A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

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To be cited as: *ChemBioChem* 10.1002/cbic.201900323

Link to VoR: http://dx.doi.org/10.1002/cbic.201900323

A Journal of

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WILEY-VCH

www.chembiochem.org

10.1002/cbic.201900323

Generation of a stand-alone tryptophan synthase α-subunit by mimicking an evolutionary blueprint

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10.1002/cbic.201900323

Abstract

The αββα tryptophan synthase (TS), which is part of primary metabolism, is a paradigm for allosteric communication in multi-enzyme complexes. In particular, the intrinsically low catalytic activity of the α-subunit TrpA is stimulated several hundred-fold by the interaction with the β-subunit TrpB1. The BX1 protein from *Zea mays* (zmBX1), which is part of secondary metabolism, catalyzes the same reaction as its homologue TrpA, but with high activity in the absence of an interaction partner. We found that the intrinsic activity of TrpA can be significantly increased by the exchange of several active site loop residues, mimicking the corresponding loop in zmBX1. The subsequent identification of activating amino acids in the generated "stand-alone" TrpA contributes to the understanding of allostery in TS. Moreover, our findings suggest an evolutionary trajectory that describes the transition from a primary metabolic enzyme regulated by an interaction partner to a self-reliant stand-alone secondary metabolic enzyme.

Allosteric communication is a central mechanism for the regulation of protein-based biological systems. A well-characterized model system for studies on allosteric communication within enzymes is the tryptophan synthase (TS). The TS, an $\alpha\beta\beta\alpha$ hetero-tetrameric complex that is crucial for primary metabolism in archaea, bacteria, and plants[1], consists of a central dimer of β-subunits (TrpB1) and two peripheral α-subunits (TrpA). TrpA catalyzes the cleavage of indole-3-glycerol phosphate (IGP) into glyceraldehyde 3-phosphate (GAP) and indole. The latter is channeled to the active site of TrpB1 where the cofactor pyridoxal-5'-phosphate (PLP) facilitates its condensation with L-serine to the final product L-tryptophan[2]. TrpA and TrpB1 mutually stimulate each other (**Figure 1a**) but the underlying allosteric pathways are not fully understood. Recently, a partial comprehension of the activation of TrpB1 by TrpA has been achieved by the directed evolution towards a "stand-alone" TrpB1 subunit, which contains amino acid exchanges that mimic its stimulation by TrpA in wild-type TS[3].

Interestingly, a blueprint for a "stand-alone" TrpA protein already exists in nature. This protein is named BX1 and is a paralogue of TrpA that plays a role within the secondary metabolism of, for example, plants like *Zea mays*. BX1 shares with TrpA the ubiquitously encountered $(\beta\alpha)_8$-barrel fold[4] and the enzyme from *Z. mays* (zmBX1) shares a sequence identity of 63.3% with zmTrpA (**Figure S1**). Remarkably, BX1 catalyzes the same IGP lyase reaction as TrpA (**Figure 1a**) using the identical active site residues[5], but with high efficiency in the absence of an interaction partner [6]. Indole generated by BX1 is not used for tryptophan biosynthesis but is fed into the benzoxazinoid pathway where it is finally converted to the plant protective agent DIMBOA-Glc[7].

It has been postulated that enzymes of secondary metabolism have evolved by gene duplication from enzymes of primary metabolism[8]. In line with this hypothesis, the high sequence identity as well as structural and functional similarities between the two IGP lyases suggest that zmTrpA is the progenitor of zmBX1. Based on this hypothesis, we reasoned that it should be possible to remove the zmTrpB1-dependence of zmTrpA by amino acid exchanges that mimic the situation in zmBX1. The resulting "stand-alone" zmTrpA variant should provide insights into the allosteric communication of the TS complex and into the evolutionary relationship between zmTrpA and zmBX1. In order to identify crucial

