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Directed evolution mimics allosteric activation by stepwise tuning of the conformational ensemble

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Abstract

Allosteric enzymes contain a wealth of catalytic diversity that remains distinctly underutilized for biocatalysis. Tryptophan synthase is a model allosteric system and a valuable enzyme for the synthesis of non-canonical amino acids (ncAA). Previously, we evolved the β -subunit from *Pyrococcus furiosus*, *PfTrpB*, for ncAA synthase activity in the absence of its native partner protein *PfTrpA*. However, the precise mechanism by which mutation activated TrpB to afford a stand-alone catalyst remained enigmatic. Here, we show that directed evolution caused a gradual change in the rate-limiting step of the catalytic cycle. Concomitantly, the steady-state distribution of intermediates shifts to favor covalently bound Trp adducts, which is associated with increased thermodynamic stability of these species. The biochemical properties of these evolved, stand-alone TrpBs converge on those induced in the native system by allosteric activation. High resolution crystal structures of the wild-type enzyme, an intermediate in the lineage, and the final variant, encompassing five distinct chemical states, show that activating mutations have only minor structural effects on their immediate environment. Instead, mutation stabilizes the large-scale motion of a sub-domain to favor an otherwise transiently populated closed conformational state. This increase in stability enabled the first structural description of Trp covalently bound in a catalytically active TrpB, confirming key features of catalysis. These data combine to show that sophisticated models of allostery are not a prerequisite to recapitulating its complex effects via directed evolution, opening the way to engineering stand-alone versions of diverse allosteric enzymes.

Graphical abstract

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Notes

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ASSOCIATED CONTENT

Complete materials and methods, as well as supplemental figures and crystallographic information are provided in the supporting information. This material is available free of charge on the ACS Publications website via <http://pubs.acs.org>.

Keywords

biocatalysis; enzymology; allostery; pyridoxal phosphate; non-canonical amino acid

INTRODUCTION

Biocatalysis is emerging as a powerful methodology for green, sustainable chemical synthesis.^{1,2} This development relies on decades of biochemical insights into enzyme structure and mechanism to guide engineering of natural enzymes into convenient synthetic tools. While active-site mutagenesis of non-essential residues is a facile way to alter substrate specificity and improve function, residues throughout a protein's structure influence catalytic activity. These remote mutations are reminiscent of allosteric effects, where binding of a ligand or partner protein (effector) distant from the active site alters enzyme function. Many, if not most, enzymes are allosterically regulated and are an important source of catalytic diversity.³ Unfortunately, removal of the native effector often results in loss of activity and can be a major barrier to the successful implementation of enzymes as biocatalysts.⁴ This scenario is exemplified by the enzyme tryptophan synthase (TrpS, EC 4.2.1.20), which is a model system for understanding allosteric function as well as a highly desirable biocatalyst for the synthesis of non-canonical amino acids (ncAAs).^{5–8}

TrpS is comprised of two subunits, TrpA (α -subunit) and TrpB (β -subunit) that function as a heterodimeric pair in an $\alpha\beta\alpha$ complex.⁹ In the native catalytic cycle, indole glycerol phosphate (IGP) binds in the α -subunit where a retroaldol reaction releases indole, which diffuses through a molecular tunnel into the β -subunit (Figure 1a).¹⁰ There, TrpB uses its pyridoxal phosphate (PLP) cofactor to effect the β -substitution of the L-serine (Ser) hydroxyl group for indole to yield L-tryptophan (Trp). This system evolved to coordinate the TrpB catalytic cycle with the release of indole,^{10,11} which could otherwise be lost by diffusion through the cellular membrane. When added exogenously, indole and its analogues react to produce over 30 different ncAAs.^{5–7,12–14} While this β -substitution reaction occurs wholly within the β -subunit, isolation of either subunit results in a significant decrease in catalytic efficiency.^{8,15}

Previously, we used directed evolution to identify variants of TrpB from *Pyrococcus furiosus* (P_{TrpB}) that have high stand-alone catalytic activity.¹² An intermediate from this lineage

harboring five mutations, *Pf*TrpB^{4D11}, was subsequently evolved to catalyze β -substitution with L-threonine (Thr) to yield β -methyltryptophan, resulting in the enzyme *Pf*TrpB^{2B9.13}. Recently, we discovered that TrpS is also proficient at the β -substitution of Thr.¹⁶ Hence, *Pf*TrpB^{2B9} was serendipitously evolved to exhibit a reactivity profile similar to the allosterically activated *Pf*TrpB. These engineering efforts relied predominantly on random mutagenesis and mutations in *Pf*TrpB^{2B9} are distant from the active site. Spectroscopic data showed similarities between the engineered and allosterically activated enzymes, however several important questions were outstanding. Specifically, *how did the mutations introduced by directed evolution affect the rate limiting step of the reaction and how do they perturb the structure of TrpB?* The ability to predict distal, activating mutations would enable rapid engineering of allosteric enzymes. Given the broad interest in understanding how enzyme structure and dynamics promote catalysis,^{17–22} and the specific applicability of *Pf*TrpB as a platform for synthesis of ncAAs,^{12,13} we sought a more detailed understanding of how these ‘allostery-mimicking’ mutations enhance catalytic activity.

The β -substitution reaction catalyzed by TrpB proceeds through at least nine elementary steps (Figure 1b).⁸ In its resting state, the PLP cofactor is bound to an active-site lysine (Lys82, *Pf*TrpB numbering) through a protonated Schiff-base linkage.²³ This species undergoes transimination with Ser to form an external aldimine intermediate, E(Aex₁).^{12,24} A subdomain (residues 95–161) that mediates communication between the α - and β -subunits of TrpS, named the COMM domain, undergoes a rigid-body motion upon E(Aex₁) formation into a partially closed state.²⁴ Deprotonation of C α by Lys82 forms a carbanion that is resonance-stabilized as a quinonoid, E(Q₁). Rapid elimination of the hydroxyl group forms an electrophilic amino acrylate, E(A-A), and concludes Stage I of the catalytic cycle.²⁵ These chemical steps are correlated with further motion of the COMM domain into a fully closed conformation.²⁶ In the native complex, formation of E(A-A) is tightly coupled to IGP binding and release of indole by the α -subunit.¹⁰ Stage II of the catalytic cycle is initiated when indole adds into E(A-A) to form a quinonoid, E(Q₂) that is rapidly deprotonated to restore aromaticity of the indole ring.²⁷ Stereospecific protonation at C α completes Trp formation as E(Aex₂), which is released from the enzyme via transimination to complete the catalytic cycle.²⁸

Elucidating the protonation states of the numerous titratable moieties in the TrpB active site at the various stages of catalysis is an active area of research.²⁹ Reaction with nitrogen nucleophiles, such as indoline and 2-aminophenol, stalls the catalytic cycle of TrpS from *Salmonella typhimurium* (*St*TrpS), enabling structural analysis of quinonoid intermediates.^{30–32} These studies have indicated that proton transfer between the phenolic group of PLP and the Schiff base may occur during the catalytic cycle. However, *Pf*TrpS performs a facile β -substitution with *N*-nucleophiles indoline and indazole,¹² indicating there are subtle differences in transition state stabilization between the mesophilic and thermophilic homologs. Additionally, it has long been hypothesized that indole is rendered nucleophilic via interaction with Glu104.³³ However, mutational studies have found that this residue is dispensable if compensatory allosteric ligands³⁴ or activating mutations³⁵ are added. These features notwithstanding, the β -substitution reaction of TrpB is relatively well-understood considering its complexity. Guided by this wealth of knowledge, we performed kinetic, spectroscopic, and thermodynamic analysis for each enzyme in the lineage leading to

PfTrpB^{2B9}. Comparison of these properties to the native *PfTrpS* complex revealed that evolution for stand-alone function mimics structural and mechanistic features of natural allosteric activation.

