

# Structural and functional innovations in the real-time evolution of new $(\beta\alpha)_8$ barrel enzymes

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New genes can arise by duplication and divergence, but there is a fundamental gap in our understanding of the relationship between these genes, the evolving proteins they encode, and the fitness of the organism. Here we used crystallography, NMR dynamics, kinetics, and mass spectrometry to explain the molecular innovations that arose during a previous real-time evolution experiment. In that experiment, the  $(\beta\alpha)_8$  barrel enzyme HisA was under selection for two functions (HisA and TrpF), resulting in duplication and divergence of the *hisA* gene to encode TrpF specialists, HisA specialists, and bifunctional generalists. We found that selection affects enzyme structure and dynamics, and thus substrate preference, simultaneously and sequentially. Bifunctionality is associated with two distinct sets of loop conformations, each essential for one function. We observed two mechanisms for functional specialization: structural stabilization of each loop conformation and substrate-specific adaptation of the active site. Intracellular enzyme performance, calculated as the product of catalytic efficiency and relative expression level, was not linearly related to fitness. Instead, we observed thresholds for each activity above which further improvements in catalytic efficiency had little if any effect on growth rate. Overall, we have shown how beneficial substitutions selected during real-time evolution can lead to manifold changes in enzyme function and bacterial fitness. This work emphasizes the speed at which adaptive evolution can yield enzymes with sufficiently high activities such that they no longer limit the growth of their host organism, and confirms the  $(\beta\alpha)_8$  barrel as an inherently evolvable protein scaffold.

HisA | TrpF | adaptive evolution | enzyme performance threshold

A central question in biology is how new genes, and thus new enzymes, emerge. Experimental evolution and phylogenetic sequence analysis have shown that horizontal gene transfer, de novo evolution, gene fusion/fission, and the duplication of preexisting genes with subsequent divergence all contribute to this process (1–5). One model for duplication and divergence, the IAD (innovation-amplification-divergence) model (6), posits that a candidate for duplication is a gene whose protein product not only performs its primary function, but also carries out a secondary, nonessential function (innovation). Such promiscuous proteins are common in all organisms (7). If conditions arise that make the secondary function important, then selection for increased gene copy number could satisfy the need for more of that protein (amplification). Once a mutation in one extra copy improves the secondary function of the resulting protein, the survival of that duplicate and the probability of its evolution toward specialization increases (divergence).

The IAD model was previously tested experimentally in *Salmonella enterica*, to show that new genes can evolve rapidly in bacteria to perform novel functions (8). The model system involved the *N*′-[5′-phosphoribosyl]formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) isomerase, HisA, which catalyzes an essential step in histidine biosynthesis (Fig. 1A). In an *S. enterica* strain in which the *trpF* gene was deleted from the chromosome, a spontaneous *hisA* mutation was selected that could complement

the resulting tryptophan auxotrophy. Growth rate data showed that this mutant, HisA(dup13-15/D10G), catalyzed both the HisA and TrpF (phosphoribosylanthranilate isomerase) reactions (Fig. 1A), albeit suboptimally. Using this bifunctional mutant as a starting point, continuous selection for improved HisA and TrpF activities led to the creation of new genes by duplication and divergence. In fewer than 3,000 generations of growth under selection, the ancestral *hisA* variant was amplified, and then individual gene copies acquired point mutations, allowing them to diverge to encode either TrpF specialists, HisA specialists, or generalists performing both reactions (Fig. 1B).

To understand how growth under selection honed the bifunctional HisA(dup13-15/D10G) variant during real-time evolution, we have characterized 11 enzymes—3 HisA specialists (including HisA itself), 3 TrpF specialists, and 5 generalists—using various structural and functional approaches. This has allowed us to relate changes in genotype (mutations) to changes in phenotype (enzyme kinetics, expression level, structure, and dynamics) and to organismal fitness (growth rate).

