Engineering of a thermophilic dihydroxy-acid dehydratase to enhance its dehydration ability on glycerate to pyruvate and its application in in vitro synthetic enzymatic biosystems

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Abstract

The low activity of dihydroxy-acid dehydratase (DHAD) on dehydration of glycerate to pyruvate hampers its applications in the biosystems. Protein engineering of a thermophilic DHAD from Sulfolobus solfataricus (SsDHAD) was performed to increase its dehydratation activity. A novel high-throughput method was established. A triple-mutant (I161M/Y145S/G205K) with a 10-fold higher activity on glycerate dehydration was obtained after three rounds of iterative saturation mutagenesis (ISM) based on computational analysis. The shrunk substrate-binding pocket and newly formed hydrogen bonds were the reason for the activity improvement of the mutant. For the in vitro synthetic enzymatic biosystems of converting glucose or glycerol to L-lactate, the biosystems with the mutant SsDHAD showed 3.32- and 2.34-times of the reaction rate than that of wild type, respectively. This study demonstrates the potential of protein engineering to improve the efficiency of in vitro synthetic enzymatic biosystems by enhancing the enzyme activity of rate-limited enzymes.

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Running title : dehydration ability improvement of SsDHAD

Abstract

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(SsDHAD) was performed to increase its dehydratation activity. A novel high-throughput method was established. A triple-mutant (I161M/Y145S/G205K) with a 10-fold higher activity on glycerate dehydration was obtained after three rounds of iterative saturation mutagenesis (ISM) based on computational analysis. The shrunk substrate-binding pocket and newly formed hydrogen bonds were the reason for the activity improvement of the mutant. For the in vitro synthetic enzymatic biosystems of converting glucose or glycerol to L-lactate, the biosystems with the mutant SsDHAD showed 3.32- and 2.34-times of the reaction rate than that of wild type, respectively. This study demonstrates the potential of protein engineering to improve the efficiency of in vitro synthetic enzymatic biosystems by enhancing the enzyme activity of rate-limited enzymes.

Keywords: dihydroxy-acid dehydratase, protein engineering, glycerate, dehydration, in vitro synthetic biosystem

1. Introduction

Dihydroxy-acid dehydratase (DHAD, EC 4.2.1.9) catalyzes the dehydration reactions of many carbohydrates such as 2,3-dihydroxyisovalerate, glycerate, gluconate, arabonate, xylonate, ribonate, and so on (Kim & Lee, 2006). This enzyme is involved in the pathways for biofuels and various chemicals production via in vitro biosystem (Carsten, Schmidt, & Sieber, 2015). DHAD has highly substrate promiscuity, being capable of converting a broad range of dihydroxy acids with obviously different activity level (Kim & Lee, 2006). For example, a thermostable DHAD from hyperthermoacidophilic archaeon *Sulfolobus solfataricus* (SsDHAD) exhibits high activity on converting 2,3-dihydroxyisovalerate to 2-oxoisovalerate (47 U/mg) (Carsten et al., 2015; Kim & Lee, 2006; Sperl, Carsten, Guterl, Lommes, & Sieber, 2016) and very low activity on converting glycerate to pyruvate (8 mU/mg). The extreme low activity of DHAD on glycerate hinders the production of bio-based products and biofuels by many in vitro synthetic enzymatic biosystems based on non-phosphorylative Entner Doudoroff (np-ED) pathway (Ahmed et al., 2005; Xie et al., 2018). Even though the dehydratation activity of SsDHAD was improved by 1.5-times through non-rational design protein engineering (Begander, Huber, Döring, Sperl, & Sieber, 2020), still cannot meet the requirement of further application.

Protein engineering, as an excellent strategy in the enzyme properties, can be divided into non-rational design, rational design, and semi-rational design according to the methods used for building mutant libraries (Debon et al., 2019; Packer & Liu, 2015). Semi-rational design is currently the most widely applied route because it takes advantage of sequence space and screening scale (Cheng, Zhu, & Schwaneberg, 2015). For this strategy, several hot-spot sites were selected by structure analysis, then the iterative site-saturated mutagenesis (ISM) on these sites followed by library screening was performed for improving the enzyme properties. .

In this study, the activity of SsDHAD on glycerate was sought to improve by the means of structure-guided protein engineering. Herein, guided by structural information from the homology model of SsDHAD, a triple-mutant (I161M/Y145S/G205K) with 10-times higher activity on glycerate in comparison to the wild type SsDHAD was obtained. Then this mutant was used in two in vitro synthetic enzymatic biosystems for converting glycerol and glucose to L-lactate in one pot. Under the same enzyme loading amount of SsDHAD, the reaction rates and product yields of in vitro biosystems containing the SsDHAD mutant for converting glycerol or glucose to L-lactate were much higher than that of the wild type SsDHAD. This study demonstrates the potential of protein engineering to improve the efficiency of in vitro synthetic enzymatic biosystems by improving the properties of rate-limited enzymes.