RESULTS

The rate-limiting step shifts with both directed evolution and TrpA binding

The first two evolutionary steps toward a stand-alone TrpB selected for increased activity with Ser and indole. The resultant enzyme, *PfTrpB*^{4D11}, had a 4-fold higher catalytic efficiency for indole compared to the native complex (Table 1).¹² Two subsequent rounds of evolution selected for increased activity with Thr.¹³ A lingering question from these studies was how the native activity was altered after selection for β -methyl tryptophan synthesis. Therefore, we determined the kinetic parameters of these latter variants in the native reaction with Ser. Addition of the single mutation F95L to *PfTrpB*^{4D11} decreased the rate of Trp formation (bringing it closer to that of the native *PfTrpS* complex) but also reduced the K_M for each of the substrates (Table 1). These changes were reiterated with the addition of I16V and V384A, yielding *PfTrpB*^{2B9}. This final variant had a K_M for Ser approximately two-fold lower than that of *PfTrpS*, and the K_M for indole decreased below the sensitivity of our assay. Notably, the k_{cat} is indistinguishable between *PfTrpB*^{2B9} and *PfTrpS*.

To obtain more detailed mechanistic information, we determined the rate-limiting step (RLS) of *PfTrpB* catalysis. A previous study by Lane and Kirschner probed the mechanism of the mesophilic homolog from *Escherichia coli*, *EcTrpB*, using mono-deuterated Ser at both the α - and β -positions.²⁵ They observed that C α -H deprotonation is rate limiting with a primary kinetic isotope effect (KIE) of 2.5 and no measurable secondary KIE using β -deuteroserine. We used two techniques to measure a KIE for the β -substitution reaction of *PfTrpB* using indole and either Ser or (2,3,3- d_3)-L-serine (d_3 -Ser). First, we compared the initial velocities and observed a clear primary KIE of 2.77 ± 0.09 , indicating that C α -H deprotonation is the RLS of catalysis (Table 1). We also measured the isotopic ratio of Trp products from direct competition reactions using equimolar d_3 -Ser and Ser, which indicated a KIE of 2.61 ± 0.08 . This competition experiment is sensitive to differences in K_M for the isotopes, and agreement with the KIE determined from initial velocity experiments under saturating conditions indicates that changes in K_M are negligible. Further insight into the deprotonation of E(Aex₁) was obtained with UV-vis spectroscopy. Each of the quasi-stable species in the TrpB reaction have distinct UV-vis absorption that provides a convenient spectroscopic handle for probing the catalytic cycle (Figure 1b).⁸ Upon incubation with Ser, we observed a steady-state buildup of the E(Aex₁) state that precedes the rate-limiting deprotonation (Figure 2a).

We determined the KIE for each TrpB variant in the lineage to *PfTrpB*^{2B9} by measuring initial velocities with (d_3 -) Ser. The single mutation T292S causes a significant reduction in the KIE to 1.46 ± 0.08 (Table 1), which is correlated with an approximate halving of the E(Aex₁) population in steady state (Figure 2b). This trend continued for each of the stand-alone enzymes. The KIE decreased, down to 1.12 ± 0.03 for *PfTrpB*^{2B9} with a concomitant shift in the steady-state spectra with Ser (Figure 2c–e). As the population of E(Aex₁) in steady state decreased, we observed a complementary increase in the E(A-A) peak. We also

performed these experiments with the native *Pf*TrpS complex and observed a KIE of 1.01 ± 0.04 , with no significant population of E(Aex₁) in the steady state (Figure 2f). Notably, these kinetic properties of thermostable *Pf*TrpS closely mimic those of *Ec*TrpS, which displays a KIE of 1.2 at pH 7.6 and similarly accumulates E(A-A) in the steady state.²⁵ Hence, the remote activation of TrpB either by mutation or the native allosteric effector accelerates C α deprotonation, stabilizes E(A-A), and causes a new step to become rate-limiting.

Stage II of catalysis is rate-limiting for activated enzymes

Further mechanistic insight came from analysis of the steady-state spectra when indole was present, and thus both stages of the catalytic cycle were accessible. Under these conditions, the spectrum of *Pf*TrpB was unchanged: the E(Aex₁) species, which precedes the RLS of *Pf*TrpB catalysis still accumulated in the steady state (Figure 2a). In contrast, the steady-state spectra of the activated enzymes revealed that E(A-A) no longer accumulated in solution when indole was present. Reaction with indole yields a new peak in the steady-state spectrum with $\lambda_{\text{max}} = 435$ nm, corresponding to E(Aex₂), and a shoulder at 475 nm.⁸ The quinonoid species that is initially formed, E(Q₂), is highly unstable and rearomatization of the indole ring drives the formation of E(Q₃), which we assign as the absorption band at 475 nm (Figure 2b–f).⁸ This steady-state scenario is similar to *Ec*TrpS, where rapid kinetic studies show that C–C bond formation is two orders of magnitude faster than the steady-state rate of product formation,²⁸ and that the proton transfer that converts E(Q₃) into E(Aex₂) is the RLS of catalysis. Consequently, the steady-state distribution of the *Ec*TrpS reaction has a prominent E(Q₃) peak.³⁶ The E(Q₃) shoulder that we observe for *Pf*TrpS is less pronounced, and it is not clear which step (or a combination of steps) determines the steady-state rate. While further insight into *Pf*TrpB catalysis requires rapid kinetic analysis, two points germane to the present study are clear. First, deprotonation of E(Aex₁) is the RLS of isolated *Pf*TrpB catalysis but not that of the activated enzymes, where stage II of catalysis is rate limiting. More importantly, the remote activation of TrpB by directed evolution resulted in complex, incremental kinetic changes that converged on a profile similar to allosterically-activated *Pf*TrpB.

Thermodynamic properties of stand-alone enzymes mirror *Pf*TrpS

An alternative route to probe the catalytic cycle of TrpB is to enter from the reverse direction by addition of Trp. Under these conditions, we observed no degradation of Trp and the resultant spectra represent not the steady-state distribution of intermediates, but their thermodynamic distribution. Previously, we found that isolated *Pf*TrpB binds Trp non-covalently under crystallographic conditions,¹² and the present study conforms to this observation. When saturating Trp is present, E(Aex₂) and E(Q₃) are populated at very low levels at 75 °C (Figure 3). The small change in absorption caused by addition of Trp was monitored at 475 nm and fit well to a single-site binding isotherm, indicating that each unit of the dimer functions independently (Figure S1). We observed that *Pf*TrpB binds Trp weakly ($K_D = 1.2$ mM, Table 1). The first enzyme in our evolutionary lineage of stand-alone TrpBs contains the single T292S mutation and binds Trp ~ 7.1 -fold tighter ($K_D = 168$ μ M) and, surprisingly with a larger fraction of covalently bound E(Aex₂) and E(Q₃) adducts. We repeated this experiment and found that each generation binds Trp more tightly than the one

preceding it, down to $K_D = 12 \mu\text{M}$ for $Pf\text{TrpB}^{2B9}$ and the population of Trp that binds covalently increased. The spectrum of the native $Pf\text{TrpS}$ complex has $K_D < 10\mu\text{M}$ and a slightly larger fraction of Trp covalently bound compared to stand-alone enzymes (Figure 3).