differences between zmTrpA and zmBX1, we compared the amino acid sequences of the central catalytic $(\beta\alpha)_8$-barrel domains, without considering the N-terminal subcellular localization sequences. An initial analysis showed that the differences in amino acid sequence were not clustered but distributed over the whole proteins (**Figure S1**). We then focused on sequence stretches from Loop2 (residues 56-76) and Loop6 (residues 174-189 for zmBX1 and residues 178-193 for zmTrpA) (**Figure 1b**), because these two loop regions are known to be important for the catalytic activity and the allosteric activation of TrpA[2, 9]. Moreover, both loops together form a lid covering the active site (**Figure 1c**). Amino acids in Loop2, which carries one of the two conserved catalytic residues (D62), differ in several positions between zmTrpA und zmBX1. However, these differences seem to be rather insignificant with respect to the physico-chemical properties of the corresponding residues (i.e. A61 and V65 in zmTrpA vs. I61 and I65 in zmBX1) or are rather remote from the active site (i.e. Y56 and K76 in zmTrpA vs. C56 and S76 in zmBX1). Loop6 contains a conserved threonine residue (T184 in zmTrpA, T180 in zmBX1), which is known to form a crucial hydrogen bond with D62[9-10]. There are six positions in Loop6 that differ between zmTrpA and zmBX1. The two most prominent ones are T186 in zmTrpA vs. P182 in zmBX1 as well as G192 in zmTrpA vs. P188 in zmBX1. In addition, the crystal structure of zmBX1 (PDB ID 1TJR, chain A) shows a fully resolved Loop6. In contrast, crystal structures of TrpA enzymes display a poorly defined Loop6, both in the presence and in the absence of TrpB1. In light of these findings, it has been argued that the higher IGP lyase activity of zmBX1 compared to TrpA is based on the stabilization of a specific conformation of Loop6[5]. We speculated that the two proline residues in zmBX1 are responsible for this stabilization and hence for the increased catalytic activity of zmBX1 compared to zmTrpA. In order to test this hypothesis, we generated in zmTrpA the T186P and G192P exchanges, separately and in combination. In addition, we replaced the entire Loop6 from zmTrpA with Loop6 from zmBX1 (**Figure 1d**).

Wild-type and mutant genes were cloned (see **Table S1** for mutagenesis primers) and expressed in *Escherichia coli*. The recombinant proteins (**Table S2**) were purified and analyzed by size-exclusion chromatography (SEC) and native mass spectrometry (MS). In SEC experiments, each of the proteins

eluted as a single peak indicating a well-defined oligomeric state (**Figure S2**). MS showed that zmTrpA and its variants are monomers, and that zmBX1 and zmTrpB1 are dimers (**Figure S3**, **Figure S4**). Steady-state enzyme kinetics were recorded for the enzymes zmBX1, zmTrpA, zmTrpA_T186P, zmTrpA_G192P, zmTrpA_T186P_G192P, and zmTrpA_L6zmBX1 (**Table 1**). The determined turnover numbers (k_{cat}) and Michaelis constants for IGP (K_m^{IGP}) of zmBX1 and zmTrpA are comparable to reported values[6b, 8c] with zmBX1 exhibiting a k_{cat} (2.8 s^{-1}) that exceeds the k_{cat} of zmTrpA by three orders of magnitude. In comparison to zmTrpA, the k_{cat}-values of the single mutants zmTrpA_T186P and zmTrpA_G192P were enhanced 10-15 fold. The k_{cat}-value of the double mutant zmTrpA_T186P_G192P was enhanced 40-fold, while the k_{cat}-value of zmTrpA_L6zmBX1 was enhanced 520-fold. Thus, the k_{cat}-value of zmTrpA_L6zmBX1 is close to those of zmBX1 as well as zmTrpA in complex with its activating binding partner zmTrpB1. While all zmTrpA variants show a significantly enhanced k_{cat}-value, they also display an increased K_m^{IGP} value compared to zmBX1 and zmTrpA (**Table 1**). These results demonstrate that the exchanges T186P and G192P as well as the incorporation of the whole Loop6 of zmBX1 are sufficient to accelerate turnover rates for zmTrpA, albeit negatively affect the binding of the substrate IGP.

