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Review

Directed evolution of $(\beta\alpha)_8$-barrel enzymes

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Abstract

Natural molecular evolution supplies us with manifold examples of protein engineering. The imitation of these natural processes in the design of new enzymes has led to surprising and insightful results. Well-suited for design by evolutionary methods are enzymes with the common and versatile $(\beta\alpha)_8$-barrel fold. Studies of enzyme stability, folding and design as well as the evolution of $(\beta\alpha)_8$-barrel enzymes are discussed.

Keywords: $(\beta\alpha)_8$-Barrel; TIM-barrel; Directed evolution; Random mutagenesis

1. Introduction

Adaptation of structure and function to the changing environments is frequently observed in nature. Enzymes evolve when proteins acquire new catalytic or binding properties that give organisms improved fitness. Mechanisms of natural molecular evolution serve well as examples for laboratory evolution and protein design. Vice versa, enzyme evolution in the laboratory can lead to a deeper understanding of natural evolution.

Thus, in the last couple of years, 'directed evolution' techniques that use random mutagenesis and/or recombination of genes combined with the selection or screening for new improved properties of the protein products became increasingly important. Diverse methods for randomization have been developed such as error-prone PCR [1], DNA-shuffling [2], StEP [3] or ITCHY [4], to name a few. Most design efforts by directed evolution aim at changing the function or stability of a protein. In other cases, it has been used to better understand protein folding or to develop systems that reveal the structural determinants of protein–protein interactions.

A scaffold that has caught a lot of attention is the $(\beta\alpha)_8$-barrel fold (also known as TIM-barrel). About 10% of all the enzymes with known structure adopt this fold. $(\beta\alpha)_8$-barrels are catalytically versatile containing representatives from five of the six enzyme classes [5,6]. The canonical $(\beta\alpha)_8$-barrel consists of an inner core of eight parallel β-strands surrounded by eight α-helices (Fig. 1). The active site is always located at the C-terminal ends of the β-strands and within the loops that link β-strands with α-helices, while stability is maintained by the opposite side of the barrel [7]. This arrangement gives the functional groups structural independence, making it possible to change catalytic activities without compromising stability [8]. Therefore, $(\beta\alpha)_8$-barrels are considered to be ideal tools for the study of enzyme stability, folding and design as well as for natural and directed enzyme evolution, which are the topics of this review.

2. Folding and folding units

Silverman et al. [9] examined the robustness of the $(\beta\alpha)_8$-barrel fold by testing the amino acid sequence restrictions at 182 out of 250 positions in yeast triose phosphate isomerase (TIM). Degenerate libraries, using only seven amino acids in binary polar/non-polar patterns, and randomly mutagenized libraries were constructed by the use of oligonucleotide assembly and gene shuffling. The libraries were then investigated by functional selection in the TIM-deficient

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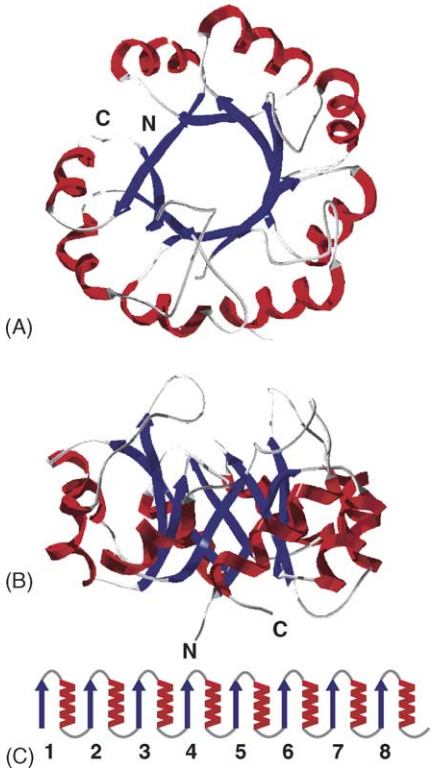

Fig. 1. The $(\beta\alpha)_8$-barrel fold. A ribbon diagram of the phosphoribosyl anthranilate isomerase from *Thermotoga maritima* (1nsj.pdb) is shown with (A) a top view and (B) a side view of the central β-barrel. The typical secondary structure composition of the canonical $(\beta\alpha)_8$-barrel is depicted in (C) a topology model.