Intriguingly, the population of Trp adducts for all activated TrpBs is slightly higher under steady-state conditions than at equilibrium (Figure S2), consistent with the hypothesis that multiple-steps in stage II contribute to rate limitation. However, the population difference between steady-state and equilibrium for $Pf\text{TrpS}$ are negligible, suggesting a subtle difference in the RLS. Generally, though, the biochemical data provided here establish that directed evolution and allosteric activation increase the activity of $Pf\text{TrpB}$ by accelerating $\text{C}\alpha\text{--H}$ deprotonation of $\text{E}(\text{Aex}_1)$ and stabilizing covalently-bound Trp adducts.

These data provide a rich picture of the complex effects induced by mutational activation. Given that all mutations are distal to the active site, we were particularly interested in what structural changes were caused by directed evolution. Because many of the catalytic intermediates are observable through X-ray crystallography, we hypothesized that structural comparison of $Pf\text{TrpB}$ to the stand-alone enzymes in the presence of substrate or product would provide insight into of how allostery-mimicking mutations influence these states and thereby control activity.

Conformational changes of $Pf\text{TrpB}$ can be observed crystallographically

We have previously grown crystals of native $Pf\text{TrpB}$ and determined high resolution structures with Ser and Trp bound to the enzyme.¹² This crystal form features two copies of the TrpB dimer in the asymmetric unit, providing four unique snapshots of the active site in each structure. Alignment of the four distinct $Pf\text{TrpB}$ protamers shows they are largely identical ($\text{RMSD} = 0.32 \pm 0.05 \text{ \AA}^2$). In the absence of a ligand, $Pf\text{TrpB}$ crystallizes in an ‘open’ state with K82 covalently bound to the cofactor as $\text{E}(\text{Ain})$ (Figure 1b, 4a).^{12,37} The degree of ‘openness’ of TrpB enzymes refers to the large rigid-body motion of the COMM domain as well as movement of several sidechains flanking the active site (F274, H275, D300, Y301), which together comprise ~25% of the protein sequence. Previously, we showed Ser binds to $Pf\text{TrpB}$ as $\text{E}(\text{Aex}_1)$ and triggers a partial closure of the COMM domain (Figure 4a), demonstrating that $Pf\text{TrpB}$ can enter the catalytic cycle *in crystallo*. The $Sf\text{TrpS}$ complex, has been the model system for understanding structural aspects of TrpS allostery and catalysis. There, it was shown that that population of $\text{E}(\text{A-A})$ is coupled to closure of the COMM domain³⁸ and that the closed state is highly stabilized by the α -subunit.³⁹ While our solution data so far conform to this paradigm, counterintuitively, the structure of $Pf\text{TrpS}$ displays a more *open* β -subunit compared to $Pf\text{TrpB}$ alone (Figure S3). This “extended open” conformation closely matches both the recently reported structure of $Pf\text{TrpB}$ with a single molecule of $\beta\text{-MeTrp}$ bound inside a molecular tunnel (Figure 4, S3),¹³ as well as the structure of the tryptophan synthase complex from *Mycobacterium tuberculosis* with a potent allosteric inhibitor bound in the tunnel.⁴⁰ These observations support assignment of this conformation as functionally important for the transport of substrate and product between solution and the active site.

***Pf*TrpB^{4D11} has perplexingly similar structural features to *Pf*TrpB**

Crystallization of the activated variants proved far more challenging than was anticipated. Extensive optimization of crystallization conditions for *Pf*TrpB^{4D11} yielded crystals that grew sporadically under conditions similar to wild type, from which we determined a structure at 1.93 Å resolution. Surprisingly, *Pf*TrpB^{4D11} has a comparable degree of closure as *Pf*TrpB. The similarity held for the local environment of each ‘allostery-mimicking’ mutation (Figure S4). Of the five mutations that separate *Pf*TrpB^{4D11} from wild type, the only notable change we observed was adjacent to T292S, in residue D300. In native *Pf*TrpB, D300 populates two conformations and is either hydrogen-bonded to T292 or rotated into the active site where it is poised to hydrogen bond to the hydroxyl of the E(Aex₁) intermediate (Figure 4b). D300 is not resolved in every subunit of the *Pf*TrpB^{4D11} structure, attesting to its flexibility, but when present it does not form the analogous hydrogen bond with S292.

We could not initially produce enough crystals of *Pf*TrpB^{4D11} for soaking experiments. However, cross-seeding with microcrystals of native *Pf*TrpB provided a reliable route to crystallization of mutant enzymes. Ser was soaked into pre-formed crystals of *Pf*TrpB^{4D11} and we determined a 1.63-Å co-crystal structure. As described above, *Pf*TrpB^{4D11} binds Ser predominantly as E(A-A) in solution (Figure 2c). We were therefore surprised to observe that Ser bound crystallographically as E(Aex₁) (Figure 4c). Nevertheless, observation of this catalytic intermediate afforded a clean comparison with *Pf*TrpB. Most of the structure is unchanged upon ligand binding (Figure S5), except for the large rigid body motion of the COMM domain (Figure 4a), which moves ~3.0 Å to produce an active site that is indistinguishable from the wild-type enzyme (Figure S6). One small difference between *Pf*TrpB and *Pf*TrpB^{4D11} was observed at the extremity of the COMM domain, residues 150–165, where the E(Aex₁) state of *Pf*TrpB^{4D11} forms an incrementally more closed state (~0.3 Å). Comparison to the *Sf*TrpS structures suggests a further 2.5-Å shift is needed to adopt a fully closed state. The structural similarity between *Pf*TrpB^{4D11} and the native enzyme was reiterated when we determined the structure of the Trp bound enzyme at 1.97 Å. Whereas solution data suggested that Trp would bind covalently as the E(Aex₂) intermediate (Figure 3), we instead obtained a non-covalently bound Trp structure of *Pf*TrpB^{4D11} that was virtually identical to the corresponding state for native *Pf*TrpB (Figure 5a, S7).

