Biochemical Engineering Journal 109 (2016) 1–8

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Regular article

Positional effects of hydrophobic non-natural amino acid mutagenesis into the surface region of murine dihydrofolate reductase on enzyme properties

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ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form
12 December 2015
Accepted 18 December 2015
Available online 22 December 2015

Keywords:
Amino acid
Artificial enzyme
Biocatalysis
Enzyme technology
Non-natural amino acid mutagenesis
Enzyme surface

ABSTRACT

The ability to incorporate non-natural amino acids during protein biosynthesis has greatly broadened the amino acid sequence space available for protein engineering. The increasing number of non-natural amino acids provides novel sidechain chemistries, structures, and functionalities for engineered proteins including enzymes. Therefore, understanding how non-natural amino acid incorporation (non-natural amino acid mutagenesis) affects protein function is crucial. This study investigates the positional effects of hydrophobic non-natural amino acid mutagenesis of the enzyme surface on the enzyme structure and function with the ultimate goal of identification of permissive sites for non-natural amino acid mutagenesis. A bulky, hydrophobic non-natural amino acid, 3-(2-naphthyl)-alanine (2Nal), was site-specifically incorporated at the solvent-exposed surface of a murine dihydrofolate reductase (mDHFR) enzyme. Incorporation of 2Nal was performed at six solvent-exposed sites (V43, E44, F142, E143, F179, and E180), generating six mDHFR variants. Incorporation of 2Nal into F142 or F179 did not exhibit any substantial change in the secondary structure and catalytic activities, whereas 2Nal incorporation at hydrophilic Glu sites significantly changed the secondary structure and catalytic activities. Such different responses upon 2Nal incorporation at hydrophobic and hydrophilic residues were likely due to the structural changes caused by the hydrophobicity change upon mutation. A non-conservative mutation of V43 with 2Nal significantly reduced the catalytic activities suggesting that a substantial size change of sidechain also causes the structural changes. These results suggest that even hydrophobic, bulky non-natural amino acids can be incorporated at the enzyme surface without compromising the enzymatic activities.

1. Introduction

Protein engineering has been used extensively to create proteins with improved or expanded function. The amino acid sequence space available for protein engineering is critical for successful protein engineering. Development of technology to genetically encode and incorporate non-natural amino acid (NAA) into proteins has greatly expanded the amino acid sequence space by increasing the number and diversity of building blocks available for protein biosynthesis. In nature, most proteins are made of only 20 natural amino acids. So far over 100 NAAs have been successfully incorporated into diverse protein targets, including therapeutic proteins, fluorescence proteins, and enzymes [1–13]. With the increasing number of NAAs available for protein biosynthesis, understanding how non-natural amino acid incorporation (non-natural amino acid mutagenesis) affects protein function becomes crucial. Enzymes, which are proteins with catalytic properties,

Abbreviations: 2Nal, 3-(2-naphthy)-alanine; AFWK, pheylalanine/tryptophan/lysine triple auxotrophic *E. coli*; DHB, 2,5-dihydroxybenzoic acid; DHF, dihydrofolate; DHFR, dihydrofolate reductase; Glu, glutamic acid; LB, Lria-Broth; Lys, lysine; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; NAA, non-natural amino acid; NADPH, Nicotinamide adenine dinucleotide phosphate; mDHFR, murine dihyrofolate reductase; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; Phe, phenylalanine; SINA, site-specific incorporation of a non-natural amino acid in vivo; TFA, trifluoroacetic acid; THF, tetrahydrofolate; Trp, tryptophan; Val, valine.

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http://dx.doi.org/10.1016/j.bej.2015.12.014
1369-703X/

are indispensable for all living organisms and play an important role in industrial biocatalytic processes [14–16]. Since the folded structures of enzymes are required for their catalytic properties, perturbation in the enzyme structure often results in loss in their catalytic properties. Site-specific incorporation of NAAs into enzymes (non-natural amino acid mutagenesis of enzymes) has gained the interest of researchers, because NAAs can be advantageously introduced into permissive sites of enzymes to add novel chemistries with minimal perturbation to structure and function [17–20]. A majority of the over 100 NAAs available for non-natural amino acid mutagenesis are phenylalanine (Phe) and tyrosine (Tyr) analogs, which have a hydrophobic aromatic sidechain [3,21]. Some of Phe and Tyr analogs have reactive groups, such as azido, alkyne, and terminal keto moieties that are commonly employed as a chemical handle for bioconjugation [17,22–24]. In order to achieve efficient bioconjugation, these reactive NAAs need to be surface-exposed [17,24]. Furthermore, most of protein–protein or protein-ligand binding occur on the protein surface. In order to modulate protein–protein or protein-ligand interactions, mutations are often introduced into the protein surface. Therefore, there are many situations where the introduction of a hydrophobic non-natural amino acid to the protein surface is required. Then, it is not surprising to ask whether the introduction of a hydrophobic non-natural amino acid to the solvent-exposed surface of proteins, which is known to have hydrophilic environment, have detrimental effects on the protein structure and function. However, this question was not yet answered.

This study evaluates the positional effects of hydrophobic non-natural amino acid mutagenesis of the enzyme surface on enzyme structure and function. The enzyme surface plays an important role in interacting with ligands and other proteins. Therefore, effects of the non-natural amino acid mutagenesis of the enzyme surface on the enzyme structure and function were investigated.