Escherichia coli strain DF502 [10]. It was observed that residues at the interface between β-sheets and α-helices, turn sequences, α-helix capping and α-helix stop motifs were extremely mutable. In contrast, residues forming the central core of the β-barrel, as well as a buried salt bridge, and β-strand stop motifs were sensitive to substitution. The substitutions that were not tolerated mainly clustered in the C-terminal quarter of the barrel (Fig. 2). In some $(\beta\alpha)_8$-barrels, this quarter has been identified as an independent folding unit [11,12]. In accordance with these findings, the detrimental effects of many of the mutations appear to result from aggregation [9].

The stability and folding of $(\beta\alpha)_8$-barrels and its substructures have been extensively studied by fragment complementation [11,13], circular permutation [14] and more recently by β/α-unit reshuffling experiments [15]. Again, the fold appeared to be highly tolerant towards these modifications. In order to identify folding unit boundaries, Hiraga et al. [16] created random fragmentation libraries from the α-subunit of tryptophan synthase (TrpA) in which randomly digested N- and C-terminal fragments were combined. Clones expressing enzymatic activity were selected in an *E. coli trp*A$^-$ strain on the medium lacking tryptophan. The results suggest that TrpA tolerates cleavage in the loops following the extra helixes 2′ and 5, suggesting that here folding unit boundaries are located. Many of the

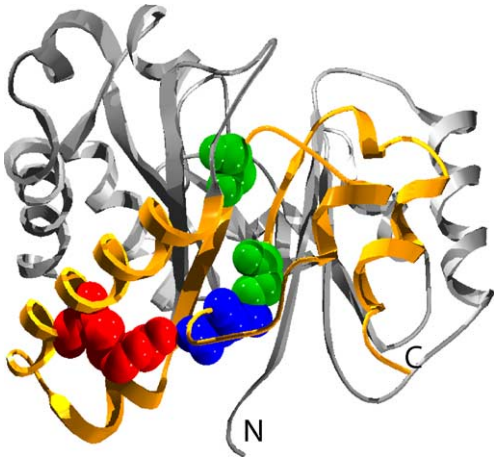

Fig. 2. The positions of non-mutable amino acids are clustered in the C-terminal part of the structure of TIM from *S. cerevisiae* (7tim.pdb) [9]. This part, which is shown in orange, has been identified as an independent folding unit in other TIM-barrel proteins [11,12], suggesting that the detrimental effects of many of the mutations might result from aggregation. The residues with most severe effects upon mutation are indicated: Arg189 in red, Asp227 in blue and Gly228 and Gly209 in green.

clones had overlapping fragments or N- and C-fragment fusions with inserted sequence duplications that occurred primarily at the cleavage sites [16]. The tolerance of overlap and insertions corresponds well with the observation that in nature, many $(\beta\alpha)_8$-barrels have accepted insertions of secondary structure elements and whole domains in the loop regions [17].

For similar reasons, phosphoribosyl anthranilate isomerase from *Saccharomyces cerevisiae* (Trp1b) has been used for the development of a so-called split-protein sensor (Fig. 3) [18]. These sensors can be used for the analysis of protein–protein interactions in living cells, the first and best working example being the split-ubiquitin system [19]. In the newly developed system, two proteins (P1 and P2) are expressed as fusion proteins with an N- and C-terminal fragment of Trp1b. Upon interaction of P1 with P2, the N- and C-terminal fragments of Trp1b are forced into a tight and functional complex that can complement an auxotrophic yeast strain EGY48 on medium without tryptophan [18]. From a library of 1600 random split-Trp clones, 11 potential candidates were selected by complementation. In only five of these, the expressed fragments were in frame with P1 and P2. By repression of N-terminal fragment expression, it was then tested whether both the Trp1b-fragments are required for complementation. One C-terminal fragment (11–224) already possessed enzymatic activity while the other four C-terminal fragments were not able to complement on their own. In the four remaining split-pairs, complex formation depended on the interaction of the proteins fused to their termini which was shown by their lost ability to complement tryptophan auxotrophy upon removal of P2 from the N-terminal fragments. Therefore, these split-Trps are all suited to sense protein interaction in the cytosol. An additional test showed that two of these split-Trps were also able to detect

