Stability, catalytic versatility and evolution of the $(\beta\alpha)_8$-barrel fold

Birte Höcker*, Catharina Jürgens*, Matthias Wilmanns† and Reinhard Sterner*‡

The $(\beta\alpha)_8$-barrel is a versatile single-domain protein fold that is adopted by a large number of enzymes. The $(\beta\alpha)_8$-barrel fold has been used as a model to elucidate the structural basis of protein thermostability and in studies to interconvert catalytic activities or substrate specificities by rational design or directed evolution. Recently, the $(\beta\alpha)_4$-half-barrel was identified as a possible structural subdomain.

Addresses
*Universität zu Köln, Institut für Biochemie, Otto-Fischer-Strasse 12-14, D-50674 Köln, Germany
†European Molecular Biology Laboratory (EMBL), Hamburg Outstation, c/o DESY, Notkestrasse 85, D-22603 Hamburg, Germany
‡e-mail: reinhard.sterner@uni-koeln.de

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Abbreviations

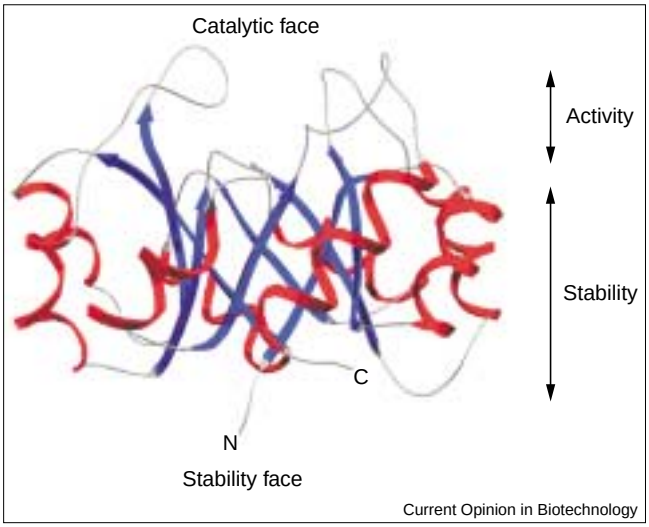
CGTase	cyclodextrin glycosyltransferase
HisA	ProFAR isomerase
HisF	imidazole glycerol phosphate synthase
TIM	triosephosphate isomerase
TrpC	indole glycerol phosphate synthase
TrpF	phosphoribosylanthranilate isomerase

Introduction

Enzymes are highly efficient and specific catalysts that accelerate a broad range of chemically diverse reactions [1]. In their natural environment, enzymes need to be stable in order to maintain their native structures but also have to be flexible to allow conformational changes during catalysis. These opposing requirements are particularly striking for enzymes from extremophiles, which must be both stable and active under extreme conditions of salt, pH and temperature [2,3]. It is important to understand not only how enzyme structure, function and stability are linked, but also to comprehend how modern enzymes have evolved from more simple precursors.

The $(\beta\alpha)_8$ (or TIM) barrel fold provides an excellent model system to address these fundamental questions, which are relevant to both basic and applied research. About 10% of all proteins with known three-dimensional structure contain a $(\beta\alpha)_8$-barrel fold [4,5]; these are versatile enzymes that act as oxidoreductases, transferases, lyases, hydrolases and isomerases [6]. The fold of the canonical $(\beta\alpha)_8$-barrel consists of a closed eight-stranded parallel β sheet, forming a central barrel, which is surrounded by eight α helices. The active-site residues are located on the catalytic face of the barrel, which comprises the C-terminal ends of the β strands and the loops that link β strands with the subsequent α helices. In contrast, the loops that link the α helices with the subsequent β strands, which are located on the opposite face of the barrel, are important for stabilising the fold (Figure 1). This 'division of labour' between the two faces of the β barrel makes it possible to change catalytic activities by mutation without compromising stability [7]. This review summarises recent progress in the use of $(\beta\alpha)_8$-barrels to investigate the relationship between thermostability and enzymatic activity. We also discuss studies to alter both catalytic efficiencies and substrate specificities and to trace the evolution of this ubiquitous fold.