2. Materials and Methods

2.1 Chemicals and materials

All chemicals were reagent grade or higher quality and purchased from Sigma-Aldrich (St. Louis, MO, USA), Aladdin (Shanghai, China), and Sinopharm (Beijing, China) unless specified. PrimeSTAR DNA polymerase was purchased from Takara (Shiga, Kusatsu, Japan). Restriction enzymes, T4 DNA ligase were purchased

from New England Biolabs (Ipswich, MA, U.S.). Kits for DNA purification and plasmid extraction were purchased from Tiangen (Beijing, China). Catalase from *Aspergillu niger* was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Strains, plasmids, and media

Escherichia coli BL21 (DE3) was used as the host for recombinant protein expression and mutant library construction. Luria–Bertani (LB) medium supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin was used for *E. coli* cell growth and recombinant protein expression.

The plasmids pET28a(+)-SsDHAD containing the SsDHAD encoding gene*dhad* from *S. solfataricus* (Genebank accession number: AKA78631.1), pET28a(+)-TaALDH containing the TaALDH encoding gene*aldh* from *Thermoplasma acidophilum* (Genebank accession number: CAC11938.1), pET28a(+)-SsGDH containing the SsGDH encoding gene*gdh* from *S. solfataricus* (Genebank accession number: SSO3003), pET28a(+)-SaKDGA containing SaKDGA encoding gene *kdga*(Genebank accession number: Saci0225), pET20b-TmLDH containing the TmLDH encoding gene *l-ldh* from *Thermotoga maritima* (Genebank accession number: TM1867), and pET20b-TsFDH containing the TsFDH encoding gene *fdh* from *Thiobacillus* sp. KNK65MA (Genebank accession number: fdh65MA) were obtained from the previous works (Song et al., 2019; Xie et al., 2018). The plasmid pETduet-SsALDO containing the ScALDO encoding gene *aldo* from *Streptomyces coelicolor* A3 (Genebank accession number: NP_630252) was constructed as described elsewhere (Li et al., 2018).

2.3 Homology modeling and docking analysis

The homology modeling of the wild-type SsDHAD was constructed by I-TASSER suite (Bashiri et al., 2019). Out of 10 top-ranked models, PDB code 5J85 (Rahman et al., 2017), shares the highest sequence identity and TM-score with SsDHAD was selected as the template (Table S1). The [2Fe-2S] cluster and Mg^{2+} ion coordinating residues are well conserved between the enzymes in IlvD/EDD family (Rahman, Andberg, Koivula, Rouvinen, & Hakulinen, 2018). Schrödinger Maestro software (Suite, 2015) was adopted to prepare the ligand structures of glycerate and [2Fe-2S] cluster. Glycerate and [2Fe-2S] cluster were docked to the pockets by Rosetta program (Richter, Leaver-Fay, Khare, Bjelic, & Baker, 2011), with the following command: rosetta_scripts.linuxgccrelease -in:file:s "protein.pdb ligand.pdb" -extra_res_fa ligand.params -out:file:scorefile dock.sc -score:weights ref2015 -ex1 -ex2 -ex1aro:level 4 -no_optH false -flip_HNQ true -parser:protocol "ligand_dock.xml" -nstruct 1000 -overwrite. The setting is in the ligand_dock.xml file which is derived from the previous study (Combs et al., 2013). After docking, the protein-ligands complex was relaxed/repacking in two rounds according to the published protocol (Park et al., 2016). The model yielded in the first round with the best score was chosen as the input to undergo the second minimization, using the following command: relax.linuxgccrelease -in:file:s <complex PDB file> -extra_res_fa <ligands parameter files> -relax:constrain_relax_to_start_coords -ramp_constraints false -relax:coord_constrain_sidechains -nstruct 1000 -ex1 -ex2 -use_input_sc -in:auto_setup_metals. The generated top 10 scored poses of the enzyme were then individually calculated the active site volume by using POVME 3.0 (Wagner et al., 2017).

2.4 Molecular dynamics (MD) simulations

The initial structure of the SsDHAD mutant was generated via the PyMol program (http://www.pymol.org) based on the wild-type model. Apo structures of wild type and the mutant enzymes were then protonated at pH 7.5 (to mimic the experimental conditions) by using H++ webserver (Anandakrishnan, Aguilar, & Onufriev, 2012). The Amber ff14SB force field (Maier et al., 2015) was employed for the protein, solvating with TIP3P water model (Jorgensen, Chandrasekhar, Madura, Impey, & Klein, 1983) up to 10 Å from any point on the protein surface. Explicit counterions (Na^+ and Cl^-) were added to neutralize the total charge of the system. Minimization of the resulting system was performed in two stages (5,000 steps for steepest conjugate and 5,000 steps for conjugate gradient) to remove steric clashes. The system was then annealed from 0 to 310 K ([?]37°C) under NVT conditions for 50 ps, and was maintained for another 50 ps of density equilibration under NPT conditions at a temperature of 310 K and pressure of 1.0 atm using Langevin-thermostat (ntt=3) with a weak restraint of 10 $kcal \cdot mol^{-1} \cdot A^{-2}$ on the protein residues. The system was

further equilibrated for 1 ns after the removal of all restraints. Subsequently, a productive MD run of 100 ns was performed for wild type of SsDHAD and R3 mutant. During all MD simulations, the covalent bonds containing hydrogen were constrained using SHAKE algorithm (Ryckaert, Ciccotti, & Berendsen, 1977) with a MD time-step of 1 fs. The trajectory file was written every 100 steps. All above MD runs were performed with GPU version of Amber16 package (Case et al., 2016).