## Results

**Functional Analyses.** We first characterized each of the nine individual enzymes that make up the evolutionary trajectory in Fig. 1B (mutation sites indicated in Fig. 1C). We purified these enzymes

## Significance

New proteins can evolve by duplication of the genes that encode them, followed by specialization of the different copies. However, how the growth rate of an organism is coupled to the changes in a protein's structure and function occurring during this process is not known. Here we show at atomic resolution how selection for the growth of a bacterium led to the evolution of HisA proteins with either a new function or two functions (old and new). We found that a distinct protein conformation is responsible for each function, and that a better enzyme leads to faster growth only up to a certain threshold. This study provides insight into how evolution works, from atomic to whole-organism levels.

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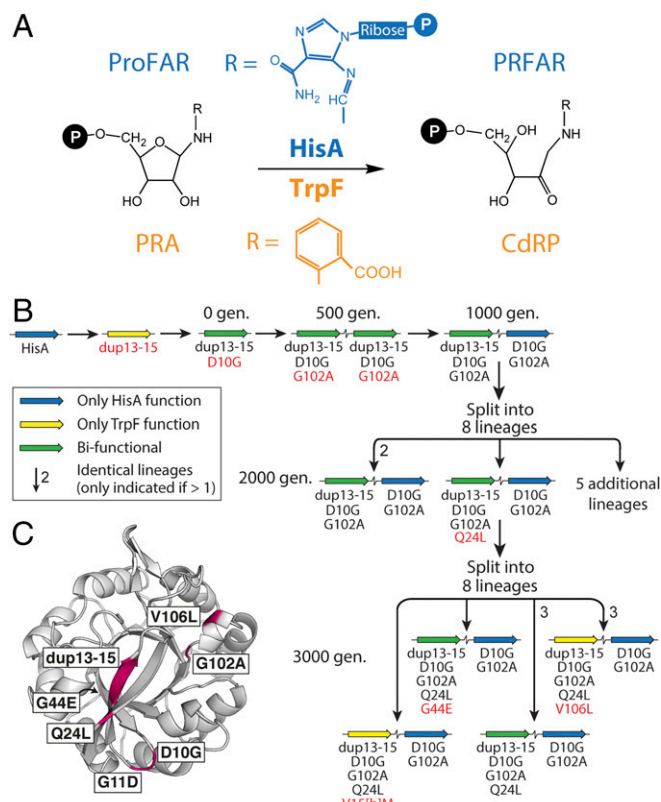
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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, [www.pdb.org](http://www.pdb.org) (PDB ID codes 5G1T, 5AC7, 5AC8, 5L9F, 5AB3, 5G1Y, 5L6U, 5G4E, 5G4W, 5G2I, 5G2W, 5G2H, and 5G5I).

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**Fig. 1.** Real-time evolution of new TrpF and HisA enzymes. (A) The analogous reactions catalyzed by HisA (blue) and TrpF (orange). HisA converts ProFAR into  $N'$ -[(5'-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (PRFAR). TrpF converts PRA to CdRP. (B) Mutational trajectory for real-time evolution of HisA into a bifunctional ancestor [HisA(dup13-15/D10G)] and then during continuous selection for improved TrpF and HisA activities (8). Amino acid substitutions arising in each population are shown below the gene symbols, with new mutations identified at each step shown in red. (C) Structure of a HisA variant, HisA(D7N/dup13-15/D10G), with the locations of all mutations from B shown in purple.

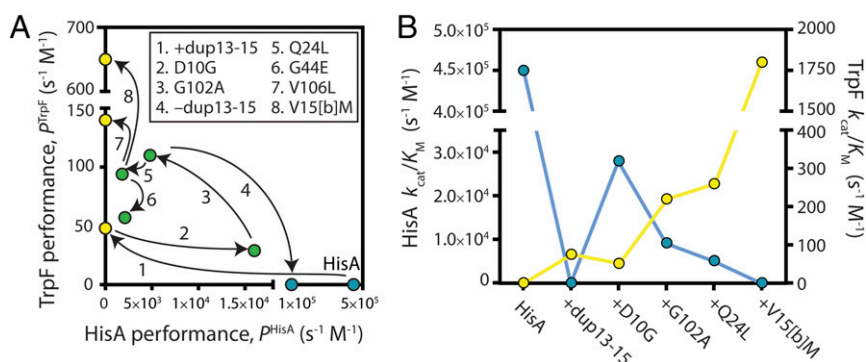
and measured their Michaelis-Menten kinetic parameters *in vitro*. In parallel, we used a multiplexed tandem mass tagging approach to determine their relative expression levels during exponential-phase growth. This allowed us to determine a performance parameter for each enzyme,  $P = (k_{\text{cat}}/K_M) \times (\text{relative enzyme level})$ , which determines the relative rate of intracellular product formation. The