The closed conformation of *Pf*TrpB is destabilized at low temperature

Small conformational differences between solid and solution state structures are common, and it has been observed that domain orientation can be impeded *in crystallo*.⁴¹ Cryogenic temperatures can also impact the crystallographic ensembles.^{42,43} This crystal form of TrpB, however, is quite flexible and the observation of an altogether different active-site species was puzzling. Previous studies established that *Pf*TrpB displays a non-linear Arrhenius effect, suggesting that the kinetic mechanism may be temperature-dependent.³⁷ A similar observation was made with *Sf*TrpB, where rigorous studies have shown the relative stability of the catalytic intermediates and the RLS of catalysis are indeed temperature-dependent, causing non-linear Arrhenius kinetics.⁴⁴ It was hypothesized that these changes are driven by a redistribution of the conformational ensemble from closed to open states as temperature decreases. Notably, our biochemical studies were performed at 75 °C, whereas crystals were

grown at room temperature, a difference of over 50 °C. We repeated the Ser- and Trp-binding experiments with *Pf*TrpB^{4D11} at 25 °C and observed a marked change (Figure 5b, S9). Ser bound predominantly as E(Aex₁) and Trp bound non-covalently, which agreed with our crystallographic observations. Although structural analysis of *Pf*TrpB^{4D11} yielded no obvious structural changes caused by mutation, it remained possible that mutation exerts a considerable effect on the closed state of TrpB, but that this effect is hidden by the large decrease in temperature required for crystallization. Indeed, analogy to *Sf*TrpS suggested that the elusive E(A-A) and E(Aex₂) states could only be observed crystallographically if the closed state of TrpB were sufficiently stable. We assayed the Ser- and Trp-binding properties of *Pf*TrpB^{2B9} at 25 °C and, although the population of E(A-A) and E(Aex₂) clearly decreases at lower temperature (Figure 5e, S8), there remained a significant population that might be crystallographically observable.

Ligand binding to *Pf*TrpB^{2B9} induces a fully closed conformational state

Cross-seeding with *Pf*TrpB was required to produce crystals of *Pf*TrpB^{2B9}, allowing determination of a ligand-free structure at 1.47-Å resolution. Again, the local structural features of this mutant enzyme are almost identical to wild type in the absence of a substrate (Figure S4), despite the introduction of eight mutations that cause a dramatic change in catalytic activity. As with *Pf*TrpB^{4D11}, a minor change was observed near the T292S mutation (Figure 4b). We soaked crystals of *Pf*TrpB^{2B9} with Ser and determined a 1.67-Å structure that displayed, in three of the four protomers of the asymmetric unit, Ser bound as E(Aex₁) in a manner indistinguishable from native *Pf*TrpB and *Pf*TrpB^{4D11} (Figures S5, S6). Again, the COMM domain was slightly more closed, this time ~0.3 Å more so than *Pf*TrpB^{4D11} at its maximum extent. In one subunit, however, we were pleased to observe density that clearly indicated the E(A-A) intermediate was formed in the active site (Figure 4d). This subunit showed a further 3.3-Å rigid-body movement of the COMM domain towards the active site to form a fully closed state (Figure 4a). This is the first report of the closed conformation of an isolated TrpB enzyme and it closely matches structures of E(A-A) in *Sf*TrpS, which required addition of strong allosteric effectors to stabilize the otherwise fleeting conformation (Figure S9).^{26,38} We also attempted to determine a structure with Ser and the non-reactive substrate analog skatole bound in the active site. While no skatole was observed in the 2.09-Å structure, we did observe E(A-A) formation and COMM closure in three out of four protomers of the asymmetric unit and E(Aex₁) in the last. Together these structures showcase the flexibility afforded TrpB within this crystal form and provide multiple snapshots of chemical and structural states leading up to E(A-A) formation.

Structural characterization of the Trp external aldimine

Lastly, we determined the structure of *Pf*TrpB^{2B9} at 1.83-Å with Trp soaked into the active site. Two of the protomers bound Trp non-covalently in a manner analogous to the Trp-soaked structures of *Pf*TrpB and *Pf*TrpB^{4D11} (Figure S7). Intriguingly, when Trp was added to native *Sf*TrpS crystals formed under conditions that stabilize the closed conformation, only non-covalent Trp binding was observed.³² We were therefore gratified to see density in two subunits of *Pf*TrpB^{2B9} that clearly show Trp covalently bound as the E(Aex₂) intermediate (Figure 5d). This observation was particularly exciting because, despite the extensive characterization of TrpS, the only previous structure of the E(Aex₂) state was

determined using an inactive variant generated by mutation of the catalytic Lys to Thr.²⁴ Compared to the structure of the inactive enzyme, Ca of E(Aex₂) in *PTrpB*^{2B9} is displaced by 0.6 Å (Figure 5c, S10) and the carboxylate moiety is rotated ~40° such that the hydrogen bond network now matches the orientation in the E(Aex₁) state. This orientation also places the Ca-H bond periplanar to the π -system, in position to form a quinonoid state upon deprotonation. The indole sidechain is tilted ~35° and forms van der Waals contacts with L161, which forms a strained partially-eclipsed conformation ($\chi_2 = 130^\circ$) in one of the two protomers, attesting to the tight steric complementarity in the active site. Lastly, we affirm that the E(Aex₂) forms with the COMM domain in a fully closed state (Figure 5f).

Mutational activation shifts the conformational ensemble of TrpB

As discussed above, each crystal structure reported here contains four molecules of TrpB in the asymmetric unit. While there were no significant changes in the local environment of each activating mutation, alignment of four copies of each state revealed modest changes in the orientation of the COMM domain in the ligand-free structures. We used the extended-open state of *PTrpB* (Figure 4a) as a reference point from which to measure the ‘openness’ of each protomer. Alignment to this common reference state shows that all structures match very well, with RMSD < 0.35 Å² when using outlier rejection and we measured the displacement of Ca of each residue from the reference state. Most regions show little variation (Figure S11). In some areas, such as the termini of the protein and the COMM domain, there were clear differences in the position of the residues among aligned subunits of the same crystal structure (Figure 6a). We calculated the standard deviation of the distance of each residue from the reference state (Figure 6b), which constitutes an experimental measurement of crystallographic conformational heterogeneity. Addition of Ser results in E(Aex₁) formation, partial closure of the COMM and a decrease in the conformational heterogeneity (Figure 6b). We repeated this analysis for each structure and found that, surprisingly, *PTrpB*^{2B9} in its ligand-free state has a significant increase in the conformational heterogeneity compared to wild-type *PTrpB* (Figure 7). This heterogeneity does not appear to be oriented randomly but is along the trajectory of closure (Figure 7a). Comparison to the E(Aex₁) and E(A-A) intermediates showed the heterogeneity diminishes once *PTrpB*^{2B9} enters the catalytic cycle (Figure 7b). The significance of these changes is not immediately obvious. Previous studies have focused extensively on the role of changes in conformational dynamics during evolution.^{45–47} Often, B-factor analysis is used as a proxy for protein dynamics with the assumption that larger B-factors indicate higher conformational motion.⁴⁷ We performed B-factor analysis for each structure and found a surprisingly poor correlation with the heterogeneity that is best illustrated with *PTrpB*^{2B9} (Figure S12). B-factors were highest in a loop that forms several contacts with the α -subunit of the native complex but is highly mobile and not resolved in all structures of TrpB (Figure 8). In contrast, heterogeneity is largest at in COMM domain, suggesting caution in interpretation of B-factors as a proxy for dynamics.