Figure 1



Division of labour within the $(\beta\alpha)_8$-barrel fold. The monomeric TrpC from *S. solfataricus* is illustrated as an example [12]. (For clarity, the N-terminal sequence extension to the barrel is not shown.) β Strands are in blue and α helices in red. The C-terminal ends of the β strands and the loops that connect the β strands with the subsequent α helices form the 'catalytic face' of the barrel, which harbours the active site. The remainder of the fold, including the opposite face of the barrel ('stability face'), is important for conformational stability.

Probing the structural basis of thermostability

In the tryptophan biosynthetic pathway, three enzymes that catalyse subsequent steps are folded as $(\beta\alpha)_8$-barrels, namely phosphoribosylanthranilate isomerase (TrpF), indole glycerol phosphate synthase (TrpC), and the α subunit of trytophan synthase (TrpA) [8].

TrpF is monomeric and labile in most mesophiles, but homodimeric and extremely thermostable in *Thermotoga maritima* [9,10]. The dimer is stabilised by multiple hydrophobic interactions. Most strikingly, two long symmetry-related loops that connect helix α2 with strand β3 at the stability face of the β barrel protrude reciprocally into cavities of the other subunit (Figure 2). In order to test the role of dimerisation for function and stability, monomeric variants were generated by site-directed mutagenesis at the

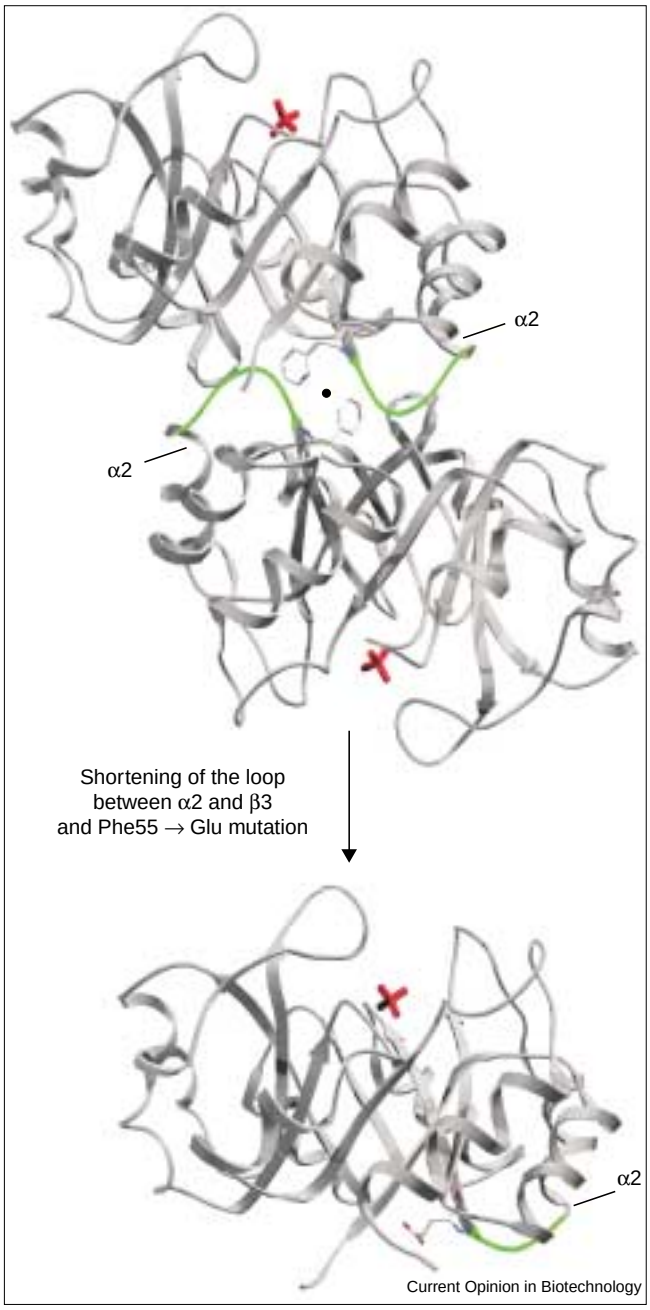
dimer interface [11•]. To this end, the loops connecting helix α2 with strand β3 were shortened by deleting two residues at their tips. The reciprocal hydrophobic interactions were further weakened by replacing Phe55, which is located close to the twofold symmetry axis of the dimer, with either glutamine or glutamate. The Phe55Glu variant is purely monomeric, apparently due to electrostatic repulsions between the two adjacent negative charges. In contrast, the Phe55Gln variant exists in equilibrium between monomers and dimers; increasing the protein concentration, that is increasing the fraction of dimers, results in higher thermostability. Dimerisation, therefore, significantly stabilises the Phe55Gln variant and, by inference, the wild-type TrpF. The monomeric variants were catalytically as active as the wild-type enzyme, indicating that extensive modifications at the stability face of the β barrel are not transmitted to the active site at its catalytic face.