2.5 Iterative saturation mutagenesis

To establish a mutant library, linearized pET28a(+)-SsDHAD was generated by PCR using the plasmid pET28a(+)-SsDHAD as a template. The primers used to introduce saturation mutagenesis at specific positions are listed in Table S2, where each selected amino acid site was randomized using NNK degeneracy. The PCR reaction solution with a total volume of 50 μL containing 3 ng/μL plasmid pET28a-DHAD, 0.05 U/μL the PrimeSTAR DNA polymerase and 0.4 μM primer pairs. The PCR reaction was conducted as follows: initial denaturation (98 °C for 3 min); 30 cycles of denaturation (98 °C for 30 s), annealing (60 °C for 30 s) and extension (72 °C for 1 min); and a final extension (72 °C for 10 min).

PCR products were analyzed on agarose gel, digested by DpnI, and purified through a DNA purification kit (Tiangen, Beijing, P. R. China). The linearized pET28a(+)-SsDHAD (20 ng/μL) were phosphorylated with 0.2 U/μL T4 polynucleotide kinase, 5 mM ATP, at 37 °C for 30 min, followed by ligation with T4 Quick Ligase at 25 °C for 20 min, transformation into *E. coli* BL21 (DE3) competent cells, and spread on LB agar plates containing 50 μg/mL kanamycin and grown overnight at 37 °C.

2.6 Screening for the improved activity of SsDHAD

The colonies of *E. coli* BL21 (DE3) carrying the SsDHAD mutants from LB plates were cultured in 96 deep-well plates with LB medium containing 50 μg/mL kanamycin (0.7 mL per well) at 37°C for overnight. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added with a final concentration of 100 μM and the cultivation temperature was decreased to 16 °C for ˜16 h. The cell pellets were harvested and washed with 300 μL 100 mM HEPES buffer (pH 7.5). The cell pellets in each well were resuspended in 300 μL 100 mM HEPES buffer (pH 7.5) containing 1 mg/mL lysozyme, and incubated at 37 °C with shaking for 1 h to lyse the cells. The cell debris was removed by centrifugation at 4 °C. 90 μL of the cell lysis supernatant was transferred into a 96-well plate, and mixed with 30 μL the reaction mixture in 100 mM HEPES buffer (pH 7.5), containing 10 mM glycerate, 5 mM $MgCl_2$ in final concentration, and incubated at 70°C for 1 h for the dehydration of glycerate. The generated pyruvate was analyzed with the modified method of 2,4-dinitrophenylhydrazine (Flint, Emptage, Finnegan, Fu, & Johnson, 1993). 30 μL mixture was mixed with 30 μL 2,4-dinitrophenylhydrazine solution (in 2 M HCl), followed by mixed with 150 μL 1.5 M NaOH solution successively. The absorbency increase at 520 nm due to pyruvate formation was measured. These colonies with higher absorbency at 520 nm were picked out, followed by an analysis of the activity toward glycerate. Finally, the mutants with enhanced activity toward glycerate were selected.

2.7 Protein expression, purification, and determination of specific activity and kinetic parameters

The SsDHAD and the mutants were expressed at 16 °C for 20 h in 50 mL LB medium containing 50 μg/mL kanamycin as described above. The cells were lysed by ultrasonication. After centrifugation at 10,000 g for 10 min at 4 °C, the supernatant of cell lysate was heated at 70 °C for 20 min for purification. SDS-PAGE analysis (12% acrylamide) was carried out for checking the successful purification of enzymes.

The dehydration activity on glycerate for SsDHAD was assayed in the tube at 70 °C in 100 mM HEPES buffer (pH 7.5) containing 5.0 mM $MgCl_2$, 10 mM glycerate, and a certain amount of enzyme. The formation of pyruvate was determined as described in the 2.6 section in Methods. An increment in absorbance at 520 nm due to the formation of pyruvate was measured by a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of product per minute. Unless otherwise stated, each measurement was conducted in triplicate.

The activity of SsDHAD toward gluconate was assayed at 50 °C. The activity assay system containing

100 mM HEPES buffer (pH 7.5), 5 mM $MgCl_2$, 20 mM gluconate, 0.27 mM NADH, 1 U/mL SaKDGA and 1 U/mL TmLDH. The generated 2-keto-3-deoxygluconate was converted into L-lactate by SaKDGA and TmLDH. The reduction in absorbance at 340nm due to the decrease of NADH was measured by a spectrophotometer (ε_{NADH}=6.22 mmol/$L^{-1}cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme that consumed 1 μmol of NADH per minute.