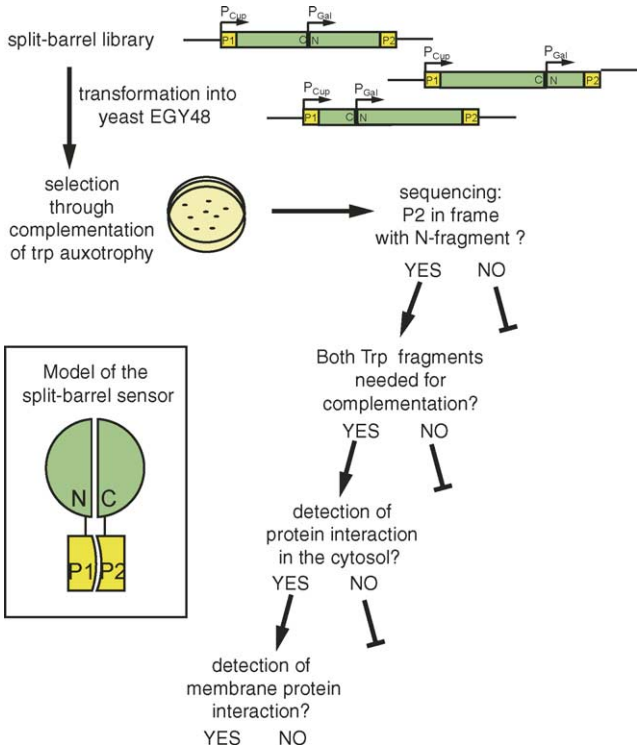

Fig. 3. Development of a split-Trp system (flow sheet) [18]. A library of genes coding for randomly split Trp1b-variants (in green, original N- and C-termini are indicated) was searched for functional variants by complementation of a Trp-auxotrophic yeast strain. The two proteins P1 and P2 (shown in yellow) are fused to the Trp1b fragments and force them into an active complex that would not form without the interaction between P1 and P2. In order to detect protein–protein interactions, P1 and P2 are replaced by candidate proteins (see text for detail).

the interaction of membrane proteins. The fragmentation sites were then mapped onto the known crystal structures of homologous enzymes in *E. coli* and *Trypanosoma maritima* by sequence alignment: the position of one of the splits (Trp44) was found to be very close to the active site while in another variant (Trp204), a loop of eight highly conserved residues is deleted that was thought to be important for binding of the phosphate group of the substrate [20]. These positions would not have been predicted to tolerate disruption, which illustrates the advantage of this evolutionary approach.

3. Catalytic activity

There is a great interest in increasing the catalytic power of enzymes using directed evolution. Knowles and co-workers asked how easy it would be to improve the catalytic potency of an enzyme [21]. They randomly mutated the gene of a sluggish variant (Glu165Asp) of the dimeric chicken TIM by the use of ''spiked'' oligonucleotide primers [22]. The 10 oligonucleotides that were synthesized with a predetermined level of altered phosphoramidites spanned the whole gene. Each of these was used in a standard oligo-directed mutagenesis procedure, thereby, producing 10 different libraries with mutations in the respective oligonucleotide window. Variants with improved catalytic activity compared to TIM-Glu165Asp were selected by their ability to complement a TIM-deficient strain on selective growth medium containing glycerol. Six variants were isolated that were not revertants to the wild-type TIM and had one amino acid substitution in addition to Glu165Asp. All the amino acid substitutions that improve the specific catalytic activity are located close to the catalytic center at the sites that are highly conserved among the species [21]. Since different single amino acid substitutions increased the catalytic activity of the sluggish TIM variant, it can be concluded that there are multiple ways to improve the catalytic power of this particular enzyme.