We next used SEC and activity titrations to test whether the activated zmTrpA variants are still able to bind zmTrpB1. The results showed that all variants form a complex with zmTrpB1 (**Figure S5**). The apparent dissociation constants (K_D^{app}) as determined by measuring TrpA activity as a function of zmTrpB1 concentration are between 0.3 µM and 0.7 µM, comparable to the K_D^{app} value of 0.5 µM for the zmTrpA:zmTrpB1 complex (**Table S3**). The effect of zmTrpB1 binding on the steady-state kinetic parameters of zmTrpA and its activated variants are summarized in **Table 1**. Wild-type zmTrpA shows a 2000-fold increased k_{cat}-value in the presence of zmTrpB1 while the K_M^{IGP}-value is unaffected. The variants zmTrpA_T186P, zmTrpA_G192P, and zmTrpA_T186P_G192P display 30-100-fold improved k_{cat}-values in the presence of zmTrpB1 while the K_M^{IGP}-values are decreased 70-110-fold. Interestingly, the variant zmTrpA_L6zmBX1 displays a two-fold reduced k_{cat}-value while the K_M^{IGP}-value is lowered 20-fold. Apparently, an optimal catalytic activity for zmTrpA is achieved by the exchange of its loop6

with that of zmBX1 and cannot be further increased by the presence of zmTrpB1. It has been shown for TrpA from *Salmonella typhimurium* that the transition from a rather inactive to a highly active conformation is the rate-limiting step for the IGP lyase reaction[11]. This conformational change, which is triggered by the formation of the aminoacrylate intermediate in TrpB1, includes loop6. It is therefore plausible to assume that zmTrpA_L6zmBX1 has become independent of zmTrpB1, since loop6 stemming from zmBX1 constitutively adopts the active conformation. In contrast, substrate binding as reflected by the K_M^{IGP}-value is impaired in the zmTrpA variants. It can, however, be restored to wild-type level by the interaction with zmTrpB1, for reasons that are unclear at this point.

The improved catalytic activities of the variants zmTrpA_T186P and zmTrpA_G192P raised the question whether residues other than proline have a similar activating effect at positions 186 and 192. We therefore tested, at both positions, a set of amino acids that sample a range of diverse physico-chemical properties[12]. Mutagenesis at position 186 led to two variants, zmTrpA_T186M and zmTrpA_T186I, that display increased IGP lyase activity (**Figure 2**). Comparable to zmTrpA_T186P, zmTrpA_T186M and zmTrpA_T186I show a 10-30 fold enhanced k_{cat}-value, but also a 20-fold increased K_m^{IGP}-value compared to zmTrpA (**Table 1**). Analytical SEC and MS demonstrated that both variants are homogeneous monomers (**Figure S2, Figure S3)** that form a complex with zmTrpB1 (**Figure S5**). Screening of position 192 identified no activating amino acid exchanges other than G192P.

Our work establishes that zmTrpA can be significantly activated by the exchange of two residues in Loop6 with the corresponding residues of zmBX1. This activating effect is further enhanced when the entire Loop6 of zmTrpA is replaced with Loop6 from zmBX1. Thus, our findings reinforce the importance of Loop6 for the allosteric activation of TrpA by TrpB1[2]. However, although the turnover number of zmTrpA_L6zmBX1 is close to that of zmBX1, the Michaelis constant for IGP is increased by several orders of magnitude. Currently, the structural basis for a weaker substrate affinity of the activated variants is unknown and might result from other sequence differences between zmTrpA and zmBX1[6a]. Remarkably, high substrate affinity is restored by the interaction of zmTrpA variants with zmTrpB1, illustrating the decoupling of stand-alone activity and allosteric activation.