Thermostability studies of SsDHAD toward glycerate were carried out with 5 mg/mL of enzyme incubated in a water-bath at 70 °C for 30 min to 12 h and were kept on ice for 20 min before enzyme assay.

The effects of substrate (glycerate) concentration (0.1-6 mM solution) on the ration rate of SsDHAD and the mutants were analyzed by measuring the released pyruvate. The kinetic parameters ofK_m and k_{cat} andk_{cat} /K_m were obtained by performing a nonlinear regression analysis of the Michaelis-Menten equation using SigmaPlot 10.0. The parameter $k_{cat}$was calculated according to the following equation:k_{cat} =Vmax/(E), in which (E) means the molar concentration of the enzymes.

2.8 One-pot biosynthesis of L-lactate from glycerol or glucose

One-pot biosynthesis of L-lactate from glycerol was conducted in a 1.0 mL reaction system containing 100 mM HEPES (pH 7.5), 12 mM glycerol, 5.0 mM NADH, 5.0 mM $MgCl_2$, alditol oxidase (ScALDO, 0.3 U/ml), glyceraldehyde dehydrogenase (TaALDH, 0.3 U/ml), catalase from*A. niger* (1000 U/ml), L-lactate dehydrogenase (TmLDH, 0.3 U/mL), formate dehydrogenase from *Thiobacillus* sp. KNK65MA (TsFHD, 5 U/mL), and equal protein quality loading of SsDHAD (0.01 U/mL, 0.33 mg/mL) or R3 mutant (0.1 U/mL, 0.33 mg/mL), at 50 °C (**Table 1**).

One-pot biosynthesis of L-lactate from glucose was conducted in a 1.0 mL reaction system containing 100 mM HEPES (pH 7.5), 10 mM glucose, 5.0 mM NAD^+, 5.0 mM $MgCl_2$, glucose dehydrogenase from *S. Solfataricus* (SsGDH, 0.3 U/mL), 2-keto-3-desoxy gluconate aldolase from *S. acidocaldarius*(SaKDGA, 0.3 U/mL), glyceraldehyde dehydrogenase *Thermoplasma acidophilum* (TaALDH, 0.3 U/mL) from, L-lactate dehydrogenase from*T. maritima* (TmLDH, 0.3 U/mL), wild type SsDHAD (0.1 U/mL, 1 mg/mL) for conversion of gluconate to 2-keto-3-deoxygluconate and equal enzyme quality loading of SsDHAD (0.1 U/mL, 0.33 mg/mL) or R3 varient (0.01 U/mL, 0.33 mg/mL) at 50 °C (**Table 1**).

An aliquot (65 μn) of the reaction sample was withdrawn and mixed with 35 μL of 1.88 M $HClO_4$ to stop the reaction. The pH value of the reaction solution was then adjusted to neutral with 13 μL of 5.0 M KOH. The supernatant of the reaction mixture was analyzed with high-performance liquid chromatography (HPLC) (Shimadzu, Japan) equipped with a refractive index detector, and a Bio-Rad HPX-87H column with 5.0 mM H_2SO_4 as a mobile phase.

3. Results and Discussion

3.1 Molecular modeling and rational design

SsDHAD, posses a extremely low activity toward glycerate, shares relative high structural similarities with other IlvD/EDD family enzymes (**Table S1**), a homology model of SsDHAD was constructed by using the crystal structure of a bacterial L-arabinonate dehydratase (PDB code 5J85, 37.5% identity with SsDHAD, 0.98 TM-score, **Table S1**) as a template. After several rounds of homology modeling and molecular docking based on computer algorithms, a reliable 3D model of SsDHAD with [2Fe-2S] cluster and glycerate inside was constructed, where the [2Fe-2S] cluster and glycerate bound in the reactive pose were situated in the cofactor binding pocket and substrate binding pocket, respectively (**Figure 1a**). On account of previous study that the mutagenesis of residues for binding [2Fe-2S] cluster could dramatically affect enzyme activity (Carsten et al., 2015). Thus, a total of ten hotspots, including the three residues (Y145, G147, and T194) at the entrance of [2Fe-2S] binding pocket, and the four residues (G122, A201, N202, and G205) inside of the pocket together with the three residues (Y93, I161, and H165) lining the glycerate binding pocket, were selected for site-saturation mutagenesis (**Figure 1b**), to indirectly enhance the activity by altering the interactions between SsDHAD and [2Fe-2S] cluster.

3.2 Site-saturation mutagenesis for enhancing activity

The procedure of protein engineering of SsDHAD and the high-throughput screening method was developed as shown in **Figure 2a** . The cells culture and cells lysis procedure for *E. coli* BL21 (DE3) cells harboring SsDHAD mutant library were described in the Method section. The cell lysate and glycerate were incubated at 70 °C for one hour for the dehydration of glycerate to pyruvate. The concentration of generated pyruvate was determined by a method based on the chemical derivatization of pyruvate into colorant products. The concentrations of up to 4 mM pyruvate were proportional to the values of absorbance at 520 nm (**Figure S1a, S1b**). The screended mutants with a higher absorbance reading, was identified gene sequencing, enzyme purification, and characterization. It was believed that the developed screening protocol and the glycerate assay method can be applicable to other dehydratase type assays.