performances of the enzymes, plotted along the trajectories in which they evolved, are shown in Fig. 2A. Kinetic parameters and relative expression levels of each enzyme are presented in Table 1.

The first innovations in the previous evolution experiment generated a gene encoding the weakly bifunctional ancestor, HisA (dup13-15/D10G). Growth experiments previously showed that duplication of codons 13–15 (encoding amino acids V15[a]-V15[b]-R15[c]) imparted TrpF activity, but destroyed HisA activity (8). Assays of purified HisA(dup13-15) confirmed that its TrpF activity was weak but measurable ( $k_{\text{cat}}/K_M = 75 \text{ s}^{-1} \text{ M}^{-1}$ ), whereas it was inactive as a HisA enzyme (Table 1). The introduction of D10G further decreased TrpF activity, to  $k_{\text{cat}}/K_M = 51 \text{ s}^{-1} \text{ M}^{-1}$ , but restored HisA activity to 6% of the wild-type (WT) level. The intracellular performances of HisA(dup13-15) and HisA(dup13-15/D10G) were further diminished by their expression levels, which were reduced to ~60% of HisA (Table 1).

Starting from HisA(dup13-15/D10G), duplication and divergence yielded a series of new genes encoding enzymes with one or both of the activities under selection. In the first 500 generations of growth under selection, amplification of the gene for HisA (dup13-15/D10G) was accompanied by fixation of the additional mutation, G102A (Fig. 1B). G102A increased TrpF performance fourfold, while diminishing HisA performance by a similar amount (mutation 3 in Fig. 2A). Thus, the ratio of the specificity constants,  $(k_{\text{cat}}/K_M)^{\text{HisA}} \div (k_{\text{cat}}/K_M)^{\text{TrpF}}$ , dropped from 550 in HisA(dup13-15/D10G) to 42 in HisA(dup13-15/D10G/G102A). As expected, this change in kinetic parameters was accompanied by significant improvements in organismal growth rates under conditions requiring TrpF activity or both TrpF and HisA activity (SI Appendix, Table S1). Despite its reduced HisA activity, HisA(dup13-15/D10G/G102A) also conferred improved growth when only this activity was required (i.e., in tryptophan-supplemented medium). Further studies are needed to explain this observation.

Divergence was first observed after 1,000 generations of evolution, with emergence of the gene encoding HisA(D10G/G102A) (Fig. 1B). Loss of the three-residue VVR duplication ablated TrpF activity in this enzyme. The bacterial cells maintained HisA(D10G/G102A) as their HisA specialist ( $k_{\text{cat}}/K_M$  for the HisA reaction =  $1.6 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$ ) for the remainder of the evolution experiment, whereas a gene duplicate acquired further mutations that modulated TrpF and HisA activities. Among the gene variants, the three mutations that effected the largest improvements in TrpF activity (dup13-15, G102A, and V15[b]M) either significantly decreased or abolished HisA activity (Fig. 2B). The variant with the greatest TrpF activity was HisA(dup13-15/D10G/G102A/Q24L/V15[b]M). This variant reached fixation in one lineage after 3,000 generations of growth under selection (Fig. 1B), and had a



**Fig. 2.** Genotype-phenotype landscapes. (A) Mutational effects on HisA and TrpF performance. Points connected by an arrow differ by a single mutation, as shown in the inset. The intracellular performance of each enzyme,  $P$ , is defined as the product of its catalytic efficiency ( $k_{\text{cat}}/K_M$ ) and its relative expression level during exponential-phase growth, with the expression level of WT HisA set to 1. Blue, HisA specialists; green, generalists; yellow, TrpF specialists. (B) Activity trade-offs along a trajectory of evolving HisA variants. Blue, HisA activity; yellow, TrpF activity.