The catalytic activity of a monomeric version of the homodimeric TIM from *T. brucei* has also been improved by directed evolution [23]. By shortening loop 3, the main component of the dimer interface, a stable monomeric variant was designed that was still functional but possessed lower activity (turnover number) [24]. Two strategies were followed to recover the activity of monoTIM: randomization of loop 2 to reinforce intrasubunit interaction in order to compensate for the loss of loop 3 and random mutagenesis of the entire gene. Interestingly, both the strategies led to two exchanges at neighboring positions: the replacement of alanine 43 by a proline and the substitution of threonine 44 by either alanine or serine [23]. This result is significant considering the extremely low probability of randomly mutating two specific positions in the same gene. Kinetic characterization of the two activated variants showed a 11-fold increase in k_{cat} and a four-fold reduction in K_m.

An enzyme with a wide range of applications, whose catalytic power has been improved, is the bacterial phosphotriesterase (PTE) [25]. It degrades highly toxic organophosphates that are commonly used in insecticides and chemical warfare agents, however, with varying effectiveness. Rational design on the basis of the known X-ray structure was used to drastically change the stereo-selectivity of PTE for several racemic substrates [26]. Furthermore, by the use of directed evolution techniques, the catalytic activity of PTE was increased [25]. Libraries of cell surface displayed PTEs were screened for the hydrolysis of methyl parathion, based on the detection of the colored product *p*-nitrophenol. From two rounds of DNA-shuffling, several improved variants were isolated, of which the best displayed a 25-fold increased catalytic turnover over the wild-type enzyme [25]. This improved variant contained seven amino acid substitutions, of which only one (His257Tyr) is positioned in the active site. This exchange apparently reduces the size of the binding pocket which allows better accommodation of the relatively small substrate. The other alterations are located away from the catalytic center. Their influence on the catalytic activity is difficult to explain.

Besides recovering and increasing enzyme activities, directed evolution was used to generate a new catalytic site

in a $(\beta\alpha)_8$-barrel. Wymer et al. [27] set out to improve *E. coli* 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase activity by randomizing residues 131–168, which harbor the active site. Unexpectedly, they found a variant with an altered substrate specificity profile, in which the active site lysine was shifted from β-strand 6 (Lys133) to β-strand 7 (Lys161). The double mutant Lys133Gln/Thr161Lys was catalytically active and displayed Michaelis-Menten kinetics. In contrast, the single mutant Lys133Gln did not show any aldolase activity. Moreover, lysine 161 was shown to form a Schiff base by reductive alkylation [27].

4. Cold adaptation

Enzymes from hyperthermophilic organisms are often barely active at mesophilic temperatures which is probably due to their conformational rigidity [28]. To better understand the relationship between stability, flexibility and catalytic activity in $(\beta\alpha)_8$-barrels, Merz et al. [29] used in vivo selection in *E. coli* to isolate indoleglycerol phosphate synthase (TrpC) variants from the thermophile *Sulfolobus solfataricus* that were catalytically more active at 37 °C than the wild-type enzyme. Several single and double amino acid substitutions improved the turnover rate albeit at the cost of substrate and product affinity. Fast kinetic measurements showed that in the wild-type protein, substrate turnover is limited by product release. However, in the selected TrpC variants, the product is released more rapidly, shifting the rate-limiting step of the reaction to the preceding chemical step. Protease digestion suggested that a susceptible loop, which is located close to the active site, is more flexible in the activated variant than in the wild-type enzyme. However, the thermostability of most of the tested variants was comparable to that of the wild-type enzyme.

In a similar experiment, a library of β-glucosidase CelB variants from the hyperthermophile *Pyrococcus furiosus* was generated through random mutagenesis and screened for improved hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside at room temperature [30]. Multiple candidates with up to three-fold increased turnover rates were identified and ten of them were further characterized. It was found that the increased turnover at low temperatures was achieved at the expense of activity at 90 °C. The amino acid substitutions were found near the active-site region, at subunit interfaces, enzyme surface and buried in the interior of the monomers. This shows that the increase in activity at low temperature can be achieved in multiple ways including altered substrate specificity and increased flexibility by overall destabilization of the enzyme.

