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Engineering dihydropteroate synthase (DHPS) for efficient expression on M13 phage

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Abstract

Phage display is a commonly used selection technique in protein engineering, but not all proteins can be expressed on phage. Here, we describe the expression of a cytoplasmic homodimeric enzyme dihydropteroate synthetase (DHPS) on M13 phage, established by protein engineering of DHPS. The strategy included replacement of cysteine residues and screening for periplasmic expression followed by random mutagenesis and phage display selection with a conformation-specific anti-DHPS antibody. Cysteine replacement alone resulted in a 12-fold improvement in phage display of DHPS, but after random mutagenesis and three rounds of phage display selection, phage display efficiency of the library had improved 280-fold. Most of the selected clones had a common Asp96Asn mutation that was largely responsible for the efficient phage display of DHPS. Asp96Asn affected synergistically with the cysteine replacing mutations that were needed to remove the denaturing effect of potential wrong disulfide bridging in phage display. Asp96Asn alone resulted in a 1.8-fold improvement in phage display efficiency, but in combination with the cysteine replacing mutations, a total of 130-fold improvement in phage display efficiency of DHPS was achieved.

Keywords: Dihydropteroate synthase; Phage display; Protein expression; Protein engineering; Cysteine residue

1. Introduction

Phage display [1] is the most commonly used technique in the selection of specific targets from large peptide or protein libraries [2]. In M13 filamentous phage display, the displayed protein or peptide is most often fused to the major coat protein pVIII [3] or the minor coat protein pIII [4,5]. In phagemid system [6,7], both wild type coat protein and the fusion protein is incorporated onto phage.

Antibody fragments were one of the first proteins that were displayed on filamentous phage [8]. Antibodies have traditionally been used for molecular recognition in biotechnology and since its discovery phage display has been widely used as a method of selection in antibody engineering [9]. However, antibodies are not optimal for binding small molecules or haptens which themselves are not immunogenic and there is an increasing interest towards alternative protein scaffolds for molecular recognition [10]. TIM ($(\beta\alpha)_8$) barrel could be an interesting alternative scaffold for binding haptens. It is a stable fold and the most frequently occurring folding motif in proteins [11–13]. Many TIM barrel proteins catalyze enzymatic reactions of small molecules and thus they can be considered as natural hapten binders. Active center in TIM barrel proteins is usually located at the loop regions that should be rather freely mutable without affecting the framework structure. This could be exploited in engineering the ligand binding site for new specificity and recognition of different small molecules.

Enzymatic activity and specificity of several TIM barrel proteins have been modified [14–17], but the mutants have been selected by screening. In the selection of binders, use

Abbreviations: DHPS, Dihydropteroate synthetase; Mab, Monoclonal antibody; WT, Wild type

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of a display technique such as phage display would, however, be very practical. To date, numerous proteins also other than antibody fragments have been displayed functionally on filamentous phage [18]. Most of these proteins are normally extracellular and secreted. Intracellular proteins may have free cysteines that limit their phage display [19], but there are also examples of cytoplasmic proteins that have been displayed successfully on phage [20–22]. In some cases, phage display of a cytoplasmic protein has been improved by optimizing the signal peptide that directs proteins to periplasm [23].

We were interested in using a homodimeric, cytoplasmic TIM barrel protein dihydropteroate synthase (DHPS, EC 2.5.1.15) [24,25] as a binding scaffold but found that DHPS could not be displayed on M13 phage. Despite the abundance of proteins with TIM barrel fold, their phage display has not been widely described and it is not known whether the inability to be displayed on phage is more common among TIM barrel proteins. As TIM barrel proteins are naturally expressed in cytoplasm, secretion to periplasm with oxidizing conditions that is needed in most display systems might cause problems [26]. Here, we describe the engineering of DHPS for efficient phage display indicating that phage display can be established by protein engineering of the displayed protein.