DISCUSSION

The relationship between the protein conformational ensemble and allostery is one of profound importance in biochemistry and to the engineering of allosteric enzymes.^{22,48–50}

However, this relationship can be difficult to study and, as a result, previous investigations have focused on enzymes that catalyze comparatively simple reactions, typically hydrolysis and phosphoryl or hydride transfer. Enzymes that catalyze multi-step transformations, such as TrpB, require the formation of multiple unique conformational states for catalysis. This implicit need for heterogeneity during the catalytic cycle further complicates analysis of the links between evolution, allostery and the ensemble.^{45,51–53} The present study addresses each of these factors by combining kinetic, thermodynamic, and structural data for a lineage of TrpB enzymes that mimic allosteric activation.

Evolutionary tuning of the conformational equilibrium of the COMM domain drives allosteric mimicry

Many studies on allostery have focused on the importance of discrete networks of residues that are involved in signal propagation upon effector binding.^{48,54,55} Such networks have also been identified in the *S*TrpS complex, where remote mutations can shift the ensemble to favor open conformational states by disrupting hydrogen bonds⁵⁶ or salt bridges⁵⁷ that form upon COMM domain closure, thereby impeding catalysis. Mutations at the subunit interface can be similarly disruptive.⁵⁸ Based on these studies, we initially hypothesized that activating mutations found agnostically through directed evolution would function along complementary lines by forming discrete stabilizing interactions, such as salt bridges and hydrogen bonds unique to the closed state of TrpB. However, analysis of multiple conformational states of the stand-alone enzymes by X-ray crystallography decisively failed to find any such obvious stabilizing effects (Figure S4, S5, S7). Understanding the structural consequences of mutation required integration of multiple lines of evidence that connect catalytic effects with the underlying conformational ensemble.

We observed three distinct trends in our lineage of evolved enzymes. First, the RLS of catalysis shifts away from deprotonation of C α , as indicated by KIE measurements (Table 1). Unambiguous assignment of the new RLS requires rigorous pre-steady state analysis to probe the rates of the five chemical steps succeeding E(A-A) formation and is outside the scope of this investigation. Secondly, UV-vis spectroscopy revealed that activated, stand-alone enzymes accumulate E(Q₃) and E(Aex₂) in steady state. Given the conservation of structural and mechanistic features between *Pf*TrpS and its mesophilic homologs, literature precedence suggests proton transfer *en route* to E(Aex₂) and/or release of product are the new RLS.^{28,59} Finally, these kinetic changes are associated with a ~100-fold increase in the affinity for Trp and accompanied by a significant increase in the population of covalently bound Trp adducts at equilibrium. Each of these relationships is summarized by the reaction coordinate diagram in Figure 9.

The structures of ligand-bound *Pf*TrpB^{2B9} conclusively show that formation of the E(A-A) and E(Aex₂) intermediates is coupled to formation of a fully closed conformational state (Figure 4a, 5f). The solution data demonstrate that population of these catalytic intermediates steadily increases throughout evolution, both at equilibrium and in steady state (Figure 3). Combined, these data establish that *directed evolution caused a stepwise change in the conformational coordinate to favor more closed states upon ligand binding*.

Structural and mechanistic comparison of the stand-alone and allosterically activated TrpBs

Whereas the E(Aex₂) structure reported here is unique, the closed conformational state of *Pf*TrpB^{2B9} E(A-A) matches that determined for *Sf*TrpS (Figure S9). Our studies have revealed diverse, detailed mechanistic features of TrpB/TrpS catalysis that are shared by distant homologs. First, Trp is synthesized from IGP and Ser without accumulation of an indole intermediate (Figure 1a).¹⁶ Deuteration of the Ser α -carbon produces a negligible KIE (~ 1.0 for *Pf*TrpS), and the steady-state population consists of covalently bound Trp adducts (Table 1, Figure 2f). Hence, the structure-function relationship between COMM domain closure and TrpB catalysis was conserved during the divergent evolution that separated *P. furiosus* and *S. typhimurium*. Previously, we showed that activating mutations discovered in *Pf*TrpB were transferrable to distantly related homologs, including TrpB from *E. coli*.¹⁴ We hypothesize that this transferability works because the mutations are altering the energetics of this conserved feature of the ensemble.

While remote mutations activate *Pf*TrpB by stabilizing the closed conformational state, we lack understanding of how mutation accomplishes this feat. In principle, mutations either decrease the energy of the closed state or increase the energy of the open state. We suspect a combination of both factors is at play and the observation that the ground state of *Pf*TrpB^{2B9} has increased conformational heterogeneity would be consistent with raising its energy. In contrast, the native allosteric effector (*Pf*TrpA) causes similar kinetic changes, but forms a complex with very little conformational heterogeneity.¹² Another, finer, distinction between *Pf*TrpB^{2B9} and *Pf*TrpS is the increased abundance of Trp-adducts in steady state (Figure S2). Given that the chemical and conformational states of TrpB are tightly coupled, we hypothesize that this difference between the engineered and allosterically-activated TrpBs is due to a difference in the *rate* of COMM domain motion.

Such rates are invisible to both crystallographic and thermodynamic measurements but are highly relevant, given the conformational cycling that occurs during TrpB catalysis (Figure 4a). Such conformational effects may also underlie a previously perplexing observation: addition of *Pf*TrpA to the engineered ‘allostery-mimicking’ enzymes does not result in further activation. Instead, the native β -substitution reaction slows down, a phenomenon we call ‘allosteric inversion’.¹² A simple open-closed model, where the closed state is responsible for high catalytic activity, would suggest the effects of allosteric and mutational activation would be additive. We hypothesized that the observed allosteric inversion could occur if activation causes the same mechanistic changes, that is, decreasing the transition state barrier for the initial RLS while raising a new one. This scenario is borne out by the present study and simply adding these transition state effects could result in a higher barrier for the new RLS than for the original RLS of *Pf*TrpB, causing mutant complexes to become sluggish catalysts.

Mutational activation increases conformational heterogeneity

The directed evolution from *Pf*TrpB^{4D11} to *Pf*TrpB^{2B9} selected for increased total turnovers using Thr as the amino acid substrate. The data reported here show these mutations caused minimal loss of catalytic efficiency for the native reaction with Ser (Table 1). This contrasts

with the previous efforts to study the evolution of new function. Previously, a phosphotriesterase was engineered into an aryl esterase, and the dynamics of the ensemble were probed by B-factor analysis.⁴⁷ The parent hydrolase is highly specific for the native reaction, and has relatively little ground state heterogeneity. It was proposed that conformational dynamics first increase as the enzyme becomes a ‘generalist’, capable of performing both reactions. Conformational heterogeneity then decreases in later generations that are specialized for the novel reaction.⁴⁷ This is a parsimonious explanation for the evolution of simple reactions. The present study highlights how a more sophisticated model will be required to account for multi-step reactions. For example, the k_{cat} of the engineered TrpB enzymes after the first and last evolutionary steps are indistinguishable, despite dramatic differences in catalytic efficiency and Trp affinity. Also, *P*TrpB^{2B9} operates efficiently with a greater range of substrates.^{13,35} Some of these differences may also reflect changes in the rate of a lyase reaction that is suppressed during the native reaction discussed here, but observable when substrate analogs are employed.^{13,16,35}

In general, enzymes that catalyze multi-step reactions (i.e. TrpB) require population of multiple discrete conformational states for each iteration of the catalytic cycle and freezing out a single state would be deleterious to catalysis. Indeed, the full range of motion displayed in Figure 4a must be accomplished for each turnover. Future work to understand how mutational perturbation of the ensemble influences substrate specificity will require accounting for the rate of exchange between conformational states and may benefit from emerging molecular dynamics techniques.^{21,60}