TrpC is monomeric both in mesophilic and hyperthermophilic organisms; however, the hyperthermophilic TrpC variants from *T. maritima* and *Sulfolobus solfataricus* contain twice the number of potentially stabilising salt bridges compared with the mesophilic TrpC from *Escherichia coli* [12,13]. For example, the additional N-terminal helix α_0 of *T. maritima* TrpC is fixed to the surface of the $(\beta\alpha)_8$-barrel by a salt bridge between Arg2 and Asp184; this salt bridge is missing in TrpC from *E. coli*. Another additional salt bridge in *T. maritima* TrpC that is formed between Glu73 and Arg241 cross-links helices α1 and α8, providing an electrostatic clamp across the noncovalent closure of the barrel. The decreased thermostabilities of the Asp184Ala and Arg241Ala variants, which each have one of these salt bridges disrupted, showed that both salt bridges stabilise *T. maritima* TrpC, albeit to different extents [14].

Triosephosphate isomerases (TIMs) provide another example of enzymes that contain a $(\beta\alpha)_8$-barrel fold. In most known TIMs, the sidechain of a glutamine residue, which is located in the loop connecting strand β3 with helix α3, is completely buried within the dimer interface and involved in a conserved intersubunit hydrogen-bonding network. In TIM from *Leishmania mexicana* this glutamine residue is replaced by a glutamate, and therefore the hydrogen-bonding network is disrupted to some extent. Establishing this network in *L. mexicana* TIM by exchanging the glutamate for a glutamine residue resulted in an increase of the melting temperature from 57°C to 83°C, with practically unchanged catalytic efficiency at 25°C [15•].

These case studies show how thermophilic $(\beta\alpha)_8$-barrels are stabilized by various means, including an increased association state, additional salt bridges or hydrogen bonds [3]. They also demonstrate that the thermal stability of enzymes can be drastically increased without necessarily loosing catalytic power at mesophilic temperatures. The combination of high intrinsic stability with high catalytic activity at low temperatures would be useful for the industrial application of enzymes [16].