ISM experiments were carried out on the 10 amino acid residues (Y93, H165, I161, A201, N202, G122, Y145, G205, G147, and T194). In the first round of ISM, the three residues Y93, H165, and I161 situated at the substrate-binding pocket were initially investigated. After screening these three site-saturated mutant libraries, only one mutant R1 (I161M) showed higher activity than the wild type (**Figure 2b**). After purification, the R1 mutant displayed a 4.7-fold increase in specific activity (155 mU/mg) than the wild-type SsDHAD (33.1 mU/mg) (**Table 2**). In the second round of ISM, three site-saturated mutant libraries regarding these three residues (Y145, G147, and T194) at the entrance of [2Fe-2S] cluster were built based on I161M mutant, respectively. After screening, a double mutant R2 (I161M/Y145S) was found with a better performance in the colorimetric assay than R1 mutant. After purification, the specific activity of the R2 mutant was increased to 191 mU/mg, which was 1.2-times of R1 mutant (**Table 2**). Then based on the I161M/Y145S mutant, four site-saturated mutant libraries based on G122, A201, N202, and G205 inside of the [2Fe-2S] cluster, which are located inside of [2Fe-2S] cluster pocket, were constructed for screening mutants with higher activity. As a result, the triple mutant R3 (I161M/Y145S/G205K) was obtained (**Figure 2b**). After purification, the specific activity of the R3 mutant was 344 mU/mg, which was 1.8-times of R2 mutant (**Table 2**). Finally, the R3 mutant with 10-times higher specific activity than the wild type SsDHAD was obtained. The activity improvement level was much higher than that of the previous study of Begander et al. by a random mutation which gave an activity enhancement on glycerate by 1.5-times (Begander et al., 2020). The semi-rational design enabled the enhancement of dehydration activity of SsDHAD toward glycerate to pyruvate based on computer algorithms.

It was known that SsDHAD possesses catalysis activity toward many aldonic acids. Gluconate, an intermediate of glucose to pyruvate, is another substrate of SsDHAD. Therefore, the activity of the wild-type SsDHAD and mutants on gluconate were also determined. The activity of the wild type of SsDHAD on gluconate was 0.203 U/mg, while the activities of R1, R2, and R3 mutants were 0.206, 0.067, and 0.018 U/mg, respectively. This result indicated that mutation sites of Y145S and G205K hamper the performance of SsDHAD on gluconate.

3.3 Characterization of kinetic parameters of the SsDHAD mutants

The purified wild-type SsDHAD and the mutants were subjected to the determinations of kinetic parameters. As shown in **Table 2** ,thek_{cat} value of the R1 mutant was 2.6-fold of that of the wild type SsDHAD, but its K_{m} value was also increased by about 2-fold. Thus the$k_{\text{cat}}/K_{\text{m}}$ values of the R1 mutant and the wild type of SsDHAD were almost the same. Compared with the R1 mutant, the R2 mutant exhibits a lower $K_{\text{m}}$and higher k_{cat} value. The$k_{\text{cat}}/K_{\text{m}}$ value of R2 mutant was 1.35-fold and 1.40-fold higher than that of wild type and R1 mutant of SsDHAD. The k_{cat} value of the R3 mutant was 6.6-fold that of the wild-type SsDHAD. Although theK_{m} value of the R3 mutant was 2.57-fold of the wild type, $k_{\text{cat}}/K_{\text{m}}$ value of the R3 mutant was 2.58-fold higher than that of wild type SsDHAD. The activity level of the R3 mutant is appropriate to boost enzymatic cascades that rely on glycerate dehydration.

3.4 Thermostability analysis of the SsDHAD mutants

Usually, increasing the enzyme activity always decrease the enzyme thermostability,(Lori, Anne, Per-Ola, & Arnold, 1998)-(Siddiqui, 2017) and higher thermostability would benefit the in vitro Biosystems (Zhou,

Huang, Zhu, & Zhang, 2018). The thermostability values of wild-type SsDHAD and mutants were determined by measuring their residual activities after incubated at 70°C for different lengths of time. Compared with the wild-type SsDHAD, the thermostability of the R1 mutant at 70°C was increased obviously (**Figure 3**). While the thermostability values of R2 and R3 mutants were in equivalent level with the wild type of SsDHAD. The $t_{1/2}$ of the wild-type SsDHAD at 70 °C was 1.58 h. The$t_{1/2}$ of the R1 mutant at 70 °C was prolonged to 4.12 h, which was 2.61-times of the wild-type SsDHAD (**Table 2**). The $t_{1/2}$ values of the R2 and R3 mutant were both shortened to 0.98 h, which was lower than that of the wild-type SsDHAD.

