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Manipulating the substrate specificity of murine dihydrofolate reductase enzyme using an expanded set of amino acids

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ABSTRACT

As the number of reactions requiring biotransformation continues to grow, manipulating the enzyme substrate specificity becomes very important. Complementary to conventional enzyme engineering techniques based on only natural amino acids, we hypothesized that the site-specific incorporation of a non-natural amino acid in vivo into an enzyme can be used to re-design the active site for the altered substrate specificity. To test our hypothesis, we introduced the non-natural amino acid *L*-2-naphthylalanine (2Nal) into the active site of the model enzyme murine dihydrofolate reductase (mDHFR). We explored whether the substrate specificity of the enzyme could be switched from the good substrate dihydrofolate (DHF) to the poor substrate folate (FOL). We used two protein design programs (RosettaLigand and RosettaDesign) to calculate ligand docking and conformational stability, respectively, for the evaluation of multiples sites in the mDHFR. From the calculations, position 31 was predicted as an optimal 2Nal incorporation site. One mDHFR variant containing 2Nal at position 31 ($mDHFR^{2Nal31}$) was expressed in the *Escherichia coli* expression host cells equipped with the engineered yeast phenylalanyl-tRNA and phenylalanyl-tRNA synthetase pair. As expected, the kinetic assays of purified mDHFR variant revealed that $mDHFR^{2Nal31}$ has the enhanced binding affinity toward FOL and also exhibits 7.6-fold enhanced catalytic efficiency of FOL over DHF compared to $mDHFR^{WT}$.

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1. Introduction

As the number of chemical reactions requiring biocatalysis is rapidly increasing, the demand to transform existing enzymes or discover new enzymes catalyzing new reactions is also increasing. In order to avoid the laborious screening of new enzymes, altering substrate specificity of existing enzymes has often been attempted [1–7]. However, the main issue in changing the enzyme substrate specificity is difficulty in fine tuning the active site accommodating a new or poor substrate. Therefore, various enzyme engineering techniques, including rational design and directed evolution, have been used to change the substrate specificity. However, as biocatalysis applications have quickly diversified, there are situations where conventional protein engineering techniques based on natural amino acids may not be very effective in generating enzymes with desirable properties.

As an emerging technique, enzyme engineering utilizing non-natural amino acids as well as natural amino acids have been explored (reviewed in [8]). Such an expanded set of amino acids expands the protein sequence space beyond the chemical/physical limits set by nature [9]. Several techniques using non-natural amino acids have developed [9–26]. Residue-specific incorporation of non-natural amino acids is recently demonstrated as a very useful tool for redesign of industrial enzymes [27], or bio-catalysts in general [28], and even useful to perform enzyme reactions in hostile environments [29]. Alternatively, site-specific incorporation in vivo techniques including stop codon suppression in vivo (SCS) shows great potential for enzyme engineering. SCS incorporates a non-natural amino acid into a single site of a target protein in expression hosts equipped with a heterologous orthogonal pair of suppressor tRNA/aminoacy-tRNA synthetase. If the incorporation site of a non-natural amino acid is appropriately chosen, the perturbation of the enzyme structure and function can be minimized. So far, numerous

Abbreviations: 2Nal, *L*-2-naphthylalanine; AFWK, phenylalanine/tryptophan/lysine triple auxotrophic *E. coli*; DHF, dihydrofolate; DHFR, dihydrofolate reductase; FOL, folate; LB, Luria-Broth; Lys, lysine; NADPH, nicotinamide adenine dinucleotide phosphate; mDHFR, murine dihyrofolate reductase; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; Phe, phenylalanine; SCS, stop codon suppresion; TFA, trifluoroacetic acid; THF, tetrahydrofolate; Trp, tryptophan.

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non-natural amino acids have been introduced into a recombinant protein expressed in industrially important expression hosts, such as *Escherichia coli*, yeast, and mammalian cells in a site-specific manner [8,11]. Recently, over 500 mg/L of human growth hormone was produced in over 1000-L scale using SCS [30]. Furthermore, very recently, non-natural amino acids synthesized from α-keto acids in *E. coli* cells were directly utilized for SCS [31]. This new technique is expected to substantially lower production cost of enzymes containing non-natural amino acids. These advances in SCS demonstrate the feasibility of SCS for industrial applications.

Although SCS has shown a great potential to engineer critical properties of enzymes, it has rarely been used to improve enzyme's catalytic properties. As a pioneering work, Jackson et al. reported that *E. coli* nitroreductase variants containing *p*-nitrophenylalanine in the active site exhibited a 2- to 4-fold increase in the catalytic activity compared to wild-type nitroreductase [32]. Recently, we also demonstrated that SCS can be used to control the competitive inhibition of murine dihydrofolate reductase without compromising the substrate binding affinities [33]. However, other important enzyme properties, such as substrate specificity and product regio- and stereo-selectivity have not yet been explored using SCS technique. Herein, we report our efforts to change the enzyme substrate specificity using an expanded set of the genetically encoded amino acids with the aid of computational protein design tools.

We used two molecular modeling programs, RosettaLigand and RosettaDesign, version 3.4 for the computational design [34–37]. RosettaLigand takes a receptor structure and a small molecule ligand, and calculates the Rosetta score to identify a conformation and relative orientation of the receptor and ligand. The Rosetta score is a linear sum of a 6–12 Lennard–Jones potential, the Lazaridis–Karplus implicit solvation model, an empirical hydrogen bonding potential, backbone-dependent rotamer probabilities, a knowledge-based electrostatic energy potential, amino acid probabilities based on particular regions of ϕ/ψ space, and a unique reference energy for each amino acid [35]. For a given crystal structure, RosettaDesign uses simulated annealing to scan through a large number of rotamers to minimize the energy score with Monte Carlo optimization [38].