Based on our experimental findings, we propose a plausible model for the evolution of zmBX1 from a progenitor zmTrpA (**Figure 3**). In this model, gene duplication allows for the evolution of one copy towards zmBX1 while the other copy of zmTrpA maintains its role in the canonical TS. On the route towards zmBX1, initial single exchanges at key positions 186 and 192 in Loop6 would have increased the turnover number. The combination of such activating exchanges plus the accumulation of further substitutions would have led to a complete rearrangement of Loop6 with a further drastic increase of activity. For the final transition from zmTrpA to zmBX1, two major changes in properties were necessary: the improvement of affinity for IGP and the formation of the dimer. It is unclear whether these changes in properties occurred in a stepwise manner or along with the improvement of the k_{cat}-value. In any case, our model is in accordance with the situation found in extant *Zea mays*. Here, a zmTrpA enzyme is activated by zmTrpB1 in the canonical TS whereas a zmBX1 enzyme displays a high IGP lyase activity without an interaction partner. Taken together, our findings demonstrate that a small number of amino acid exchanges can be sufficient to drive the evolution of an enzyme from primary metabolism into one of secondary metabolism with altered catalytic and regulatory properties [13].

Acknowledgments

This work was supported by a grant of the Deutsche Forschungsgemeinschaft to R.S. (STE 891/ 11-1) and a grant by the National Institutes for Health to V.H.W. (NIH P41GM128577). The cDNA of *Zea mays* W22 was a generous donation of Dr. Kevin Begcy (University of Regensburg). We thank Sonja Fuchs, Christiane Endres, and Jeannette Ueckert for expert technical assistance, and Sandra Schlee, Patrick Babinger, and Rainer Merkl for critical reading of the manuscript.

10.1002/cbic.201900323

Table 1. Steady-state enzyme kinetic parameters for the IGP lyase reaction of zmBX1 and zmTrpA and its variants, in the absence and the presence of zmTrpB1.

Protein(s)	k_{cat} / s^{-1}	K_m^{IGP} / mM	k_{cat}/K_m^{IGP} / $M^{-1}s^{-1}$
zmBX1	2.8 ± 0.1	0.005±0.001	$5.4\times10^{5}\pm 9.4\times10^{4}$
zmTrpA	0.0020 ± 0.0001	0.170±0.025	$1.2\times10^{1}\pm 2.2\times10^{0}$
zmTrpA + zmTrpB1	3.9 ± 0.1	0.175±0.014	$2.2\times10^{4}\pm 2.2\times10^{3}$
zmTrpA_T186P	0.021 ± 0.001	3.2±0.3	$6.5\times10^{0}\pm 8.0\times10^{-1}$
zmTrpA_T186P + zmTrpB1	2.3 ± 0.1	0.043±0.005	$5.5\times10^{4}\pm7.6\times10^{3}$
zmTrpA_G192P	0.031 ± 0.004	4.0±1.2	$7.7\times10^{0}\pm 3.3\times10^{0}$
zmTrpA_G192P + zmTrpB1	1.34 ± 0.04	0.051±0.006	$2.6\times10^{4}\pm 4.0\times10^{3}$
zmTrpA_T186P_G192P	0.08 ± 0.01	4.01±0.75	$2.0\times10^{1}\pm5.2\times10^{0}$
zmTrpA_T186P_G192P + zmTrpB1	2.4 ± 0.1	0.036±0.006	$6.8\times10^{4}\pm 1.4\times10^{4}$
zmTrpA_L6zmBX1	1.04 ± 0.05	1.8±0.3	$5.7\times10^{2}\pm1.2\times10^{2}$
zmTrpA_L6zmBX1 +zmTrpB1	0.46 ± 0.01	0.080±0.006	$5.8\times10^{3}\pm 5.8\times10^{2}$
zmTrpA_T186M	0.057±0.001	3.9±0.2	$1.5\times10^{1}\pm1.1\times10^{0}$
zmTrpA_T186I	0.024±0.001	3.0±0.3	$8.1\times10^{0}\pm1.1\times10^{0}$