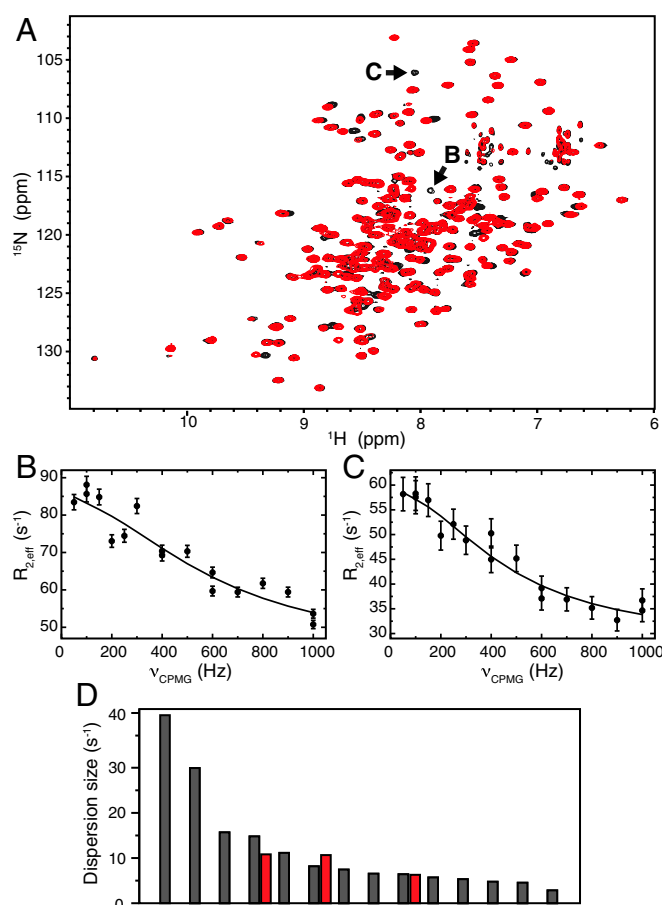
significantly, HisA(dup13-15/D10G/G102A/G11D/G44E) had the highest measured  $K_M$  for the HisA substrate, ProFAR ( $K_M^{\text{ProFAR}} = 100 \mu\text{M}$ ; Table 1).

**Structural Analyses.** We determined a total of 13 apo and complexed crystal structures of the evolved HisA variants, all of which form similar  $(\beta\alpha)_8$  barrels (SI Appendix, Figs. S1–S3 and Table S2). The long loops on the catalytic face of WT HisA enclose and trigger a structural change in ProFAR before catalysis (Fig. 3A) (13). In the apo structures of the evolved variants, active site loops 1, 5, and 6 are partly disordered or open, and phosphate or sulfate ions from the crystallization buffer often mimic the two phosphate groups of ProFAR in the active site. Selected mutants, including some with disabling mutations of the catalytic base (Asp7), also were subjected to cocrystallization or soaking with either ProFAR or the TrpF product analog, reduced 1'-(2'-carboxyphenylamino)-1'-deoxyribulose 5'-phosphate (rCdRP). This resulted in some structures in which the active site loops are more ordered (SI Appendix, Fig. S1 and Table S2) allowing us to observe closed, presumably active conformations. Owing to the low affinity of rCdRP, its ligand density was always too poor for unambiguous interpretation. Apart from G44E, all mutations are on the substrate-binding, catalytic face of the barrel (Fig. 1C). Several mutations are on loop 1 and thus are only visible in some of the structures.