We chose murine dihydrofolate reductase (mDHFR) and its two substrates as a model system. DHFRs catalyze, the conversion of (1) a relatively poor substrate folate (FOL) into dihydrofolate (DHF); and (2) a relatively good substrate DHF into tetrahydrofolate (THF) using NADPH as a co-factor in each step (Fig. 1A). Although the crystal structure of human DHFR (hDHFR) complexed with FOL is available for computational design, the activity of hDHFR using FOL as a substrate is very low (30 times slower than that of rat DHFR) [39], which makes the kinetic analysis of hDHFR quite difficult. The amino acid sequence of mDHFR differs from that of hDHFR by about twenty amino acids. All of these, except two amino acids, are located near enzyme surface strongly indicating that the active site structures of mDHFR and hDHFR are essentially identical [40]. Therefore, we chose catalytically more efficient mDHFR and constructed the structural models of mDHFR variants using the crystal structure of hDHFR. Although the chemical structure of FOL differs slightly from that of DHF, the binding affinity of FOL to DHFRs is significantly lower than that of DHF [39,41]. As a model system, DHFR and FOL/DHF have several favorable features. First, developing DHFR variants that efficiently catalyze FOL has clear benefits in biomedical applications. THF is a co-factor critical in the metabolism of amino acids and nucleic acids. The deficiency in THF (possibly caused by the deficiency in FOL) leads to megalobalstic anemia [42]. Therefore, the delivery of human DHFR variants efficiently catalyzing the conversion reaction of FOL or the human DHFR genes into megaloblastic anemia patients is a potential therapeutic strategy. Second, the crystal structures of DHFRs bound to substrates are readily available facilitating the identification of key residues for the substrate binding/computational active site design [43–46]. Third, both DHF and THF are much more expensive than FOL, restricting their therapeutic applications. Therefore, DHFR variants with a higher FOL conversion rate can serve as a potential biocatalyst facilitating the commercial production of DHF and THF. Fourth, the expression of DHFRs in *E. coli* and kinetic assay conditions is well established [5,47,48]. The enhanced substrate specificity for FOL of *E. coli* DHFR (eDHFR) achieved using enzyme engineering has been reported [49]. Based on the fact that chicken DHFR is approximately 400 times more specific for FOL than eDHFR, the insertion of additional residues in one loop region of chicken DHFR into eDHFR led to the 13-fold enhanced specificity for FOL [49]. Although such a loop modification approach is very effective in enhancing the specificity for FOL of eDHFR, its application to mammalian DHFRs including murine DHFR seems limited due to very high amino acid sequence homology among mammalian DHFRs. Therefore, altering the substrate specificity of mammalian DHFRs requires an alternative strategy, such as active site engineering using computational protein design tools.

Theoretically, any non-natural amino acid could be tested to alter substrate specificity of mDHFR. The non-natural amino acid, 2Nal was used in this study to incorporate into mDHFR due to its favorable features. 2Nal has a hydrophobic, bulkier side chain compared to other natural amino acids and was successfully used to control mDHFR inhibition [33]. The rotamer library of 2Nal was already developed for computational protein design using Rosetta programs [34]. Since the introduction of a polar amino acid into inside region of a protein likely perturbs the folded structure, a non-natural amino acid with a non-polar side chain, such as 2Nal was chosen in this study. We have demonstrated the site-specific incorporation of 2Nal in *E. coli* using the orthogonal pair of yeast phenylalanyl-tRNA suppressor variant and phenylalanyl-tRNA synthetase with high specificity toward 2Nal [33,50]. However, generating and testing multiple mDHFR variants containing 2Nal at different sites are not trivial. Therefore, RosettaLigand and RosettaDesign [34–37], two computational protein design tools calculating ligand docking and conformational stability scores respectively, were utilized to identify an optimal 2Nal incorporation site in the active site.

2. Materials and methods

2.1. Materials

Twenty natural amino acids, antibiotics, isopropyl-β-D-thiogalactopyranoside (IPTG), *L*-(−)-fucose, 2,5-dihydroxybenzoic acid (DHB), dihydrofolate (DHF), folate (FOL), and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma (St. Louis, MO). *L*-2-naphthylalanine (2Nal) was obtained from Chem-Impex (Wood Dale, IL). The nickel-nitrilotriacetic acid affinity column (Ni-NTA column) was purchased from Qiagen (Valencia, CA). Sequencing grade modified trypsin was procured from Promega (Madison, WI). ZipTip pipette tips containing C_{18} reversed-phase media was obtained from Millipore (Billerica, MA). All other chemicals, unless otherwise noted, were purchased from Sigma (St. Louis, MO), and were used without further purification.

2.2. Methods

2.2.1. Computational protein design

There is no crystal structure of the wild-type mDHFR bound to either DHF or FOL. Alternatively, considering that human DHFR is highly homologous to mDHFR with greater than 95% of amino acid sequence similarity, the crystal structure of human DHFR (PDB ID: 2W3M), ligand structures, and the rotamer library of 2Nal was utilized to construct the structural models of mDHFR variants containing 2Nal [34,36,37,51–53]. Docking of mDHFR variants with

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