Experimental conditions: 100 mM EPPS/KOH (pH 7.5), 180 mM KCl, 40 mM PLP, 6 mM NAD^+, 20 mM $NaAsO_4$, varying concentrations of IGP, 100 mM L-serine (in presence of zmTrpB1), and 5.5 µM GAP dehydrogenase from *Thermotoga maritima*. The reaction was followed at 30 °C by monitoring the cleavage of IGP to GAP and indole with a coupled enzymatic assay[14]. The mean and the standard error were calculated from at least three independent measurements.

10.1002/cbic.201900323

Figure Captions

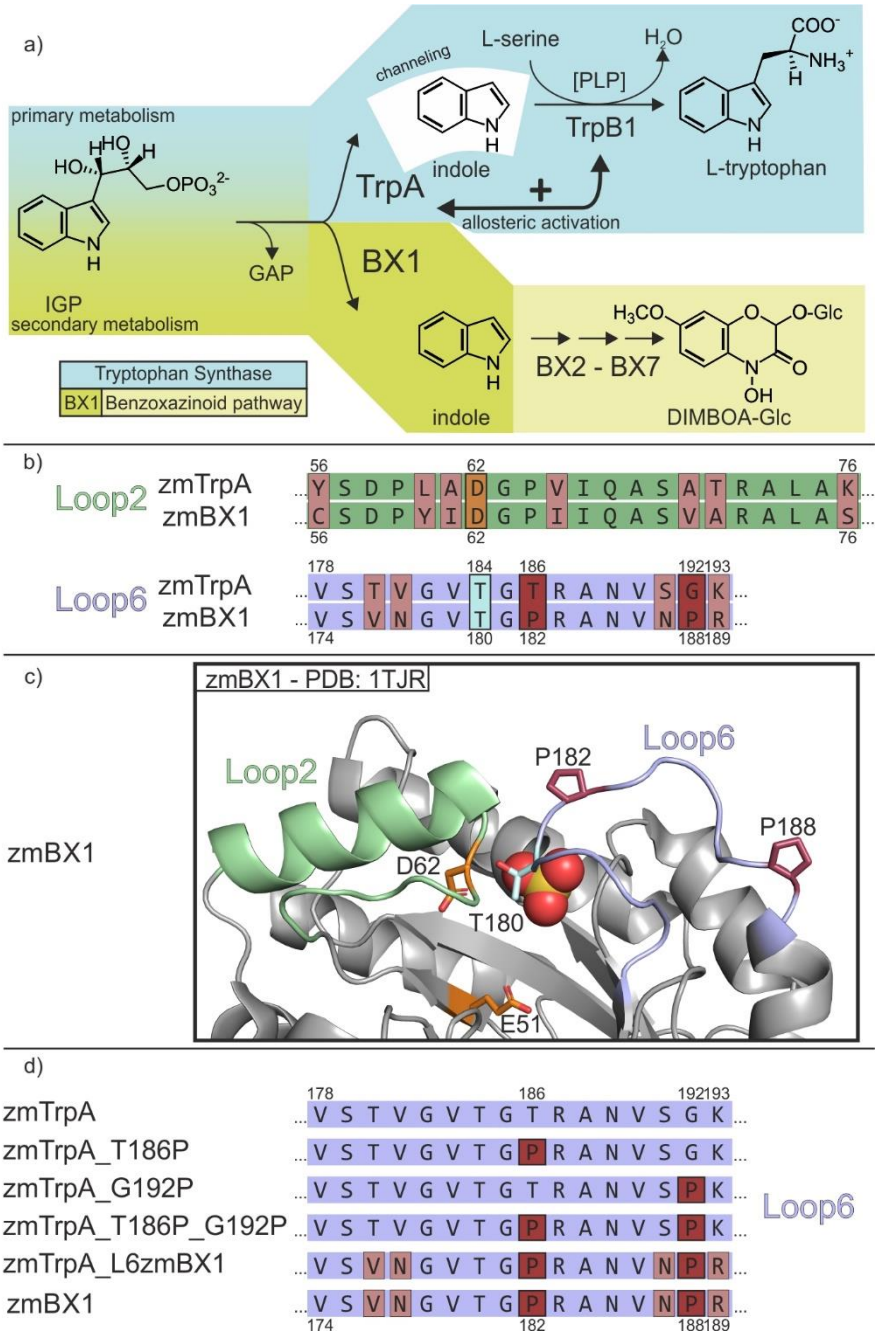

Figure 1. a) The cleavage of IGP by the paralogous enzymes TrpA and BX1 in *Zea mays* marks a branch point between primary (tryptophan biosynthesis) and secondary metabolism (benzoxazinoid biosynthesis). In primary metabolism, TrpA is part of the tryptophan synthase and is allosterically activated by the TrpB1 subunit. In secondary metabolism, BX1 is independent of an activating interaction partner[7]. **b)** Sequence comparison of Loop2 and Loop6 from zmTrpA and zmBX1. In Loop2 the catalytic residue D62 is colored orange. In Loop6 the conserved T184 is colored cyan. The residues