The VVR duplication in loop 1, which initially provided TrpF activity, induces a new, extended conformation of loop 1 toward loop 5 that is observed in structures of two bifunctional enzymes and one TrpF specialist, in three different crystal forms (SI Appendix, Fig. S1). In this presumably TrpF-active conformation, the duplicated arginine, Arg15[c], is positioned in the active site, available for interaction with the TrpF substrate PRA (Fig. 3B). A different arginine, Arg143, has an equivalent interaction with rCdRP in the active site of PriA (11). Another *S. enterica* mutant with TrpF activity, HisA(L169R) (8), places Arg169 in a similar position (Fig. 3C and SI Appendix, Table S2), as does TrpF itself (14). The appropriate positioning of an arginine appears to be a shared innovation for PRA binding and a catalytically competent active site (11, 14) in the emergence of TrpF activity on the  $(\beta\alpha)_8$  barrel scaffold.

HisA activity is abolished in HisA(dup13-15) because the extended conformation of loop 1 blocks Trp145 (on loop 5) from interacting with ProFAR (Fig. 3D). A HisA(W145A) mutant was unable to rescue the histidine auxotrophy of *S. enterica*  $\Delta\text{hisA}$ , showing that the Trp145–ProFAR interaction is essential for ProFAR isomerase activity. Mutagenesis of the equivalent tryptophan in PriA also destroys HisA activity (11). The D10G mutation in loop 1, which reintroduced HisA activity into HisA(dup13-15), does not induce any changes in the crystal structure, but is predicted to allow more flexibility of loop 1, so that loop 5 can compete for the active site (Fig. 3D). To test this hypothesis, we performed NMR relaxation dispersion experiments, which demonstrated that D10G leads to increased conformational dynamics (Fig. 4). Significant microsecond-millisecond motions were detected at 14 backbone  $^{15}\text{N}$  positions for HisA(dup13-15/D10G), compared with only three positions for HisA(dup13-15) (Fig. 4D; and SI Appendix, Fig. S4). Importantly, the two resonances with the largest dispersions are unique to HisA(dup13-15/D10G) (Fig. 4A–C). Thus, the D10G mutation, which confers bifunctionality in presence of dup13-15, leads to increased dynamics of HisA, supporting our hypothesis.

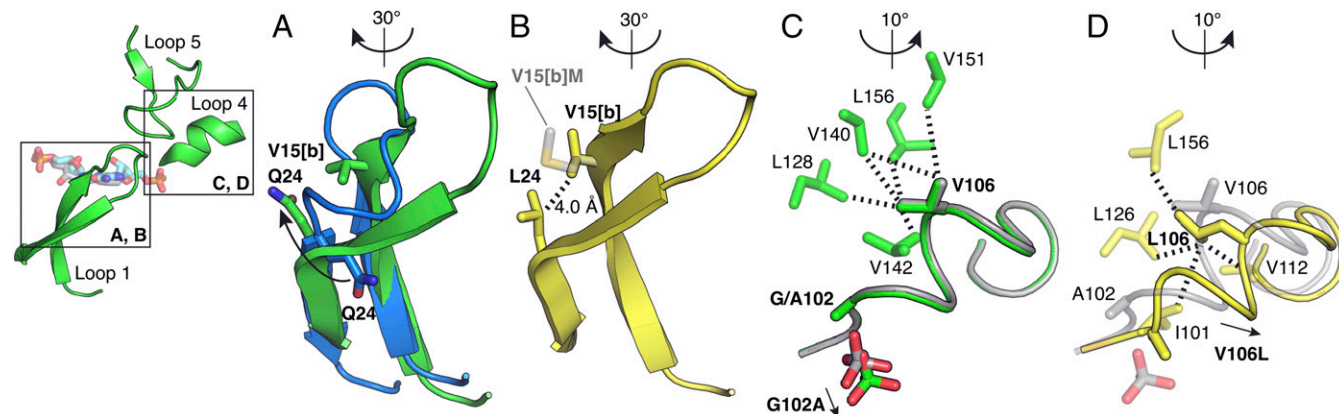
These data suggest that the HisA and TrpF reactions are catalyzed by enzymes adopting mutually exclusive loop conformations, where either loop 1 or loop 5 forms a critical interaction with the substrate (i.e., Arg15[c] with PRA or Trp145 with ProFAR; Fig. 3D). Further support for this model is provided by structures containing the Q24L mutation. In what we assume is the TrpF-active conformation of loop 1, the Gln24 side chain flips from its native position, inducing a backbone turn that brings it close to Val15[b] of the duplication, which is on the other strand of the