CONCLUDING REMARKS

Lineages of evolved enzymes have emerged as a rich resource for studying the connection between sequence and function. Spectroscopic data collected during our initial effort to evolve TrpB enzymes into efficient, stand-alone catalysts suggested that mutational activation operated in a similar fashion to native allosteric activation. The present study demonstrates that activating mutations in *P*TrpB alter the conformational ensemble to favor closed states, which in turn shifts the RLS of catalysis. These are the precise effects induced by the native allosteric effector, despite no explicit selection for shared conformational properties. We hypothesize this seemingly serendipitous convergence is a manifestation of the fact that allosteric enzymes are poised to respond to perturbation.⁶¹ This special sensitivity of their conformational ensemble likely also contributes to the unusually high frequency of activating mutations (over 1% of randomly mutated variants of *P*TrpB) that were found during directed evolution. Moreover, conservation of the allosteric activation mechanism throughout divergent evolution explains why activating mutations are so readily transferable between distant homologs. Hence, we believe that it may be feasible or even facile to engineer stand-alone versions of other allosteric enzymes by directed evolution without prior knowledge of the native allosteric mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Pf	<i>Pyrococcus furiosus</i>
Ec	<i>Escherichia coli</i>
St	<i>Salmonella typhimurium</i>
TrpS	tryptophan synthase
TrpA	tryptophan synthase α -subunit
TrpB	tryptophan synthase β -subunit
RLS	rate-limiting step
IGP	indole-3-glycerol phosphate
Ain	internal aldimine
Aex	external aldimine
A-A	amino acrylate

References

1. Bornscheuer UT, Huisman GW, Kazlauskas RJ, Lutz S, Moore JC, Robins K. Nature. 2012; 485:185. [PubMed: 22575958]
2. Sheldon RA, Woodley JM. Chem Rev. 2018; 118:801. [PubMed: 28876904]
3. Motlagh HN, Wrabl JO, Li J, Hilser VJ. Nature. 2014; 508:331. [PubMed: 24740064]
4. Gao X, Jiang W, Jiménez-Osés G, Choi MS, Houk KN, Tang Y, Walsh CT. Chem Biol. 2013; 20:870. [PubMed: 23890005]
5. Francis D, Winn M, Latham J, Greaney MF, Micklefield J. ChemBioChem. 2017; 18:382. [PubMed: 28005309]
6. Smith DRM, Willemse T, Gkotsi DS, Schepens W, Maes BUW, Ballet S, Goss RJM. Org Lett. 2014; 16:2622. [PubMed: 24805161]
7. Phillips RS. Tetrahedron: Asymmetry. 2004; 15:2787.
8. Dunn MF. Arch Biochem Biophys. 2012; 519:154. [PubMed: 22310642]
9. Hydesg CC, Ahmedliiii SA, Padlans EA, Milesli EW, Daviess DR. J Biol Chem. 1988; 263:17857. [PubMed: 3053720]
10. Anderson KS, Miles EW, Johnson KA. J Biol Chem. 1991; 266:8020. [PubMed: 1902468]
11. Dunn MF, Niks D, Ngo H, Barends TRM, Schlichting I. Trends Biochem Sci. 2008; 33:254. [PubMed: 18486479]

12. Buller AR, Brinkmann-Chen S, Romney DK, Herger M, Murciano-Calles J, Arnold FH. *Proc Natl Acad Sci*. 2015; 112:14599. [PubMed: 26553994]
13. Herger M, Van Roye P, Romney DK, Brinkmann-Chen S, Buller AR, Arnold FH. *J Am Chem Soc*. 2016; 138:8388. [PubMed: 27355405]
14. Murciano-Calles J, Romney DK, Brinkmann-Chen S, Buller AR, Arnold FH. *Angew Chemie Int Ed*. 2016; 55:11577.
15. Lee SJ, Ogasahara K, Ma J, Nishio K, Ishida M, Yamagata Y, Tsukihara T, Yutani K. *Biochemistry*. 2005; 44:11417. [PubMed: 16114878]
16. Buller AR, Van Roye P, Murciano-Calles J, Arnold FH. *Biochemistry*. 2016; 55:7043. [PubMed: 27935677]
17. Dodani SC, Kiss G, Cahn JKB, Su Y, Pande VS, Arnold FH. *Nat Chem*. 2016; 8:419. [PubMed: 27102675]
18. Fraser JS, Clarkson MW, Degnan SC, Erion R, Kern D, Alber T. *Nature*. 2009; 462:669. [PubMed: 19956261]
19. Fenwick RB, van den Bedem H, Fraser JS, Wright PE. *Proc Natl Acad Sci*. 2014; 111:E445. [PubMed: 24474795]
20. Wu S, Acevedo JP, Reetz MT. *Proc Natl Acad Sci*. 2010; 107:2775. [PubMed: 20133612]
21. Latallo MJ, Cortina GA, Faham S, Nakamoto RK, Kasson PM. *Chem Sci*. 2017; 8:6484. [PubMed: 28989673]
22. Jiménez-Osés G, Osuna S, Gao X, Sawaya MR, Gilson L, Collier SJ, Huisman GW, Yeates TO, Tang Y, Houk KN. *Nat Chem Biol*. 2014; 10:431. [PubMed: 24727900]
23. Caulkins BG, Bastin B, Yang C, Neubauer TJ, Young RP, Hilario E, Huang YMM, Chang CEA, Fan L, Dunn MF, Marsella MJ, Mueller LJ. *J Am Chem Soc*. 2014; 136:12824. [PubMed: 25148001]
24. Rhee S, Parris KD, Hyde CC, Ahmed Sa, Miles EW, Davies DR. *Biochemistry*. 1997; 36:7664. [PubMed: 9201907]
25. Lane AN, Kirschner K. *Eur J Biochem*. 1983; 129:561. [PubMed: 6402361]
26. Ngo H, Kimmich N, Harris R, Niks D, Blumenstein L, Kulik V, Barends TR, Schlichting I, Dunn MF. *Biochemistry*. 2007; 46:7740. [PubMed: 17559232]
27. Cash MT, Miles EW, Phillips RS. *Arch Biochem Biophys*. 2004; 432:233. [PubMed: 15542062]
28. Lane AN, Kirschner K. *Eur J Biochem*. 1983; 129:571. [PubMed: 6402362]
29. Chang C, Huang Y, Mueller L, You W. *Catalysts*. 2016; 6:82. [PubMed: 27885336]
30. Lai J, Niks D, Wang Y, Domratcheva T, Barends TRM, Schwarz F, Olsen RA, Elliott DW, Fatmi MQ, Chang CEA, Schlichting I, Dunn MF, Mueller LJ. *J Am Chem Soc*. 2011; 133:4. [PubMed: 21142052]
31. Caulkins BG, Young RP, Kudla RA, Yang C, Bittbauer TJ, Bastin B, Hilario E, Fan L, Marsella MJ, Dunn MF, Mueller LJ. *J Am Chem Soc*. 2016; 138:15214. [PubMed: 27779384]
32. Hilario E, Caulkins BG, Young RP, Dunn MF, Mueller LJ, Fan L. 2016 PDB ID: 5CGQ.
33. Brzovic PS, Kayastha AM, Miles EW, Dunn MF. *Biochemistry*. 1992; 31:1180. [PubMed: 1346502]
34. Ruvinov SB, Ahmed SA, McPhie P, Miles EW. *J Biol Chem*. 1995; 270:17333. [PubMed: 7615535]
35. Romney DK, Murciano-Calles J, Wehrmüller JE, Arnold FH. *J Am Chem Soc*. 2017; 139:10769. [PubMed: 28708383]
36. Drewe WF, Dunn MF. *Biochemistry*. 1986; 25:2494. [PubMed: 3087420]
37. Hioki Y, Ogasahara K, Lee SJ, Ma J, Ishida M, Yamagata Y, Matsuura Y, Ota M, Ikeguchi M, Kuramitsu S, Yutani K. *Eur J Biochem*. 2004; 271:2624. [PubMed: 15206928]
38. Niks D, Hilario E, Dierkers A, Ngo H, Borchardt D, Neubauer TJ, Fan L, Mueller LJ, Dunn MF. *Biochemistry*. 2013; 52:6396. [PubMed: 23952479]
39. Pan P, Dunn MF. *Biochemistry*. 1996; 35:5002. [PubMed: 8664293]