that differ between zmTrpA and zmBX1 are colored light red. The positions that carry a proline in zmBX1 are colored dark red. The entire sequences of zmTrpA and zmBX1 are shown in **Figure S1**. **c)** Crystal structure of zmBX1 (PDB ID 1TJR, chain A). Loop2, consisting of a loop segment and a helical segment is colored green and Loop6 is colored light blue. The proline residues in Loop 6 at positions 182 and 188 are colored dark red. The catalytic residues E51 and D62[5] are colored orange. The conserved T180 is colored cyan. A sulfate ion, which is represented as sphere, marks the phosphate-binding pocket at the active site. **d)** Loop6 sequences of the experimentally characterized enzymes. For the zmTrpA variants, differences to wild-type zmTrpA are colored dark red (substitutions to proline) or light red (other substitutions).

10.1002/cbic.201900323

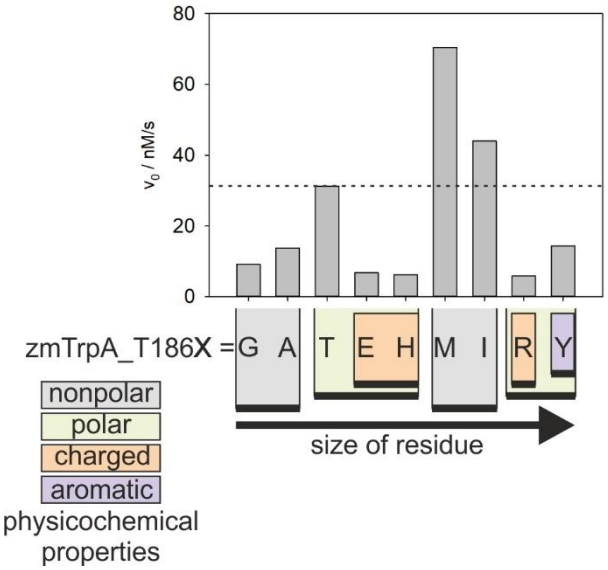

Figure 2. Reaction rates of zmTrpA variants with different amino acids at position 186. The dashed line indicates wild-type activity. Experimental conditions: 100 mM EPPS/KOH (pH 7.5), 180 mM KCl, 40 mM PLP, 6 mM NAD^+, 20 mM $NaAsO_4$, 5.5 µM GAP dehydrogenase from *T. maritima*, 5 µM zmTrpA variant. The reaction was followed at 30 °C by monitoring the cleavage of 1 mM IGP to GAP and indole with a coupled enzymatic assay[14].