Another experiment to increase cold adaptation for industrial purposes has been carried out with the xylose isomerase (XylA) from *Thermus thermophilus*, whose larger N-terminal domain folds into a $(\beta\alpha)_8$-barrel that harbors the active site [31]. A mutant library of the *xyl*A gene was generated in *E. coli* by random mutagenesis and was screened for higher activity at 30 °C by the use of a pH-indicator in the growth medium. Three mutants were identified that carried a common amino acid substitution and were found to be active over a broader pH-range that was extended into the alkaline region compared to the wild-type enzyme. Due to overall low activity at 30 °C, the catalytic parameters were compared at 60 °C. For the substrate xylose, the turnover number but not the catalytic efficiency was increased while for the substrate glucose, an increase in both k_{cat} and k_{cat}/K_M was found. The amino acid substitutions were neither affecting the substrate-binding site nor the metal-binding site. The authors suggest that the substitutions increase flexibility in areas that undergo conformational transitions during catalysis [31].

Similarly, an already rationally improved single mutant variant of the extremely thermostable *Thermotoga neapolitana* xylose isomerase was subjected to directed evolution experiments in order to further increase its activity on glucose at low temperatures and pH [32]. Two rounds of random mutagenesis were performed and transformants were screened at 60 °C (pH 7.0) and 80 °C (pH 5.2). One variant with two additional amino acid substitutions was identified to be more active on glucose at all the conditions than the parent and the wild-type protein. Therefore, activity could be increased at lower temperature and acidic pH without compromising thermal stability, suggesting that the determinants of activity and thermostability are not linked [32]. The introduced amino acid substitutions introduce a kink in an α-helix and a cavity that presumably increases the mobility of the active site.

The experiments on indoleglycerol phosphate synthase, β-glucosidase and xylose isomerase suggest that single amino acid substitutions, distant or close to the catalytic center, can lead to cold adaptation. They also demonstrate the advantage of the directed evolution approach since the identified substitutions could not have been predicted with our current knowledge of structure–function relationship.

5. Thermostability

Proteins from thermophiles have been studied in detail and compared with their homologs from mesophilic organisms in order to identify the structural determinants of thermostability [33,34]. It appears that various factors can contribute to the stabilization of thermophilic proteins, including increased association state, additional salt bridges and hydrogen bonds [34]. In extreme cases though, a single amino acid substitution can stabilize a protein considerably. For example, the melting temperature of TIM from *Leishmania mexicana* was increased from 57 to 83 °C without loosing catalytic efficiency at 25 °C by replacing a glutamine by a glutamate in the loop following β-strand 3. This loop is completely buried within the dimer interface and the substitution restores an intersubunit hydrogen-bonding network that is conserved in other TIMs [35].

However, sequence and structural data, if available at all, does not always suggest such straightforward solutions. The thermal resistance of β-glucosidase A from *Paenibacillus polymyxa* was increased by several amino acid exchanges which were introduced by directed evolution [36]. X-ray crystallography showed that a newly introduced Lys96 at the N-terminus of α-helix 2 formed a salt bridge with Asp28 which is located close to the active site [37]. The thermal stability of β-glucosidase B from the same organism was increased by random mutagenesis followed by shuffling of improved mutants. The best enzyme variant displayed a 20-fold increase in heat resistance and an eight-fold increase in catalytic activity [38]. According to a homology-based structural model, the stabilising amino acid substitutions are positioned in loops on the periphery of the $(\beta\alpha)_8$-barrel, while the only activating substitution is located on one of the β-strands of the barrel.

Also, thermostable variants of lactate oxidase were isolated by screening a library of mutants that were generated by random mutagenesis. The characterization of one of the substitutions (Glu160Gly) revealed that the reduced side chain volume in combination with lost electrostatic repulsion contributed to the thermoresistance [39].

The experiments on TIM, β-glucosidase A and B, and lactate oxidase show that thermostability can be increased by various means, as the introduction of new hydrogen bonds and salt bridges, and the substitution of conflicting side chains. This was possible without compromising activity, suggesting separate determinants of activity and thermostability.