3.5 Molecular basis for the improved activity of SsDHAD mutants

To investigate the molecular basis of improved activity of R3 mutant, the structural model of R3 mutant of SsDHAD was built (**Figure 4a**). Docking analysis and MD simulations were subsequently carried out. As shown in **Figure 4b** , position I161 mutated to methionine was anticipated to shrink the volume of the substrate-binding pocket visibly. This observation was further supported by calculating the active site volume, which revealed that the volume decreased from*ca.* 52.1 Å^3 of the wild type to 49.2 Å^3 of the R3 mutant. As a consequence, reshaping the active pocket in the R3 mutant can affect the substrate binding and orientation. In the docking result (**Figure S2**), the distance between the oxygen atom of S472 and the hydrogen atom of substrate C2 was reduced from 4.0 Å of the wild type to 3.5 Å of R3 mutant, enabling the proton abstraction more likely to occur. For G205K, new hydrogen bonds were observed between the side-chain of K205 and main-chains of residues A201 and G122 (**Figure 4c**). The distance between the nitrogen atom in the side-chain of K205 and the oxygen atom in the main-chains of A201 and G122 were well kept *ca.* 3.4 Å in R3 mutant (**Figure S3a**), suggesting that the interactions between K205 and two other residues are well maintained. Analogously, mutant Y145S can also form new hydrogen bonds with the side-chain of S218 as well as R228 (**Figure 4d**), while the interaction between S145 and R228 may be less stable than that between S145 and S218 (**Figure S3b**). Overall, the newly formed hydrogen bonds may increase the structural stability of the [2Fe-2S] cluster binding region and therefore benefit the improvement of the activity. The substrate docking analysis shed light on the possible source of activity. This work highlights the ease of elevating enzyme activity by computational analysis.

3.6 The application of SsDHAD mutant in in vitro synthetic enzymatic biosystems

DHAD plays a critical role in some in vitro biosynthetic systems for the production of value-added chemicals from glucose or glycerol via pyruvate (Gao et al., 2015; Li et al., 2018; Xie et al., 2018). In these in vitro biosystems, the reaction step of converting glycerate to pyruvate catalyzed by SsDHAD is rate-limited due to its low activity on glycerate dehydration. To investigate the effects of enhanced activity of the rate-limited enzyme on the efficiency of in vitro biosystem, the R3 mutant and the wild type SsDHAD were both applied in the in vitro synthetic enzymatic biosystems of converting glycerol or glucose to L-lactate under the same protein concentration. Therefore, the one-pot conversion of glycerol or glucose to L-lactate was implemented (Gao et al., 2015; Xie et al., 2018). The stantdard Gibbs free energy changes (ΔG^o) of the overall reaction is -312.7 kJ/mol, 192.3 kJ/mol expectively in pH 7.5 and when ionic strength is 0.1 M (*http://equilibrator.weizmann.ac.il/*) (**Figure S4a, S4b**).

For the L-lactate in vitro biosysthsis from glycerol, the generated glycerate, which was converted from glycerol by ScALDO (EC 1.1.3.41), was dehydrated into pyruvate catalyzed by SsDHAD, followed by reduction into L-lactate catalyzed by TmLDH (EC 1.1.1.27) (**Figure 5a**). The generated H_2O_2 accompanied the oxidation of glycerol to glycerate was removed by catalase. The NADH and NAD^+ were recycled with TsFDH, (EC 1.17.1.9). In the in vitro biosystem for converting 12 mM glycerol to L-lactate, the wild-type SsDHAD and the R3 mutant were both loaded at 0.33 mg/mL, which were 0.01 U/mL and 0.1 U/mL, respectively, while the other reaction conditions were the same. After 24h, almost 72% and 82% of glycerol were consumed for the biosystems containing wild-type SsDHAD and R3 mutant respectively. However, only about 2.78 mM L-lactate was generated by the biosystem containing wild-type SsDHAD after 24h, while about 9.21 mM L-lactate was generated by the biosystem containing R3 mutant (**Figure 5b**). The L-lactate yields were 23% and 77% for the biosystems containing wild type and R3 mutant of SsDHAD, respectively. The overall production rate of L-lactate by the biosystem containing R3 mutant was 0.38 mM/h, which was 3.32-times of

the biosystem containing wild-type SsDHAD (0.12 mM/h). Although higher L-lactate and D-lactate yields of 91.6% and 97.3% from glycerol were obtained from the equivalent biosynthesis cascade in the study of Li et al. (Li et al., 2018), the SsDHAD loading was 9.1 mg/mL in their reaction system, about 27-times of that in the present study. Moreover, in another study of pyruvate production from glycerol, the pyruvate yield was 93%, with the SsDHAD loading was 9.1 mg/mL as well (Gao et al., 2015).

