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Establishing catalytic activity on an artificial $(\beta\alpha)_8$-barrel protein designed from identical half-barrels

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ABSTRACT

It has been postulated that the ubiquitous $(\beta\alpha)_8$-barrel enzyme fold has evolved by duplication and fusion of an ancestral $(\beta\alpha)_4$-half-barrel. We have previously reconstructed this process in the laboratory by fusing two copies of the C-terminal half-barrel HisF-C of imidazole glycerol phosphate synthase (HisF). The resulting construct HisF-CC was stepwise stabilized to Sym1 and Sym2, which are extremely robust but catalytically inert proteins. Here, we report on the generation of a circular permutant of Sym2 and the establishment of a sugar isomerization reaction on its scaffold. Our results demonstrate that duplication and mutagenesis of $(\beta\alpha)_4$-half-barrels can readily lead to a stable and catalytically active $(\beta\alpha)_8$-barrel enzyme.

1. Introduction

Evolution has provided us with a myriad of enzymes which catalyze an amazing wealth of different reactions with tremendous selectivity and specificity. One of the most important and frequent mechanisms for the creation of new enzymes is the duplication of genes [13,28]. Following duplication, the new gene copy can be either recruited for a new function, or it can be fused with the original gene copy or with another nucleotide fragment from the genome. Especially proteins that exhibit a high degree of internal structure and sequence symmetry suggest a scenario of duplication and fusion of identical gene copies, with a conversion of symmetric oligomers into symmetric monomers [38,53]. However, we can only claim a full understanding of the underlying mechanisms if we are able to build stable and active proteins in the laboratory by reproducing proposed gene duplication and fusion events. Along these lines, recent advances in computational and rational design afforded the creation of stable chimeric proteins by fusing gene fragments from different proteins [10,16,42] or by generating completely artificial proteins from scratch [24]. However, these artificial proteins lacked measurable catalytic activity. In contrast, state-of-the-art computational approaches have allowed for the introduction of new, albeit weak enzymatic activities on existing natural protein scaffolds [19,39,43]. Importantly, these weak activities could be significantly improved by directed laboratory evolution, that is the combination of random mutagenesis with powerful selection or screening techniques [2,22,23,47,48,51].

The $(\beta\alpha)_8$-barrel is one of the oldest, most versatile and ubiquitous protein folds [52]. It is found in about 10% of all proteins with known three-dimensional structure [14]. $(\beta\alpha)_8$-Barrel enzymes can act as oxidoreductases, transferases, lyases, hydrolases and isomerases, thereby covering five of the six enzyme commission (EC) classes. The canonical barrel consists of at least 200 amino acids grouped in eight units. Each unit contains a β-strand which is connected to an α-helix via a βα-loop. The individual modules are linked via αβ-loops. The central barrel is formed by the eight β-strands and surrounded by the eight α-helices. Residuesimportant for substrate specificity and catalysis are found at the C-terminal ends of the β-strands and in the connecting βα-loops whereas the remainder of the structure including the αβ-loops on the opposite face of the barrel are important for stability [45]. The modular structure of the barrel suggests an evolutionary precursor that consisted of $(\beta\alpha)_{n<8}$-modules, and there are multiple hints indicating that modern $(\beta\alpha)_8$-barrels have evolved from $(\beta\alpha)_2$- and $(\beta\alpha)_4$-fragments via gene duplication and fusion events [6,16,25,38]. In particular, the strong fourfold and twofold internal symmetry of the $(\beta\alpha)_8$-barrel enzymes *N*′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) isomerase (HisA) and imidazole glycerol phosphate synthase (HisF), which

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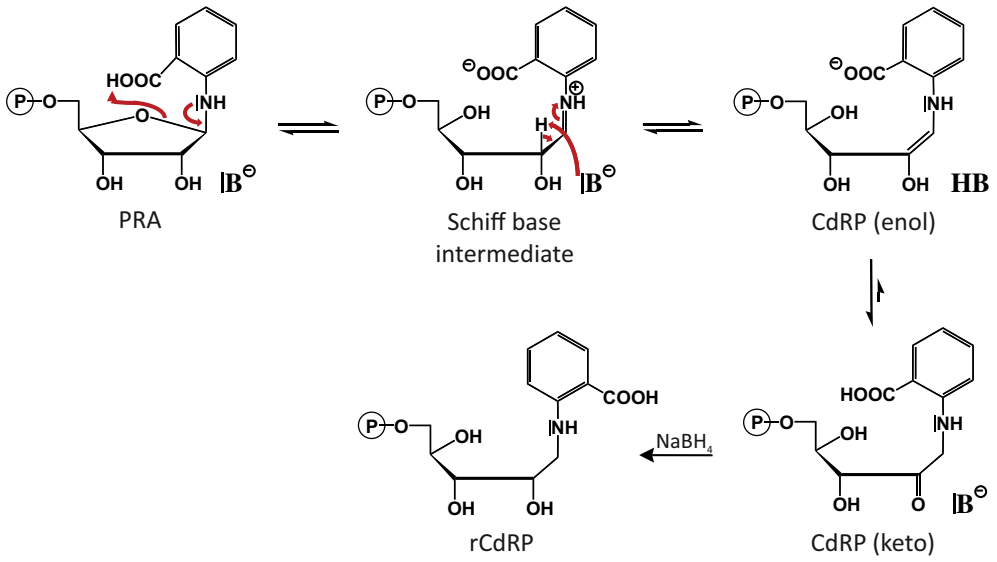