Figure 2

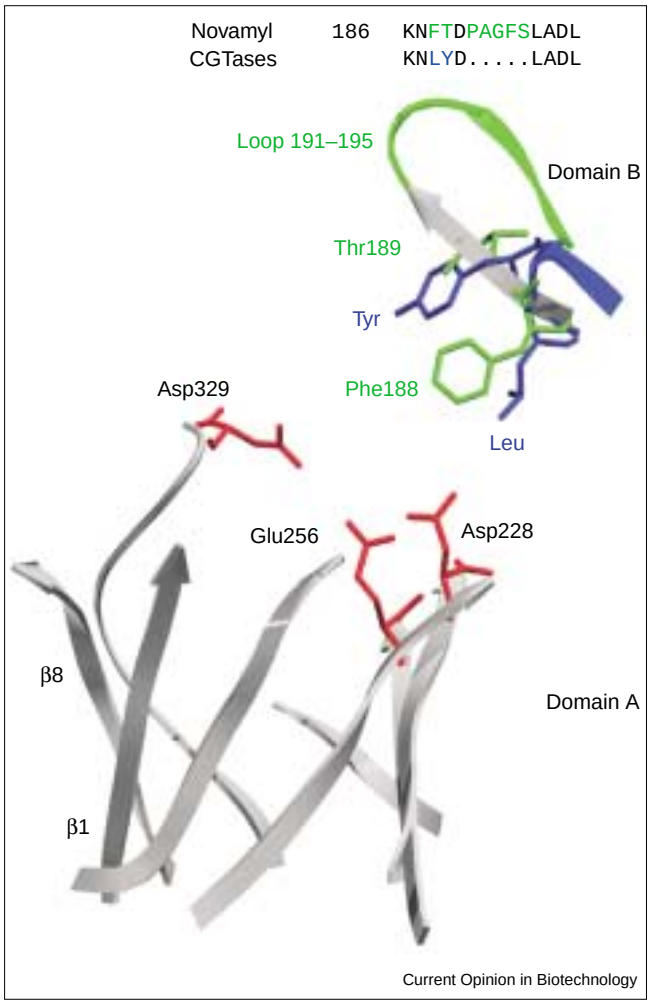


Monomerisation of the native homodimer of TrpF from *T. maritima* by rational design. Monomers were generated by shortening of the loops connecting helices α2 with strands β3 (in green), and by replacing the two Phe55 residues located close to the twofold symmetry axis (shown as a black dot) with glutamates (shown in stick format). The bound phosphate ions (red tetrahedrons) identify the active sites. The monomeric variants are catalytically as active as the dimer, but far more thermolabile [11•].

Amino acid restrictions for a stable fold

The effects of amino acid replacements on the stability of TIM were tested in a comprehensive study [17••]. Combinatorial mutagenesis, followed by selection *in vivo* for retention of catalytic activity, demonstrated that the

Figure 3



Extension of the product specificity of the glycoside hydrolase Novamyl by rational design. Compared with the consensus sequence of CGTases in domain B, Novamyl contains a loop insertion consisting of five residues (in green) and phenylalanine and threonine (in green) instead of leucine and tyrosine (in blue). To convert Novamyl into a cyclodextrin-producing enzyme, the loop insertion was deleted and the two amino acid changes Phe188Leu and Thr189Tyr were introduced [30•]. The catalytic aspartate and glutamate residues (in red) are located in close proximity within domain A, which is a $(\beta\alpha)_8$-barrel.

central core of the β barrel and a buried and invariant salt bridge are sensitive to amino acid exchanges. In contrast, exchanges at the hydrophobic interface between β strands and α helices or in loops linking α helices with β strands are more easily tolerated. It appears that, at about 80% of the investigated sequence positions, a subset of only seven amino acids would be sufficient to generate a stable and active enzyme. The results of this work might be helpful in studies aimed at the *de novo* design of $(\beta\alpha)_8$-barrels [18], which have not been successful to date [19,20].

Improving the catalytic activity

Most naturally occurring enzymes from hyperthermophiles are only marginally active at mesophilic temperatures, probably due to conformational rigidity [21]. The turnover number at 37°C of TrpC from the hyperthermophile *S. solfataricus* was improved by a combination of random mutagenesis and selection *in vivo* [22••]. Fast kinetic measurements revealed that the turnover number of the wild-type enzyme at low temperatures is limited by the dissociation of the enzyme–product complex. In contrast, selected TrpC variants release the product more rapidly, shifting the rate-limiting step of the reaction to the preceding chemical step. This switch is probably achieved by weakening the binding of the phosphate moiety of the product, owing to an increased flexibility that is caused by the introduced amino acid exchanges. The 'constipation' caused by the slow release of product, which limits the activity of wild-type TrpC at 37°C, is probably relieved at 60°C by a similar increase in flexibility.