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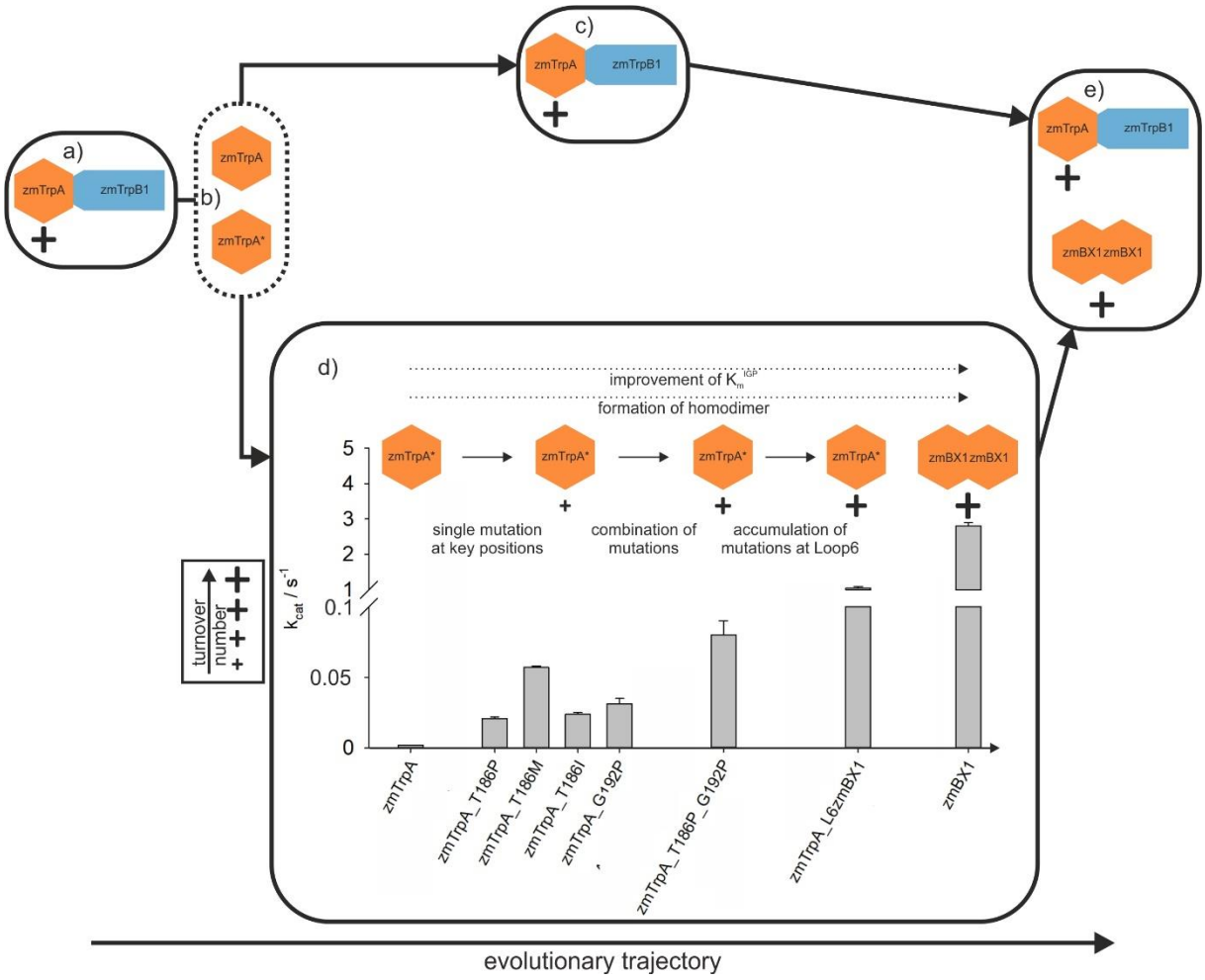
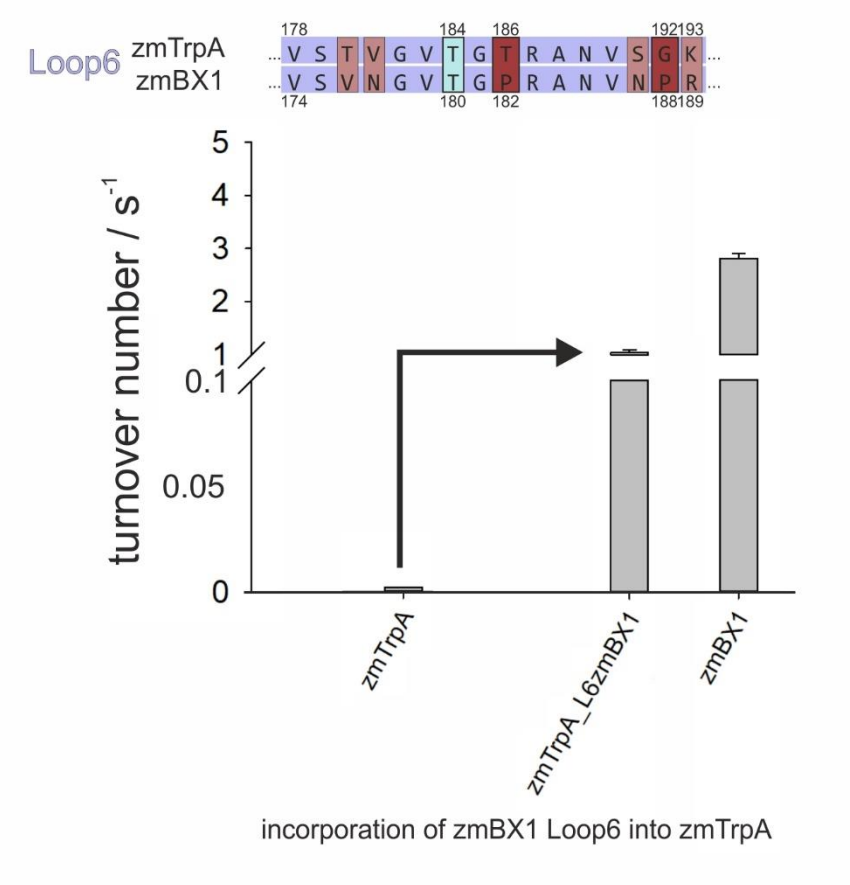