2. Materials and methods

2.1. Primers, strains, plasmids, culture media and buffers

Oligonucleotide primers WO1117 (5′-TTA CTC GCG GCC CAG CCG GCC ATG GCG CAT ATG AAA CTC TTT GCC CAG GG-3′), WO1118 (5′-GGA ATT CGG CCC CCG AGG CCT CAT AGC GTT TGT TTT CCT T-3′), WO1230 (5′-GCG GTC ATT GCC GCA ATG CAA GGA GCT CAC ATC ATT CGT GTT CAT-3′), WO1381 (5′-CTG ATG CAC ATG CAG GGA AAT CCA AAA AC-3′), WO1382 (5′-GAG CAG GCG GGT ATC GCG AAA GAG AAA TTG TTG CTC GAC-3′), WO1383 (5′-TTT CCC TGC ATG TGC ATC AG**G RY**A ACC GGT AGA CCG GTT TCT GCA GCC GC-3′), WO1384 (5′-TTC GCG ATA CCC GCC TGC TC**C RY**A CGT GCT ATT TGC TCA ATA AAG-3′), WO1385 (5′-TGC ATT GCG GCA ATG ACC GC**G RY**T GCG AGG GAC CCG CTC AG-3′) were synthesized on an automatic DNA/RNA-synthesizer (Model 394, Applied Biosystems, Foster City, CA) using standard phosphoramidite chemistry. DHPS172C (5′-TTC GCG ATA CCC GCC TGC TCG **RYA** CGT GCT ATT TGC TCA ATA AAG-3′ oligonucleotide primer was from TAG Copenhagen A/S (Copenhagen, Denmark). Mutated bases are in bold and *Sfi*I restriction sites are underlined.

Escherichia coli XL1-Blue strain (*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1, *lac* [F′ *proAB*, *lacI*^{q}*Z*ΔM15, Tn*10* (tet^{r})] Stratagene, La Jolla, CA) was used as a host for cloning, periplasmic protein expression and phage production. Vectors pAK100, pAK200 and pAK400 [27] were obtained as a gift from Andreas Plückthun (Department of Biochemistry, University of Zürich, Switzerland) and the cloned *E. coli* DHPS gene [28] from Walter S. Dallas (Glaxo Wellcome, North Carolina, USA). VCS M13 (Kan^{r}) helper phage used in phage production was from Stratagene. SB medium, SOC medium, LB agar plates and TBS buffer were prepared as described [29]. In TBT-0.05 and TBT-0.5 buffers TBS was supplemented with 1% (w/v) bovine serum albumin and 0.05% or 0.5% (v/v) Tween20, respectively. The concentrations of antibiotics on agar plates and culture media were chloramphenicol 25 μg/ml and tetracycline 10 μg/ml. DELFIA assay buffer, wash buffer and enhancement solution were products of Perkin-Elmer Life Sciences (Turku, Finland).

2.2. Antibodies

Monoclonal anti-DHPS antibodies 2H5, 3G4 and 4G7 and anti- M13 phage antibody 9E7 were produced in-house. Antibodies were biotinylated with an 80-fold molar excess of biotin-isothiocyanate [30] in 50 mM carbonate buffer (pH 9.8) for 4 h at room temperature. Unreacted biotin-isothiocyanate was removed with NAP-10 and PD-10 columns (Amersham Biosciences AB, Uppsala, Sweden). Antibodies 4G7 and 9E7 were labeled with europium according to the instructions in the DELFIA Eu-labeling kit (Perkin-Elmer Life Sciences).

Conformation specificity of anti-DHPS antibodies was studied on dot blot using native DHPS and DHPS denatured for 5 min at 90 °C in 2.5% (w/v) sodium dodecyl sulfate and 5.0% (w/v) 2-mercaptoethanol. Samples (0.4 μg/dot) were applied onto Hybond-P PVDF membrane (Amersham Biosciences) and recognition of DHPS by individual monoclonal anti-DHPS antibodies (at 2 μg/ml) was tested using ECL Western blotting system (Amersham Biosciences).

2.3. Screening cysteine-free DHPS libraries for improved periplasmic expression

Cysteine-free DHPS library K with C137T/A/V/I, C172S/A/V/M and C242T/A/V/I mutations was constructed by strand overlap extension (SOE) PCR [31] from sequentially overlapping DNA fragments that had been amplified from *E. coli* DHPS with *Pfu* DNA polymerase (Stratagene) using primer pairs WO1117–WO1383, WO1381–WO1384, WO1382–WO1385 and WO1230–WO1118. For another cysteine-free library KII (C137T/A/V/I, C172T/A/V/I and C242T/A/V/I), primer WO1384 was replaced with DHPS172C. The assembled libraries were cloned at *Sfi*I sites into vector pAK100. The libraries were transformed into *E. coli* XL1-Blue cells and plated on to LB agar plates containing chloramphenicol and tetracycline. 120 clones from each library (complexity