6. Substrate-specificity and stereo-selectivity

Another application of protein engineering with industrial relevance is the alteration of enzyme specificity, especially enantio-selectivity [40]. The $(\beta\alpha)_8$-barrel enzyme aldolase has been converted into an enzyme with improved catalytic efficiency, altered substrate specificity and stereo-selectivity. The *E. coli* KDPG aldolase was subjected to multiple rounds of random mutagenesis and DNA-shuffling, and the resulting libraries were screened for variants that are more efficient in accepting non-phosphorylated substrates as well as for the variants with altered enantio-selectivities regarding D- and L-sugars [41]. The effective substitutions were remote from the active site and did occur in non-conserved sequence regions, and the mechanistic basis for the observed functional changes remained unclear [41]. In an analogous directed evolution experiment, the enantio-selective cleavage of *N*-acetylneuraminic aldolase from *E. coli* was altered, thereby, improving its ability to form L-sialic acid [42]. All amino acid substitutions were again found outside the active site of the $(\beta\alpha)_8$-barrel. The X-ray structure of the best second generation variant showed no significant difference between the mutant and the wild-type enzyme, demonstrating that a small number of mutations outside the active site can significantly affect specificity and stereo-selectivity [42].

The enantio-specificity of L-hydantoinase from *Arthrobacter aurescens* is substrate-dependent as D-methyl-thioethyl-hydantoin is preferred over the L-enantiomer. Single amino acid substitutions identified by directed evolution experiments were found to alter the enantio-specificity of the enzyme in both directions [43]. While increased D-selectivity was easily obtained, an L-selective variant was only found after saturation mutagenesis of position 95 whose substitution had been observed to relax enantio-selectivity. The single mutation Ile95Phe was L-selective and five-fold more active than the wild-type. The X-ray structure of the wild-type hydantoinase showed that each subunit of the tetrameric protein consists of an elliptically distorted $(\beta\alpha)_8$-barrel harboring the active site and a β-sheet domain [44]. It is suggested that the mutation Val154Ala increases D-enantio-specificity by offering more space for the substrate side chain and that Ile95Phe increases L-enantio-specificity by increasing the hydrophobicity close to the methyl-thiolethyl-hydantoin side chain [44].

Based on the structural knowledge, substrate specificity of D-hydantoinase from *Bacillus stearothermophilus* was altered by saturation mutagenesis of selected positions of active site loops [45,46]. The turnover for aromatic amino acids increased gradually as the size of the amino acid side chain at position 159 decreased. Furthermore, saturation mutagenesis of two additional residues, 63 and 65, led to a 10-fold increase in activity towards the aromatic hydantoin derivative hydroxy-phenyl-hydantoin [46].

The substrate-specificity of barley α-amylase has also been altered by saturation mutagenesis of few positions in selective active site loops of the $(\beta\alpha)_8$-barrel domain followed by screens for different substrates [47,48].

The engineering of aldolases, hydantoinases and amylases show that small changes distant or close to the active site can significantly affect substrate specificity and enantio-selectivity. These effects are often difficult to predict, justifying the directed evolution approach.

7. Directed versus natural evolution

There have been numerous speculations on the evolution of the $(\beta\alpha)_8$-barrel enzyme fold. The common structural features support divergent evolution from an ancestral barrel, while versatility of function and absence of sequence similarity are in favor of convergent evolution. There have been multiple attempts to identify evolutionary links by searching for overall sequence and structural similarities [49,50], and by comparing metal and phosphate binding sites and the architecture of catalytic centers [17,51–53]. More recently, in order to trace relationships between $(\beta\alpha)_8$-barrel enzymes, it was attempted to reconstruct evolution in the laboratory.

The three $(\beta\alpha)_8$-barrel enzymes TrpF, TrpC and TrpA catalyze consecutive steps within the tryptophan biosynthetic pathway and share related phosphate-binding sites