Figure 3. Model for the evolution of zmTrpA to zmBX1. The '+' sign size indicates the relative IGP lyase activity of the depicted variants. **a)** Initial situation: TS complex with the IGP lyase activity of zmTrpA being stimulated by zmTrpB1. **b)** The gene for zmTrpA is duplicated. **c)** The TS complex remains unaltered whereas **d)** zmTrpA* sequentially accumulates beneficial mutations that enhance its IGP lyase activity towards that of zmBX1. The bars indicate the experimental determined k_{cat} values. Solid arrows describe experimentally confirmed steps, while dashed arrows indicate the steps necessary for the final evolution of extant zmBX1. **e)** Situation in extant *Z. mays*: The TS complex exists in parallel with zmBX1. For the TS complex, only zmTrpA:zmTrpB1 is shown instead of the entire zmTrpA:zmTrpB1:zmTrpB1:zmTrpA complex.

10.1002/cbic.201900323

Table-of-Contents text

ZmTrpA and zmBX1 are homologous IGP lyase enzymes from tryptophan and benzoxazinoid biosynthesis. Transplantation of a loop from zmBX1 into zmTrpA results in drastic activation, presumably mimicking the evolution of a primary into a secondary metabolic enzyme.



Keywords

enzyme • protein design • molecular evolution • primary metabolism • secondary metabolism

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