Dual substrate specificity within the enolase superfamily

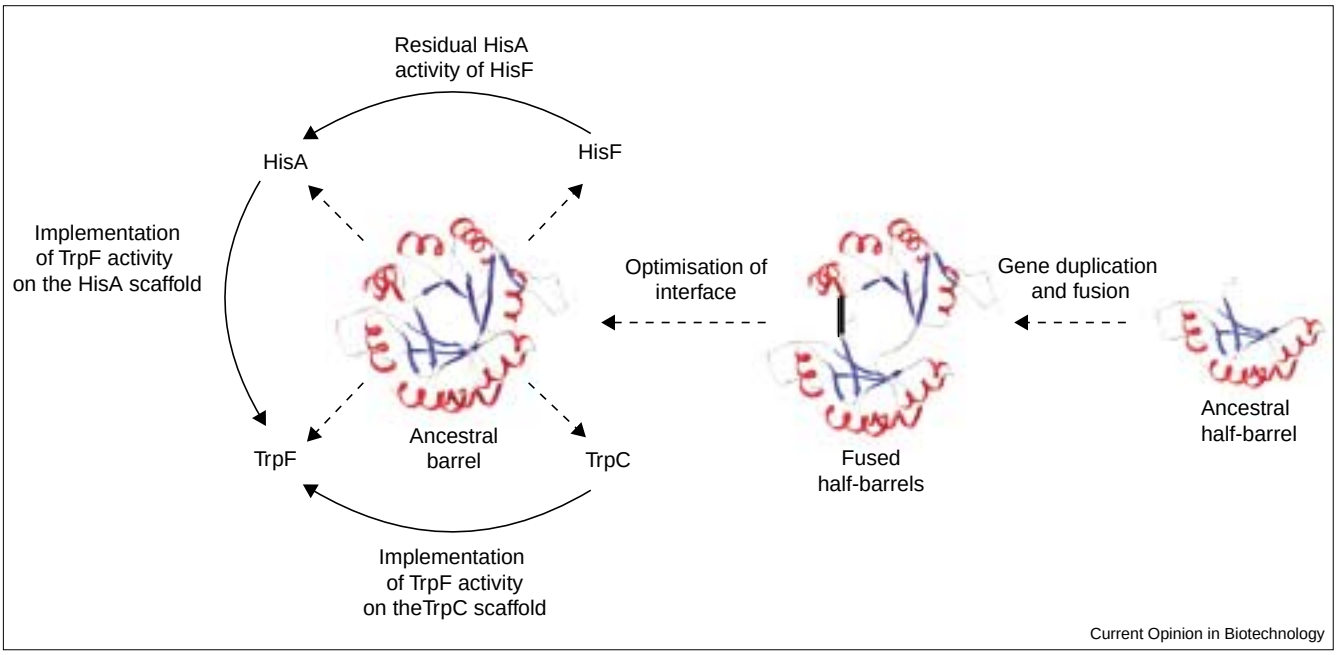
Members of the enolase superfamily [23] consist of two domains: a larger $(\beta\alpha)_7\beta$ barrel domain, which is a modified version of the $(\beta\alpha)_8$-barrel, and a mixed α/β domain that is formed by sequence extensions at the N- and C-terminal ends of the barrel. Common to the superfamily is the abstraction of the α-proton from a carboxylate anion substrate. The resulting enolate intermediate is stabilized by a metal ion that is complexed by three acidic residues located at the ends of the third, fourth and fifth β strands. The enolate intermediate then reacts to different products via different chemical intermediates in the various active sites.

