residue mutation enhanced thermostability [17]. However, to much extra salt bridges and hydrogen bonds of DA06 decreased enzyme activity due to the effect of activity-stability trade-off by enhanced the rigidity[32].

Effect of pH on DAADHs was studied. Among them, DA11 is with the widest adaptable pH range, especially in alkaline solutions (Fig. 4a). Catalytic properties the designed enzyme were verified. The effect of temperature on DAwild, DA06 and DA11 activity was studied in the range of 30 to 80 °C. The optimal temperature for DA06 and DA11 was 60 °C, while it was only 50 °C for DAwild (Fig. 4b). DA11 can maintain 93% activity in 80 °C, while it was only about 40% for DAwild. This result indicated the interifical mutations of subunits enhanced thermostability of DA11 without decreasing the activity.

3.6. Thermostability study

Thermostability of DA11 and DAwild was studied under 50 (Fig. 5). The deactivation kinetic parameters was obtained, and the half-life of DA11 was 11.2 h, almost 2-fold of DAwild (Table S1). Thermostability of DA11 was further studied under 60, 70, 80 (Figure S2). DA11 retained 52% activity under 80 after 10 h. The result indicated interfacial interactions enhancement greatly improved thermostability. Mutations of DA11 on the subunit interface were marked in Fig. 6. MDS result indicated the salt bridges formed between Lys300-Glu482, Lys341-Asp407, Arg324-Asp407, Lys346-Asp410, Lys439-Glu375, Arg351-Asp410 indicated the enhanced thermostability by interfacial interactions of subunits of DA11(Table S3). Therefore, co-evolutionary analysis are sufficient to identify higher-than-pairwise mutation patterns of DAADH, which can both increase the activity and thermostability.

3.7. Kinetic study of DAADH

Kinetic parameters of the DAwild and DA11 were calculated by Hanes-Woolf method within the phenylpyruvate concentration range of 0.625-10 mM, as shown in Fig. S3. V_{max} of DAwild and DA11 are all 0.5 mM/min, while DA11 showed a decreased K_m value as 1.00 mM (Table 6). This result indicated that the DA11 increased the affinity with substrate, and further improved enzyme catalyzed efficiency. Interfacial mutation which are outside the active sites can improved stability without decrease the enzyme activity, which can reduce the trade-off effect of activity and thermostability.

3.8. Effect of ionic liquids on the enzyme activity

Ionic liquids (ILs) is with industrially attractive advantages for biocatalysis due to their tunable chemical properties. Several ionic liquids ([EMIM]BF_4, [BMIM]Cl, [BMIM]BF_4) were selected and added into the reaction system with final concentration as 5 mM (Fig. S4). The activity of DA11 in [BMIM]BF_4 ionic liquid was 97.8%, while [EMIM]BF_4 and [BMIM]Cl reduced the relative enzyme activity of DA11. It is reported that $[BMIM]^+$ cations can strip off essential water molecules from the enzyme surface, which cause the decreased enzyme activity [33]. Ionic liquids also can affect enzyme activity through direct interaction with the enzyme and by altering the microenvironment of enzyme. Due to the conductivity and hydrophobicity, ILs are widely applied for electrode modification to obtain high heterogeneous electron transfer rates [34]. Therefore, ILs tolerant DA11 is promising for its application in biosensor.

4. Conclusion

To improve the thermostability of DAADH, several co-evolved interfacial residue pairs were identified. Based on the amino acid residue interaction network and co-evolutionary analysis, those residues were mutated for redesign the interfacial interaction of subunits. Eleven novel DAADHs were obtained. Mutations induced new salt bridges and hydrogen bonds. The average number of salt bridges of DA06 and DA11 are 1.4 and 1.2-fold of that of DAwild, respectively. Thermostability study indicated the half-life of DA11 was 2-fold of DAwild. MDS was applied to uncover the mechanism of enhanced thermostability from molecular level. These findings suggest that the dimer interface motifs are essential for the compactness and stability of DAADHs and may illuminate design based on evolution in other enzyme families.

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Abbreviations

DAADH, D-amino acid dehydrogenase; **MDS,** Molecular dynamic simulation; **RMSD,** Root mean-square deviations;**RMSF,** Root mean-square fluctuations; **PDB,** Protein Data Bank.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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