The 5-enzyme L-lactate production pathway from glucose is NADH balanced and ATP-free (**Figure 6a**). In this in vitro biosystem, glucose is converted to gluconate by SsGDH (EC 1.1.1.47) accompanying the generation of NADH from NAD^+. Gluconate is converted to 2-keto-3-deoxygluconate catalyzed by SsDHAD, then the generated 2-keto-3-deoxygluconate is converted into pyruvate and glyceraldehyde by SaKDGA (EC 4.2.1.14). With the help of TaALDH (EC 1.2.1.3), glyceraldehyde is transformed into glycerate accompanying by the generation of NADH from NAD^+. Glycerate is converted to pyruvate by SsDHAD. Finally, pyruvate is converted to L-lactate by TmLDH with the consumption of NADH. In this in vitro enzymatic biosystem, 10 mM glucose was used to produce L-lactate at 50°C. For the in vitro biosystem containing R3 mutant, due to the activity of R3 mutant on gluconate was too low (0.018 U/mg), the wild-type SsDHAD was still employed in this in vitro biosystem on the conversion of gluconate to 2-keto-3-deoxygluconate. Thus in the in vitro biosystem containing wild type SsDHAD, the loading amount of SsDHAD was 1.33 mg/mL, and in the in vitro biosystem containing R3 mutant, the loading amounts of wild type SsDHAD and R3 mutant were 0.33 and 1.00 mg/mL, respectively. To make the following description more clear, the biosystem only containing wild type SsDHAD was named as WT biosystem, and the biosystem containing wild type and R3 mutant of SsDHAD was named as R3 biosystem. After reaction for 24 h, the residual glucose concentration was 1.25 mM in the WT biosystem, while the glucose was utilized completely in the R3 biosystem (**Figure 6b)** . The final L-lactate titers were 8.14 and 19.05 mM for the WT and R3 biosystems, corresponding to the product yields of 41% and 95%, respectively. The overall production rate of L-lactate for the R3 biosystem was 0.79 mM/h, which was 2.34-times of that for the WT biosystem (0.34 mM/h). Another obvious difference between the WT and R3 biosystems was the glycerate accumulation titer. After reaction for 24h, the concentration of glycerate in the WT biosystem was 11.94 mM, while the R3 biosystem only accumulated 1.11 mM of glycerate. Compared with a previous study by Xie et al. converting glucose to L-Lactate by the same biosystem, 20 mg/mL of SsDHAD was used to achieve 90% of L-lactate product yield in the previous in vitro biosystem (Xie et al., 2018), while only 1.33 mg/mL of SsDHAD loading in the R3 biosystem could achieve more than 90% of L-lactate product yield.

In the two in vitro biosystems tested in this study, the in vitro biosystems containing R3 mutant exhibited much higher product yield, higher reaction rate, and lower intermediate accumulation than the in vitro biosystems containing the same loading amount of wild type SsDHAD.

4. Conclusions

This study demonstrated a case of protein engineering study based on computational analysis of SsDHAD with a novel screending platform to improve the catalytic activity toward glycerate. After 3 rounds of ISM, a triple-mutant (I161M/Y145S/G205K) with 10-times higher specific activity was obtained, that possess shrunke substrate-binding pocket and new fromed hydrogen bonds. For these two in vitro biosystems, the R3 mutant perofrmed more excellent than the wild type SsDHAD. The protein engineering of SsDHAD in this study will provide some useful information for the rational design and screening method establishment for activity improvement of dehydrates.

Declaration of Competing Interest

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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Tables

Table 1. The list of enzymes and their properties

NO.	Enzyme	Abbreviation	Source	EC Number	ΔrG'	T_{opt} (°C)[a]	Reaction	Sp. Act (U/mg)
1	Alditol oxidase	ScALDO	*Streptomyces coelicolor* A3	1.1.3.41	-249.3	50	Glycerol + O_2 - Glycerate + H_2O_2	0.26
2	Dihydroxy acid dehydratase	SsDHAD	*Sulfolobus solfataricus*	4.2.1.9	-38.5	70	Glycerate - Pyruvate + H_2O	0.030 (W
								0.3 (R3
					-32.7	70	Gluconate - 2-Keto-3-deoxygluconate + H_2O	0.1
3	L-lactate dehydrogenase	TmLDH	*Thermotoga maritima*	1.1.1.27	-188	85	Pyruvate+ NADH - L-Lactate + NAD^+	98
4	Glucose dehydrogenase	SsGDH	*S. Solfataricus*	1.1.1.47	-3.4	70	Glucose + NAD^+ ucose + NADus-genas-esee + Hir p	12.5
5	2-keto-3-deoxy gluconate aldolase	SaKDGA	*S. acidocaldarius*	4.2.1.14	6.2	99[1]	2-Keto-3-deoxygluconate - Pyruvate + Glyceraldehyde	1.2
6	Glyceraldehyde dehydrogenase	TaALDH	*Thermoplasma acidophilum*	1.2.1.3	-37.3	63	Glyceraldehyde + NAD+ umGlyc-erate + NADH	0.95
7	Formate dehydrogenase	TsFDH	*Thiobacillus* sp. KNK65MA	1.17.1.9	-14.1	58	Formate + NAD^+ + NA_2 + NADH	2

T_{opt}, optimal temperature; WT: wild type SsDHAD; R3: R3 mutant of SsDHAD.