An interesting member of the enolase superfamily is an enzyme from *Amycolaptosis* sp., which acts both as *N*-acyl amino acid racemase and as o-succinylbenzoate synthase [24•]. These two enzymatic activities are considerably different with regard to the substrate and the catalyzed chemical reaction, which is a racemisation and a dehydration, respectively. The recently solved structure of o-succinylbenzoate synthase from *E. coli* shows that most interactions between the bound product o-succinylbenzoate and the active site are either indirect via water molecules or via hydrophobic interactions [25]. It was speculated that this plasticity within the active site might contribute to the naturally occurring dual substrate specificity of the homologous enzyme from *Amycolaptosis* sp. [26].

Changing substrate and product specificities

The substrate specificity of TIM is limited to the natural substrates D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. An experimental approach based on modelling was used to extend the substrate specificity. The loop connecting strand β8 and helix α8 (loop 8) forms a tight-binding pocket for the phosphate moieties of the substrates and, therefore, is an important determinant of substrate specificity. Loop 8 was shortened by three amino acid residues [27]. The corresponding TIM variant was produced and shown to be stable but enzymatically inactive toward native substrate. X-ray crystallography demonstrated that loops 6 and 7, which are adjacent to loop 8 and

Figure 4



Divergent evolution of $(\beta\alpha)_8$-barrel enzymes of histidine and tryptophan biosynthesis from a half-barrel $(\beta\alpha)_4$ precursor. The ancestral half-barrel [32••,35••] was probably not a monomeric protein. Instead, in order to shield its hydrophobic surface from the polar solvent, it may have formed homodimers [4,35••]. Dashed arrows highlight putative evolutionary events. Solid arrows highlight experimental interconversions of enzymatic activities (TrpC to TrpF [31••]; HisA to TrpF [33••]) and the residual HisA activity of HisF [32••].

important for the catalytic activity, adopt wild-type conformations. However, the conformation of loop 8 has changed: the original phosphate-binding pocket has become wider and a connecting groove between the new pocket and the active site has emerged. Attempts will now be made to find a suitable new substrate for this TIM variant.

Glycoside hydrolases catalyse the cleavage of α-1,4-glycosidic linkages in starch polymers, producing various products; for example, α-amylases produce linear oligosaccharides, whereas cyclodextrin glycosyltransferases (CGTases) produce both linear and circular oligosaccharides (cyclodextrins). Cyclodextrins are composed of six, seven or eight glucose units and can form inclusion complexes with small hydrophobic molecules. These complexes have important applications in the food, pharmaceutical and other industries [28]. α-Amylases consist of three domains (A–C), whereas CGTases consist of five domains (A–E); in both classes domain A is a $(\beta\alpha)_8$-barrel that contains the residues essential for catalysis. Novamyl is a glycoside hydrolase that is composed of five domains. Moreover, its amino acid sequence and three-dimensional structure are very similar to those of CGTases [29]. However, as Novamyl only produces linear oligosaccharides, it was described as an α-amylase. In an attempt to convert Novamyl into a CGTase, an additional loop in domain B of Novamyl, which probably interferes with the cyclisation reaction, was deleted. In addition, two amino acid residues crucial for the cyclisation reaction in CGTases were introduced (Figure 3). The resulting variant efficiently produced cyclodextrins, but its α-amylase hydrolysing activity was decreased [30•].

TrpF and TrpC, as mentioned above, catalyse two successive reactions in the biosynthesis of tryptophan. As a consequence, they bind the common ligand CdRP (1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate), which is the product of TrpF and the substrate of TrpC. TrpF activity was established on the scaffold of *E. coli* TrpC using a combination of rational design and directed evolution [31••] (Figure 4). In a first step, the N-terminal extension to the $(\beta\alpha)_8$-barrel of TrpC was removed and several loops on the catalytic face of the β barrel were designed to resemble the corresponding loops in TrpF. Subsequently, random mutagenesis and selection for TrpF activity were performed. A variant was isolated that had gained high TrpF activity but lost all of its TrpC activity. The variant showed only about 30% sequence identity to *E. coli* TrpF, but retained 90% sequence identity to the parental protein.