**Fig. 4.** The solution structure of HisA(dup13-15/D10G) is similar to that of HisA(dup13-15), but more dynamic on the microsecond-millisecond time scale. (A)  $^{15}\text{N}$ - $^1\text{H}$  correlation maps of HisA(dup13-15/D10G) (black) and HisA(dup13-15) (red). (B and C) Relaxation dispersion profiles for the HisA(dup13-15/D10G)  $^{15}\text{N}$  resonances indicated with arrows in A. (D) Dispersion sizes for residues with significant  $^{15}\text{N}$  dynamics for HisA(dup13-15/D10G) (gray) and HisA(dup13-15) (red), sorted in order of decreasing dispersion size. The two leftmost gray bars correspond to the dispersions in B and C.

$\beta$ -hairpin (Fig. 5A). Q24L thus introduces a new hydrophobic interaction that stabilizes this TrpF-active loop conformation (Fig. 5B). Val15[b] is mutated to methionine in the variant with the highest TrpF activity (Table 1). Although V15[b]M is disordered in the available apo structure of this variant, based on the position of Val15 in the TrpF-active conformation, we can predict that the mutation further stabilizes the interaction with Leu24 (Fig. 5B). This seems to favor the TrpF-active conformation of loop 1 to the extent that the enzyme can no longer adopt its HisA-active conformation, and HisA activity is lost.

When HisA(dup13-15/D10G/G102A) evolved from HisA(dup13-15/D10G), its diminished performance as a HisA (Fig. 2) was related to an increase in  $K_M^{\text{ProFAR}}$  (Table 1). The structure of HisA(dup13-15/D10G/G102A) (Fig. 5C) reveals the Ala102 side chain oriented into the binding site for the second phosphate of ProFAR. This phosphate-binding site is not required for PRA binding, as the TrpF substrate is only monophosphorylated (Fig. 1A). Subsequently, fixation of the mutation V106L in one lineage, after 3,000 generations of evolution (Fig. 1B), converted the bifunctional generalist into a TrpF specialist. V106L induces a shift of loop 4 that pushes G102A even further, preventing ionic phosphate from binding to the protein in the crystal structure (Fig. 5D). The blocked phosphate binding site presumably prevents productive binding of ProFAR in the active site and leads to abolished HisA activity.



with catalytic efficiencies on the order of  $10^2$ – $10^3$  s<sup>−1</sup> M<sup>−1</sup> conferred maximal growth rates. In comparison, the *Escherichia coli* TrpF enzyme has  $k_{cat}/K_M = 6.8 \times 10^6$  s<sup>−1</sup> M<sup>−1</sup> (25) and the bifunctional *M. tuberculosis* PriA has  $k_{cat}/K_M = 1.7 \times 10^5$  s<sup>−1</sup> M<sup>−1</sup> for the TrpF reaction (11). In *E. coli*, TrpF is synthesized at a rate of ~110 molecules per generation when tryptophan is present in the medium, rising by approximately eightfold when the *trp* operon is derepressed in minimal medium (26). In our experimental system, the HisA variants were expressed from a constitutive promoter (8). Even if this artificially increased expression by 1–2 orders of magnitude (making our HisA variants among the most highly expressed metabolic enzymes in each strain), the total intracellular TrpF activities of the evolved *S. enterica* strains would still be lower than those of *E. coli* and *M. tuberculosis*. Thus, some bacteria may have TrpF enzymes that exceed their performance thresholds and have “excess capacity” at this node in the metabolic network (27).