40. Wellington S, Nag PP, Michalska K, Johnston SE, Jedrzejczak RP, Kaushik VK, Clatworthy AE, Siddiqi N, McCarren P, Bajrami B, Maltseva NI, Combs S, Fisher SL, Joachimiak A, Schreiber SL, Hung DT. *Nat Chem Biol.* 2017; 13:943. [PubMed: 28671682]
41. Wurm JP, Holdermann I, Overbeck JH, Mayer PHO, Sprangers R. *Proc Natl Acad Sci.* 2017; 114:6034. [PubMed: 28533364]
42. Fischer M, Shoichet BK, Fraser JS. *ChemBioChem.* 2015; 16:1560. [PubMed: 26032594]
43. Keedy DA, Kenner LR, Warkentin M, Woldeyes RA, Hopkins JB, Thompson MC, Brewster AS, Van Benschoten AH, Baxter EL, Uervirojnangkoorn M, McPhillips SE, Song J, Alonso-Mori R, Holton JM, Weis WI, Brunger AT, Soltis SM, Lemke H, Gonzalez A, Sauter NK, Cohen AE, van den Bedem H, Thorne RE, Fraser JS. *Elife.* 2015; 4:e07574. [PubMed: 26422513]
44. Fan YX, McPhie P, Miles EW. *Biochemistry.* 2000; 39:4692. [PubMed: 10769125]
45. Biel JT, Thompson MC, Cunningham CN, Corn JE, Fraser JS. *Structure.* 2017; 25:739. [PubMed: 28416112]
46. Jackson CJ, Foo JL, Tokuriki N, Afriat L, Carr PD, Kim HK, Schenk G, Tawfik DS, Ollis DL. *Proc Natl Acad Sci U S A.* 2009; 106:21631. [PubMed: 19966226]
47. Campbell E, Kaltenbach M, Correy GJ, Carr PD, Porebski BT, Livingstone EK, Afriat-Jurnou L, Buckle AM, Weik M, Hollfelder F, Tokuriki N, Jackson CJ. *Nat Chem Biol.* 2016; 12:944. [PubMed: 27618189]
48. Lee J, Natarajan M, Nashine VC, Socolich M, Vo T, Russ WP, Benkovic SJ, Ranganathan R. *Science.* 2008; 322:438. [PubMed: 18927392]
49. Choi JH, Laurent AH, Hilser VJ, Ostermeier M. *Nat Commun.* 2015; 6:6968. [PubMed: 25902417]
50. Schnell JR, Dyson HJ, Wright PE. *Annu Rev Biophys Biomol Struct.* 2004; 33:119. [PubMed: 15139807]
51. Buller AR, Townsend CA. *Proc Natl Acad Sci.* 2013; 110:E653. [PubMed: 23382230]
52. Smith AJT, Müller R, Toscano MD, Kast P, Hellinga HW, Hilvert D, Houk KN. *J Am Chem Soc.* 2008; 130:15361. [PubMed: 18939839]
53. Zeymer C, Zschoche R, Hilvert D. *J Am Chem Soc.* 2017; 139:12541. [PubMed: 28783336]
54. McGresham MS, Reinhart GD. *Biochemistry.* 2015; 54:952. [PubMed: 25531642]
55. Süel GM, Lockless SW, Wall MA, Ranganathan R. *Nat Struct Biol.* 2003; 10:59. [PubMed: 12483203]
56. Weyand M, Schlichting I, Herde P, Marabotti A, Mozzarelli A. *J Biol Chem.* 2002; 277:10653. [PubMed: 11756454]
57. Weber-Ban E, Hur O, Bagwell C, Banik U, Yang LH, Miles EW, Dunn MF. *Biochemistry.* 2001; 40:3497. [PubMed: 11297416]
58. Rowlett R, Yang LH, Ahmed SA, McPhie P, Jhee KH, Miles EW. *Biochemistry.* 1998; 37:2961. [PubMed: 9485448]
59. Lane AN, Kirschner K. *Eur J Biochem.* 1981; 120:379. [PubMed: 7032914]
60. Osuna S, Jiménez-Osés G, Noey EL, Houk KN. *Acc Chem Res.* 2015; 48:1080. [PubMed: 25738880]
61. Hilser VJ, Wrabl JO, Motlagh HN. *Annu Rev Biophys.* 2012; 41:585. [PubMed: 22577828]

Figure 1.

Mechanism of tryptophan synthase. a) TrpS is a heterodimeric enzyme in an $\alpha\beta\alpha$ arrangement (only one dimer is shown for clarity). In the overall catalytic cycle indole-3-glycerol phosphate binds in the α -subunit (TrpA), which catalyzes a retroaldol reaction that releases indole into an intermolecular tunnel extending into the β -subunit (TrpB), where indole condenses with L-serine in a pyridoxal phosphate (PLP)-dependent reaction to yield L-tryptophan. (b) The mechanism of TrpB catalysis with the *gem*-diamine intermediates omitted for clarity. Residues from the enzyme are shown in blue, the PLP cofactor is in black, and the intermediates are in red.

Figure 2.

Steady-state distribution of TrpB catalytic intermediates. The black line is the spectrum of each enzyme prior to the addition of substrate, and λ_{max} corresponds to E(Ain) (see Figure 1). Catalysis was initiated by the addition of 20 mM Ser and 1 mM indole. The resulting steady-state distribution is shown in the purple trace. Indole was consumed within 1–2 minutes and the steady state shifts to reflect intermediates in just stage I of catalysis, shown in the red line. Spectra were collected with 20 μM enzyme in 0.2 M potassium phosphate buffer, pH 8.0 and normalized with the value of the absorbance of the E(Ain) peak at 412 nm set to 1.0 for ease of comparison.