[51]. Based on these similarities, a common evolutionary origin was postulated, serving as an example for pathway evolution theories [54]. In histidine biosynthesis, the structures of the two closely related $(\beta\alpha)_8$-barrel enzymes ProFAR isomerase (HisA) and imidazole glycerol phosphate synthase (HisF) reveal an internal two-fold symmetry with respect to sequence and structure [55,56]. On the basis of these findings, it was postulated that the two enzymes have arisen from a common $(\beta\alpha)_4$-half-barrel precursor through a series of gene duplication events. This hypothesis is supported by the fact that the separately produced half-barrels of HisF, which predominantly form dimers, exhibit well-defined secondary and tertiary structures while upon co-expression, they assemble to a catalytically fully active heterodimeric complex [57]. Moreover, HisF is able to catalyze the HisA reaction, albeit with low efficiency [56]. Within this subset of $(\beta\alpha)_8$-barrel enzymes from histidine and tryptophan biosynthesis, evolutionary events were mimicked by interconverting one enzyme into another, that is, by changing substrate specificities and/or catalytic mechanisms. In the first successful example, TrpF activity was established on the scaffold of HisA by random mutagenesis and selection in vivo [58]. The enzyme pair was chosen because HisA and TrpF use the same reaction mechanism to convert an aminoaldose into the corresponding aminoketose (Fig. 4A) [59]. Surprisingly, a single amino acid exchange (Asp127Val) was sufficient for almost the complete interconversion of substrate specificity of HisA.