This study uses murine dihydrofolate reductase (mDHFR) as the model protein. DHFRs catalyze conversion of dihydrofolate (DHF) into tetrahydrofolate in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). Numerous crystal structures for DHFRs were resolved, and the enzyme kinetics has been extensively characterized [25–31]. The non-natural amino acid 3-(2-naphthyl)-alanine (2Nal) was used, because 2Nal has a very bulky aromatic sidechain required to change local hydrophobicity of the enzyme surface. The pair of engineered yeast phenylalanyl-tRNA/synthetase was available for site-specific incorporation of 2Nal into proteins including mDHFR [32,33]. Six sites were selected from solvent-exposed regions of mDHFR to investigate how changes in hydrophobicity at these sites affect mDHFR function (Supporting Information; Fig. S1). Catalytic properties of mDHFR variants containing 2Nal were characterized though the kinetics studies of DHF conversion into tetrahydrofolate. Effects of 2Nal incorporation on mDHFR structure were investigated by circular dichroism spectroscopy.

2. Materials and methods

2.1. Materials

The 20 natural amino acids, ampicillin, kanamycin, *L*-(−)-fucose, and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma–Aldrich (St. Louis, MO). 3-(2-naphthy)-alanine (2Nal) was obtained from Chem-Impex (Wood Dale, IL). Dihydrofolate (DHF), nicotinamide adenine dinucleotide phosphate (NADPH), and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Santa Cruz Biotechnologies (Dallas, TX). Sequencing-grade trypsin was procured from Promega (Madison, WI). Nickel-nitrilotriaceticacid (Ni-NTA) affinity column resin and plasmid pQE16 were obtained from Qiagen (Valencia, CA). C18 ZipTip pipette tips were obtained from Millipore (Billerica, MA). All other chemicals, unless otherwise noted, were purchased from Fisher Scientific (Pittsburgh, PA) and were used without additional purification.

2.2. Expression and purification of wild-type mDHFR and variants

The pQE16 plasmid containing the mDHFR gene was transformed into the AFWK host as described previously [33,34]. The cells were grown to OD_{600} = 1.0 and induced with a final concentration of 1 mM IPTG. Cells were harvested after overnight induction at 37 °C. In order to express mDHFR variants containing 2Nal, plasmids pQE16-XXAmb_mDHFR-yPheRS^{naph} containing an amber codon in a specified site were generated by PCR mutagenesis using the plasmid pQE16-mDHFR^{WT}-yPheRS^{naph} as a template [33,34]. Here XX represents the corresponding position of amber codon suppression based on PDB ID: 1U72 [26]. Each of the plasmid pQE16-XXAmb_mDHFR-yPheRS^{naph} and pREP4-ytRNA$^{Phe}_{CUA_UG}$ were co-transformed into the *Escherichia coli* AFWK host. The cells were then grown in M9-20AA media (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.4% glucose, 50 mg/L of each of the 20 natural amino acids), supplemented with 100 mg/ml ampicillin and 50 mg/ml kanamycin, until OD_{600} = 1.0. Cells were harvested and centrifuged at 4500 × *g* for 8 min. The resulting cell pellet was resuspended and gently washed with 0.9% NaCl and centrifuged again at 4500 × *g* for 8 min. The resulting cell pellet was resuspended in M9-17AA 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.4% glucose, 0.05 g/L of each of the natural amino acids except phenylalanine, tryptophan and lysine which were supplemented at 25 μM, 50 μM and 100 μM, respectively [33,34]. The M9-17AA was then supplemented with 3 mM of 2Nal. After incubation at 37 °C for 10 min, mDHFR expression was induced by the addition of IPTG to the culture at a final concentration of 1 mM. The cells were induced overnight at 32 °C. Purification of the mDHFR variants was performed via Ni-NTA affinity chromatography according to manufacturer's protocol (Qiagen, Valencia, CA), except 20 mM and 40 mM imidazole were used in the lysis and wash buffers, respectively [33,34]. The SDS-PAGE images of the purified mDHFR variants were taken using a UVP BioSpectrum imager (Upland, CA) and then analyzed with VisionWorks image analysis software. Protein concentration was determined by absorbance measurements at 280 nm, using a calculated extinction coefficient of 24,750 cm^{-1} M^{-1} [33,34].

2.3. Confirmation of in vivo 2Nal incorporation by MALDI-TOF/MS

Incorporation of 2Nal into mDHFR was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) after enzymatic digestion of each variant. Trypsin (1 μg in 50 mM acetic acid) or chymotrypsin (1 μg in 50 mM acetic acid) was added to 10 μL of 5 μM each mDHFR variant in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH = 8.0). Upon addition the sample was incubated at 37 °C overnight. 12 μL of 5% trifluoroacetic acid was added to quench the reaction. Digested peptides solutions were desalted using C18 ZipTips according to the manufacturer's protocol (EMD—Millipore, Darmstadt, Germany). DHB (20 mg/mL) and *L*-(−)-fucose (2 mg/mL) dissolved in 10% ethanol were used as the matrix for MS analysis using a Microflex MALDI-TOF/MS (Bruker, Billerica, MA) [33,34]. The several variants (mDHFR^{V43Z}, mDHFR^{E44Z}, mDHFR^{F179Z}, and mDHFR^{E180Z}) were digested with trypsin. The other variants (mDHFR^{F142Z} and mDHFR^{E143Z}) were digested with chymotrypsin.