Fig. 1. Scheme for the isomerization of phosphoribosyl anthranilate (PRA) to 1-(2-carboxy-phenylamino)-1′-deoxyribulose-5′-phosphate (CdRP) by HisF-D130V + D176V from *T. maritima*, and the reduction of CdRP to rCdRP by sodium borohydrate ($NaBH_4$). The furanose ring oxygen of PRA is protonated by the carboxylic acid of the anthranilate moiety (substrate assisted catalysis). The proton is most probably transferred via a water molecule (not shown for clarity) [37]. The C2′ atom is then deprotonated by the general base D11 facilitated by the Schiff base intermediate which acts as electron sink. The keto form of CdRP is finally formed by the spontaneous tautomerization of its enolamine form. Reduction of CdRP with sodium borohydrate yields rCdRP.

catalyze two successive steps in histidine biosynthesis, support this scenario [16,27]. We have previously reconstructed the proposed evolutionary process of gene duplication from a $(\beta\alpha)_4$-half-barrel to a $(\beta\alpha)_8$-barrel starting with the C-terminal half of HisF (HisF-C) as a model for the original half-barrel [16,17,40]. Two copies of HisF-C were fused to the HisF-CC construct, which was then stabilized by a combination of rational design and directed evolution, yielding the stable but inactive constructs Sym1 [17] and Sym2 [5].

Here we report on the next and ultimate step of reconstructing $(\beta\alpha)_8$-barrel evolution by establishing enzymatic activity on the artificial $(\beta\alpha)_8$-barrel protein Sym2. As model reaction we used the isomerization of phophoribosyl anthranilate (PRA) to 1-(2-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CdRP), a transformation that is catalyzed by the PRA isomerase (TrpF) within tryptophan biosynthesis (Fig. 1). PRA isomerization is mechanistically rather simple [15,37] and has already been established on several $(\beta\alpha)_8$-barrel enzymes including HisA, HisF [20,26], a HisAF chimera [6], as well as the α-subunit of tryptophan synthase (TrpA) [12]. In a first step, we used rational design to establish binding of reduced CdRP (rCdRP), which is a stable product analog of TrpF. Subsequent to the successful introduction of a high affinity binding pocket for rCdRP on the Sym2 scaffold, random mutagenesis with a very high error rate was performed to create PRA isomerase activity. In vivo selection of variant clones from the resulting library in *trpF*-deficient *Escherichia coli* cells identified an active Sym2 variant that differed from the parental protein at nine amino acid positions. After reducing the number of mutations upon keeping activity, we ended up with a symmetrical construct that contained only six amino acid differences between the two fused $(\beta\alpha)_4$-half-barrels.

2. Materials and methods

2.1. Cloning of Sym2 variants

Cloning of the *sym2* gene into vector pET24a(+) using *Nde*I, *Bam*HI, and *Xho*I restriction enzymes was described previously [5]. For construction of the *sym2_bindC* gene, the 3′-half-sequence of the *sym2* gene, *sym2-C*, was first cloned into plasmid pET24a(+) using BamHI and XhoI restriction sites [5] and mutated at position $D176_C$ by QuikChange™ PCR [50] using the complementary oligonucleotides 5′-CCAGTATCGACAGAGTCGGCACAAA ATCGGG-3′ and 5′-CCCGATTTTGTGCCGACT CTG TCGATACTGG-3′ as overlapping primers (nucleotide exchange underlined), yielding *sym2C-D176V*$_C$. Subsequently, the *sym2C-D130V*$_C$ + *D176V*$_C$ sequence was amplified by PCR using *sym2C-D176V*$_C$ as template. The oligonucleotide 5′-GGTCGC GGATCCCAG GCCGTTGTCGTGGCGA-TAGTTGCAAAAAGAGTGGATGGAGAG-3′ with a BamHI-site at the 5′-terminus (underlined) and a nucleotide exchange (underlined) was used as 5′-primer, and the oligonucleotide 5′-GCTAGT-TATTGCTCAGCGG-3′ was used as 3′-primer. The amplified fragment *sym2C-D130V*$_C$ + *D176V*$_C$ was cloned into pET24a(+)-sym2 N [5] using *Bam*HI and *Xho*I restriction sites, yielding pET24a(+)-*sym2_bindC*. For construction of the *sym2_bindN* and *sym2_bindNC* genes, the *sym2N-D130V*$_N$ + *D176V*$_N$ sequence was amplified by PCR using *sym2C-D130V*$_C$ + *D176V*$_C$ as template. The oligonucleotide 5′-ATACATATGCAGCGCGTT GTCG TGGCGATA-3′ with an *Nde*I-site at the 5′-terminus (underlined) was used as 5′-primer, and the oligonucleotide 5′-CTGGGATCCGAAGGTCTGT-GCGATTTGTGTGATGAGGCTCGGGTTTTCGACAGCGGCAGTATTGATA-GAGACCTTGTCAGCACCTGCCAGGAAG-3′ with a *Bam*HI-site at the 3′-terminus (underlined) was used as 3′-primer. The amplified fragment *sym2N-D130V*$_N$ + *D176V*$_N$ was cloned into pET24a(+) using *Nde*I and *Bam*HI restriction sites, yielding pET24a(+)-*sym2N-D130V*$_N$ + *D176V*$_N$. Next, *sym2-C* and *sym2C-D130V*$_C$ + *D176V*$_C$ were cloned into pET24a(+)-*sym2N-D130V*$_N$ + *D176V*$_N$ to yield pET24a(+)-*sym2_bindN* or pET24a(+)-*sym2_bindNC*, respectively. The *cpSym2_bindC* gene was cloned from a modified pTNA plasmid (see below) into a modified pET24a(+) vector using *Sph*I and *Hind*III restriction sites.

2.2. Generation of a plasmid-encoded cpSym2_bindC gene library with randomized N-terminal half

The *cpSym2_bindC* gene was generated and cloned into a modified plasmid, which was derived from pTNA [3,30] and allows for constitutive expression in *E. coli*. The plasmid was constructed in