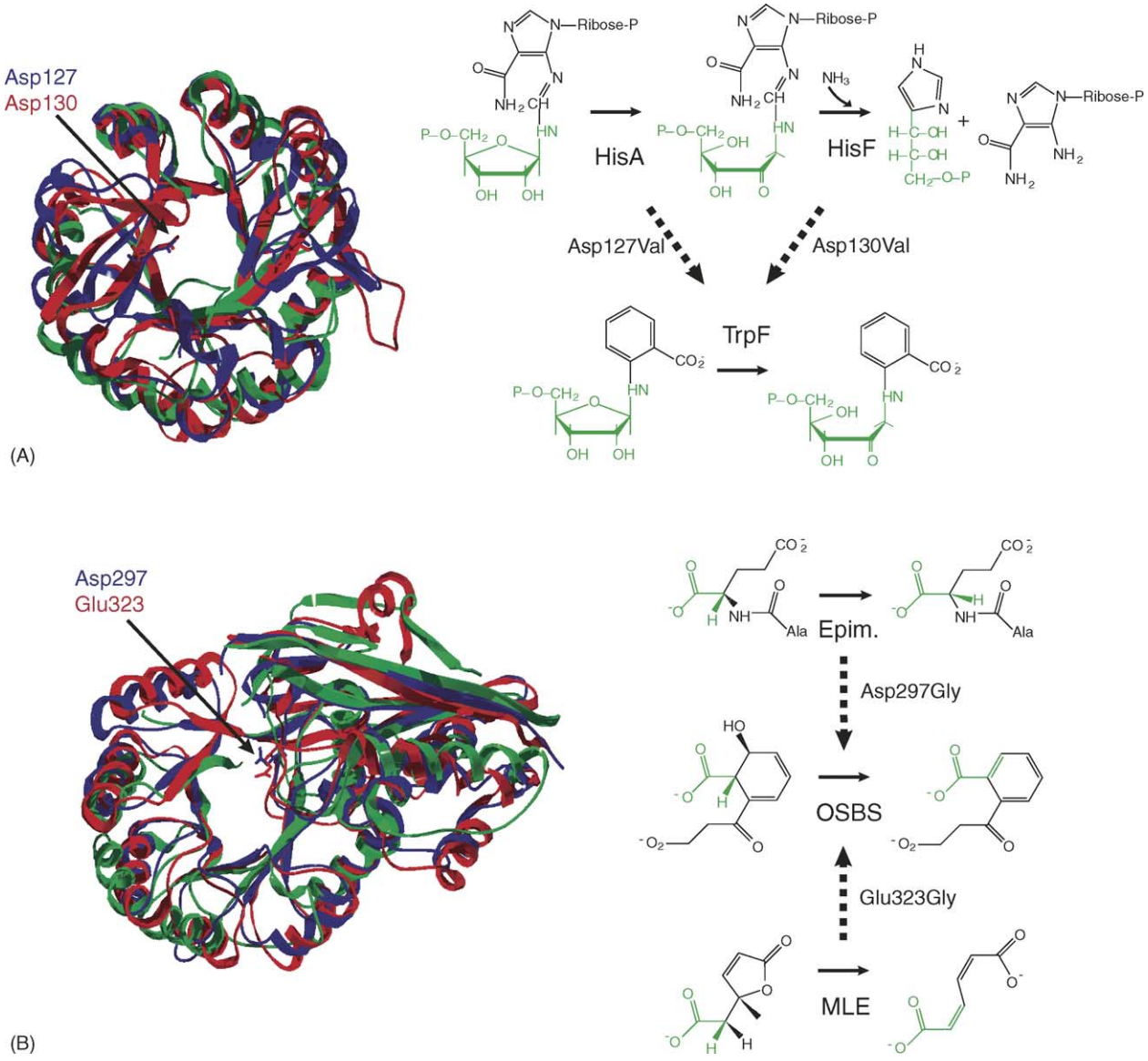

Fig. 4. Interconversion of catalytic activities of enzymes from (A) tryptophan and histidine biosynthesis and (B) the MLE subgroup of the enolase superfamily. The positions for the single amino acid substitutions that lead to the new activities are indicated in the structural superposition of (Panel A) HisA (1qo2.pdb, in blue), HisF (1thf.pdb, in red) and TrpF (1nsj.pdb, in green) and (Panel B) epimerase (1jpd.pdb, in blue), MLE (1muc.pdb, in red) and OSBS (1fhu.pdb, in green). The directions of the interconversions are indicated by broken arrows that are labeled with the identified amino acid substitutions [58,60,62].

Based on this result and the similarities between HisA and HisF [56], TrpF activity was also established on the HisF scaffold by the analogous Asp130Val exchange (Fig. 4A) [60]. Saturation random mutagenesis of position 127 in HisA and 130 in HisF showed that the removal of the negatively charged carboxylate side chain is necessary and sufficient to establish TrpF activity, presumably because this allows the binding of the negatively charged substrate of TrpF to the active site of HisA and HisF [60].

Members of the enolase superfamily consist of two domains, one of them being a modified version of the $(\beta\alpha)_8$-barrel. They possess a common catalytic strategy which in the first step comprises the abstraction of an α-proton from a carboxylate anion substrate. The resulting enolate intermediate is stabilized by a metal ion which binds to the ends of the third, fourth and fifth β-strand [61], and then converted further into the product. Three subgroups can be distinguished based on their active site residues. Within the muconate lactonizing enzyme (MLE) subgroup, the evolutionary potential of $(\beta\alpha)_8$-barrel enzymes was investigated by Gerlt and co-workers [62]. The active sites of *o*-succinylbenzoate synthase (OSBS) from *E. coli*, MLE II from *Pseudomonas spec* and L-Ala-D/L-Glu epimerase from *E. coli* contain two catalytically essential lysines at the end of β-strands 2 and 6, suggesting their evolutionary relationship. Rational design of the epimerase and directed evolution of MLE II both yielded protein variants with OSBS activity (Fig. 4B)[62]. They differed from their progenitor by a single amino acid substitution and were still able to catalyze their parental reaction, though at a slower rate. In both cases, a glycine residue was introduced in β-strand 8 replacing an aspartic and a glutamic acid, respectively (Fig. 4B). The resulting cavity presumably relaxed the substrate specificity by allowing the binding of OSBS which is sterically impeded in the wild-type enzymes [62].

The experiments suggest that in spite of low sequence similarities, the $(\beta\alpha)_8$-barrel enzymes of the amino acid biosynthesis and within the enolase superfamily are closely related. The ease with which the TrpF and OSBS activities can be established on their homologs illustrates the functional plasticity and capability of the $(\beta\alpha)_8$-barrel fold for both natural and laboratory evolution.

8. Conclusions

$(\beta\alpha)_8$-Barrel enzymes have been altered by directed evolution in various ways which illustrates both the versatility of this experimental approach and the robustness of the fold. Prominent examples are the increase of stability and catalytic activity, and the interconversion of related enzymes from the same and different metabolic pathways requiring the change of substrate specificities. Moreover, the successful combination of high stability with high catalytic activity at low temperatures and the development of specificity for non-natural substrates points to the potential industrial applications. These achievements also illustrate that directed evolution and rational design experiments perfectly complement each other and are most powerful when applied in combination. Whereas rational design makes use of the known structural and mechanistic information, directed evolution allows to search sequence space more efficiently.

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