Figure 3. Spectra of *PfTrpB* enzymes with saturating Trp. The black line corresponds to the E(Ain) state of the cofactor. Each colored spectrum corresponds to a unique TrpB enzyme saturated with Trp. The peak at ~435 nm corresponds to the E(Aex₂) intermediate and the shoulder at ~275 nm corresponds to the E(Q₃) intermediate. The spectra were normalized with the value of the absorbance of the E(Ain) peak at 412 nm set to 1.0 for cross-comparison.

Figure 4.

Effects of Ser binding on *PTrpB* enzymes (a) Overlay of TrpB structures in different states shows the correlation between the conformational state of the COMM domain (colored) and the chemical state of the active site. The extended open state (green, PDB ID: 5T6M) was observed when a single molecule of β -methyl tryptophan serendipitously bound in a cleft connecting the active site to solvent. The open state in the absence of a ligand bound is in gray (PDB ID: 5DVZ). The external aldimine of serine, E(Aex₁), is observed with a partially closed conformation in blue (PDB ID: 6AMH). Formation of the electrophilic E(A-A) intermediate is coupled to formation of a fully closed conformational state in red (PDB ID: 5VM5). (b) Comparison of the ligand free state of *PTrpB* (PDB ID: 5DVZ), *PTrpB*^{4D11} (PDBID: 6AMC) and *PTrpB*^{2B9} (PDBID: 6AM7) reveals only a minor conformational change at residue Asp300. (c) *PTrpB*^{4D11} binds Ser as E(Aex₁). Fo-Fc omit map (green) is contoured at 3.0 σ , PDB ID: 6AMH (d) A single subunit of *PTrpB*^{2B9} binds Ser as the E(A-A) intermediate. Fo-Fc omit map (green) is contoured at 3.0 σ , PDB ID: 5VM5)

Figure 5.

Tryptophan binding by stand-alone TrpB enzymes. (a) L-Trp binds non-covalently to *Pf*TrpB^{4D11} *in crystallo*. Fo-Fc omit map (green) is contoured at 3.0 σ , PDB ID: 6AMI. (b) The affinity of *Pf*TrpB^{4D11} for Trp is significantly decreased at room temperature, as is the population of covalent adducts. (c) Comparison of *Pf*TrpB^{2B9}-E(Aex₂) with the previous structure determined with an active site mutation (PDBID: 2TYS, blue) shows changes in the orientation of the intermediate. (d) Addition of L-Trp to *Pf*TrpB^{2B9} crystals shows the product covalently bound as E(Aex₂). Fo-Fc omit map (green) is contoured at 3.0 σ , PDB ID: 6AM8. (e) The affinity of *Pf*TrpB^{2B9} for Trp is decreased at room temperature, but there is observable population of the E(Aex₂) and E(Q₃) intermediates. (f) Covalent Trp binding (purple) causes COMM domain closure that is comparable to the state observed when E(A-A) is bound.

Figure 6.

Crystallographic conformational heterogeneity of *PTrpB*. (a) Structure of *PTrpB* in the extended-open conformation (PDB ID: 5T6M) is shown in light gray. The structure with no ligands bound is colored as a rainbow along the COMM domain and E(Ain) is shown in spheres. The COMM domain of *PTrpB*-E(Aex₁) is shown in dark gray. (b) Average distance and standard deviation to the extended-open conformation for each residue of *PTrpB* in a unique state. *PTrpB* with no ligands in the E(Ain) state (PDB ID: 5DVZ) is colored as a rainbow along the COMM domain. Comparison to the Ser-bound *PTrpB* (PDB ID: 5DW0) in gray shows the heterogeneity is highest at sites that undergo motion upon closure of the COMM domain, whereafter the heterogeneity decreases.

Figure 7.

Crystallographic conformational heterogeneity of *PflTrpB*^{2B9}. (a) Structure of *PflTrpB* in the extended-open conformation (PDB ID: 5T6M), *PflTrpB*^{2B9}-E(Aex1), and *PflTrpB*^{2B9}-E(A-A) (PDB ID: 6AM9) are shown in increasingly dark shades of gray. The structure of *PflTrpB*^{2B9} with no ligands bound is colored as a rainbow along the COMM domain to match panel (b). (b) Average distance and standard deviation from the extended-open conformation to each residue of *PflTrpB*^{2B9} in a unique state. *PflTrpB*^{2B9} with no ligands in the E(Ain) state (PDB ID: 6AM7) is colored as a rainbow along the COMM domain. Comparison to *PflTrpB*^{2B9}-E(Aex₁) (PDB ID: 5VM5) in light gray and *PflTrpB*^{2B9}-E(A-A) in dark grey shows the COMM domain heterogeneity decreases upon closure.

Figure 8.

Comparison of experimental conformational heterogeneity with B-factors. (a) The heterogeneity of *PflTrpB*^{2B9} is visualized with the error bars in Figure 7b mapped onto the structure and colored from low (blue, thin) to high (red, thick). (b) The B-factor of each residue in chain B of *PflTrpB*^{2B9} is colored using the same scheme as panel (a) and shows that the maximum of the heterogeneity does not coincide with the maximum B-factor.

Figure 9.

Hypothetical reaction coordinate diagram for *PTrpB* enzymes. Energy levels are drawn (not to scale) to represent the relationship between the intermediates characterized in this study. The states that are lower in energy are those that are associated with a closed COMM domain.

Table 1
Biochemical parameters of native, engineered, and allosterically activated *P*TrpB enzymes

Enzyme	Additional Mutations	k_{cat} (s^{-1})	K_M L-Ser (mM)	K_M indole (μM)	k_{cat}/K_M L-Ser ($\text{M}^{-1}\text{s}^{-1}$)	k_{cat}/K_M indole ($\text{M}^{-1}\text{s}^{-1}$)	Kinetic Isotope Effect ^a	K_D L-Trp (μM)
<i>P</i> TrpB	N/A	0.31 ± 0.02^b	1.2 ± 0.2^b	80 ± 10^b	260	3,900	2.77 ± 0.09	1200 ± 250
<i>P</i> TrpB ^{G9}	T292S	1.1 ± 0.2^b	0.84 ± 0.04^b	14 ± 3^b	1,300	78,000	1.46 ± 0.08	168 ± 12
<i>P</i> TrpB ^{4D11}	E17G, I68V, F274S, T321A	2.2 ± 0.2^b	1.2 ± 0.2^b	11 ± 2^b	1,800	200,000	1.37 ± 0.08	30 ± 4
<i>P</i> TrpB ^{4G1}	F95L	1.3 ± 0.05	0.45 ± 0.08	<10	2,900	$>130,000$	1.17 ± 0.11	16 ± 1
<i>P</i> TrpB ^{2B9}	I16V, V384A	0.94 ± 0.02	0.34 ± 0.03	<10	2,800	$>94,000$	1.12 ± 0.03	13 ± 1
<i>P</i> TrpS	N/A	1.0 ± 0.1^b	0.6 ± 0.1^b	20 ± 2^b	1,600	50,000	1.01 ± 0.04	<10

All reactions conducted with 1–60 μM enzyme in potassium phosphate buffer pH 8.0 at 75 °C.

^aKinetic isotope effect determined as the ratio of the initial velocities of product formation using Ser and d3-Ser.

^bThese values from ref. 10. See materials and methods for details. Error bars represent the standard error for each parameter determined from a non-linear fit.