In analogy to TrpF and TrpC, ProFAR isomerase (HisA) and imidazole glycerol phosphate synthase (HisF) catalyse two successive reactions in the biosynthesis of histidine. Both enzymes bind the common ligand PRFAR (*N*′-[(5′-phosphoribulosyl)formimino]-5-aminoimidazole-4-carboxamide-ribonucleotide), which is the product of HisA and the substrate of HisF. A superposition of the structures of monomeric HisA and HisF from *T. maritima* showed that the two catalytically essential aspartate

residues of HisA and HisF are located at equivalent positions at the C-terminal ends of β strands 1 and 5. Both enzymes were therefore tested for their mutual residual activities. Whereas HisA has no detectable HisF activity, wild-type HisF catalyses the HisA reaction, albeit with low efficiency [32••] (Figure 4).

HisA and TrpF catalyse mechanistically similar reactions, namely Amadori rearrangements of an aminoaldose into an aminoketose. There is now also strong experimental evidence for a close interpathway relationship between these $(\beta\alpha)_8$-barrel enzymes from histidine and tryptophan biosynthesis. Using random mutagenesis and selection, several HisA variants were generated that catalyse the TrpF reaction, and one of these variants retained significant HisA activity [33••] (Figure 4). Closer analysis revealed that a single amino acid change in the active-site region was sufficient to almost completely interconvert the substrate specificity from HisA to that of TrpF, although the enzymes share a sequence identity of only about 10%.

Evolution of the $(\beta\alpha)_8$-barrel fold from ancestral 'half-barrels'

The data presented suggest that TrpF, TrpC, HisA and HisF have evolved by divergent evolution from an ancestral $(\beta\alpha)_8$-barrel (Figure 4). Recent experiments provide evidence that this ancestral single-domain barrel evolved from a smaller subdomain comprising four $(\beta\alpha)$ units. The superposition of the N- and C-terminal halves of HisA and HisF (HisA-N, HisF-N, HisA-C and HisF-C) from *T. maritima* revealed close overall similarities with respect to structure and sequence [32••,34]. The gene segments encoding HisF-N and HisF-C were expressed in *E. coli* and the proteins purified and characterized. Individually, HisF-N and HisF-C are inactive proteins with well-defined secondary and tertiary structures, which form oligomers, predominantly $(\text{HisF-N})_2$ and $(\text{HisF-C})_2$ [35••]. Upon co-expression *in vivo* or joint refolding *in vitro*, mixtures of HisF-N and HisF-C assembled to form the catalytically fully active HisF-NC complex.

In summary, it appears that HisA, HisF, TrpF and TrpC have evolved from an ancestral half-barrel [36] by a series of gene duplication, fusion, and diversification events (Figure 4). Large-scale sequence comparisons suggest that a considerable number of the known $(\beta\alpha)_8$-barrel enzymes are evolutionarily related [37] and, therefore, might all be descendants of the half-barrel ancestor.

Conclusions

The interconversion of the catalytic activities of $(\beta\alpha)_8$-barrel enzymes from the same [31••] and from different [33••] metabolic pathways (Figure 4) show that this fold is suitable for enzyme design, and indicate that it has potential for industrial applications [30•] (Figure 3). Along these lines, the spatial separation of regions important for stability and function [11•] (Figures 1,2) will facilitate the generation of $(\beta\alpha)_8$-barrel enzymes that combine high stability with high catalytic activity at low temperatures [9,14,16].

The $(\beta\alpha)_8$-barrel is viewed as a single structural domain [38]. It was therefore quite unexpected that HisA and HisF consist of two superimposable subdomains, the half-barrels [32••,35••]. This finding should stimulate the search, in the framework of structural genomics [39], for other yet undiscovered, small ancestral domains within apparently single-domain folds.

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