Table 2. Kinetic parameters of the wild type and the mutants of SsDHAD[a]

Enzymes	Mutations	Specific activity ($mU \cdot mg^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	$t_{1/2}$ at 70 °C (h)
WT	—	33.1±0.3	0.23±0.01	0.05 ±0.0	217±10.0	1.62
R1	I161M	155±4.0	0.59±0.0	0.13±0.0	224±3.1	3.90
R2	I161M/Y145S	191±2.8	0.45±0.0	0.14 ±0.0	303±4.9	1.02
R3	I161M/Y145S/G224K	354±4.9	0.59±0.0	0.33±0.0	559±8.7	0.99

[a]: Each value represents the average ± standard deviation of triplicate independent measurements.

Figure Legends

Figure 1. The overall structure of the wild-type SsDHAD was obtained by homology modeling. (a) The substrate-binding pocket and [2Fe-2S] binding pocket are depicted by green and cyan surfaces, respectively. The inset shows the residues lining the two pockets. The substrate and [2Fe-2S] cluster are drawn in the ball and stick representation. The [2Fe-2S] cluster is shown with yellow and orange sticks. Mg^{2+} is shown as a purple sphere; (b) Representation of hotspot residues for site-saturation mutagenesis. The hotspots lining substrate-binding pocket as well as [2Fe-2S] cluster binding pocket are indicated by green and cyan cycles, respectively. Hydrogen bonds are shown in yellow dashed lines, while the iron metal coordinate bonds as black dotted lines.

Figure 2. Schematic presentation of SsDHAD evolution for dehydration activity. (a) Overview of the procedure of protein engineering of SsDHAD and high screening method established based on 2,4-dinitrophenylhydrazine; (b) Colorimetric assay to detect the dehydration activity of the wild type SsDHAD and mutants based on 2,4-dinitrophenylhydrazine methods and photo images of the color reaction of 0 to 2.0 mM pyruvate standard solution.

Figure 3. Thermostability of the wild type of SsDHAD and mutants (R1, R2, and R3). Residual activities were measured after heat treatment at 70 °C for various times. Reactions were done in triplicate.

Figure 4. Structural comparison of the wild-type SsDHAD and R3 mutant. (a) Overlay of wild type of SsDHAD (gray) and R3 mutant (cyan); (b) The substrate-binding pockets of wild type of SsDHAD and R3 mutant are shown as gray meshes and green meshes, respectively; (c) Interactions between the side-chain of K205 with the main-chains of A201 and G122 in the R3 mutant; (d) Interactions between the side-chain of S145 with side-chains S218 and R228 in the R3 mutant. The [2Fe-2S] clusters are shown with yellow and orange sticks. $Mg^{2+}$is shown as a purple sphere.

Figure 5. L-lactate production from glycerol by in vitro synthetic enzymatic biosystems containing wild-type or R3 mutant of SsDHAD. (a) Schematic of the in vitro synthesis pathway of lactate from glycerol. The enzymes are alditol oxidase from *S. coelicolor* A3 (ScALDO), dihydroxy acid dehydratase from *S. solfataricus*(SsDHAD), L-lactate dehydrogenase from *T. maritime* (TmLDH), catalase from *A. niger* , formate dehydrogehyde from *Thiobacillus* sp. KNK65MA (TsFDH); (b) Profiles of L-lactate production from glycerol as time. The reaction was performed at 50 °C in 100 mM HEPES buffer (pH 7.5) containing 12 mM glycerol, 5.0 mM $MgCl_2$, 5.0 mM NADH, 0.3 U/mL ScALDO, 0.3 U/mL TaALDH, 1000 U/mL catalase, 0.3 U/mL TmLDH, 5 U/mL TsFDH, and 0.33 mg/mL SsDHAD (0.01 U/mL) or 0.33 mg/mL R3 mutant (0.1 U/mL). Results are means of three parallel replicates.

Figure 6. L-lactate production from glucose by in vitro synthetic enzymatic biosystems containing wild-type or R3 mutant of SsDHAD. (a) Schematic of the in vitro synthesis pathway of lactate from glucose. The enzymes are glucose dehydrogenase from *S. solfataricus* (SsGHD), dihydroxy acid dehydratase from *S. solfataricus* (SsDHAD), 2-keto-3-deoxy gluconate aldolase from *S. acidocaldarius* (SaKDGA), L-lactate dehydrogenase from *T. maritime* (TmLDH), glyceraldehyde dehydrogenase from *T. acidophilum* (TaALDH); (b) Profiles of L-lactate production from glucose as time. The reaction was performed at 50 °C in 100 mM

HEPES buffer (pH 7.5) containing 10 mM glucose, 5.0 mM $MgCl_2$, 5.0 mM NAD^+, 0.3 U/mL SsGHD, 0.3 U/mL SaKDGA, 0.3 U/mL TaALDH, 0.3 U/mL TmLDH, 0.1 U/mL SsDHAD (1 mg/mL) for conversion of gluconate to 2-keto-3-deoxygluconate, and 0.33 mg/mL SsDHAD (0.01 U/mL) or 0.33 mg/mL R3 mutant (0.1 U/mL) for glycerate dehydration. Results are means of three parallel replicates.