The (β $\alpha$ )<sub>8</sub> barrel is the most common enzyme fold. Its extraordinary functional diversification is generally ascribed to the mutability of its active site-forming loops. Experimental tests of this hypothesis have focused on engineering by point mutagenesis or loop swaps (28). Our real-time evolution experiment yielded a different functional innovation, the VVR duplication in loop 1, which would have been difficult to discover with currently used design algorithms and engineering methods. The duplication places Arg15[c] in an analogous (but not homologous) position to functionally equivalent arginines in HisA(L169R), PriA and TrpF (Fig. 3C), whereas D10G allows conformational switching between the two mutually exclusive loop orientations that are necessary for TrpF and HisA activities. This emphasizes the important contribution of loop mutability to (β $\alpha$ )<sub>8</sub> barrel evolvability (29, 30), as well as the potential of this fold’s overall loop architecture to enable convergent evolutionary solutions.

Our detailed study of a real-time evolution process explains how mutations acquired under continuous selection can result in structural and functional innovations in the encoded enzymes over

a relatively small number of generations. The result is a rich—and at present, largely unpredictable—landscape of evolved proteins.

## Materials and Methods

The materials and methods used in this study are summarized below. More detailed information is provided in *SI Appendix, Materials and Methods*.

**Molecular Biology.** The cloning of *hisA* mutants and site-directed mutagenesis were carried out as described previously (13).

**Protein and Ligand Preparation.** All proteins were purified as described previously (13). ProFAR was synthesized as reported previously (13), and rCDRP was purchased from Chemir.

**Enzyme Kinetics.** HisA assays were conducted as described previously (13). TrpF activities were quantified using a coupled spectrophotometric assay (31).

**Mass Spectrometry.** Relative expression levels of each HisA variant were determined using multiplexed tandem mass tagging, as described previously (32).

**Structure Determination.** Crystallization conditions for each mutant are listed in *SI Appendix, Table S2*. Data were collected at the European Synchrotron Radiation Facility (Grenoble, France), Diamond Light Source (Didcot, U.K.), and PETRA (Hamburg, Germany), and structures were solved by molecular replacement, as described for WT HisA (13). Data and refinement statistics are provided in *SI Appendix, Table S2*.

**NMR.** <sup>15</sup>N Carr–Purcell–Meiboom–Gill relaxation dispersion experiments were recorded for <sup>15</sup>N-labeled HisA(dup13–15) and HisA(dup13–15/D10G), as described in *SI Appendix, Materials and Methods*.

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- Nelson-Sathi S, et al. (2015) Origins of major archaeal clades correspond to gene acquisitions from bacteria. *Nature* 517:77–80.
- Carvunis A-R, et al. (2012) Proto-genes and *de novo* gene birth. *Nature* 487:370–374.
- Rogers RL, Hartl DL (2012) Chimeric genes as a source of rapid evolution in *Drosophila melanogaster*. *Mol Biol Evol* 29:517–529.
- Ohno S (1970) *Evolution by Gene Duplication* (Springer, New York).
- Dittmar K, Liberles D (2010) *Evolution After Gene Duplication* (Wiley, New York).
- Berghorsson U, Andersson DI, Roth JR (2007) Ohno’s dilemma: Evolution of new genes under continuous selection. *Proc Natl Acad Sci USA* 104:17004–17009.
- Kheronsky O, Tawfik DS (2010) Enzyme promiscuity: A mechanistic and evolutionary perspective. *Annu Rev Biochem* 79:471–505.
- Näsvall J, Sun L, Roth JR, Andersson DI (2012) Real-time evolution of new genes by innovation, amplification, and divergence. *Science* 338:384–387.
- Bar-Even A, et al. (2011) The moderately efficient enzyme: Evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry* 50:4402–4410.
- Claren J, Malisi C, Höcker B, Sterner R (2009) Establishing wild-type levels of catalytic activity on natural and artificial (β $\alpha$ )<sub>8</sub>-barrel protein scaffolds. *Proc Natl Acad Sci USA* 106:3704–3709.
- Due AV, Kuper J, Geerlof A, von Kries JP, Wilmanns M (2011) Bisubstrate specificity in histidine/tryptophan biosynthesis isomerase from *Mycobacterium tuberculosis* by active site metamorphosis. *Proc Natl Acad Sci USA* 108:3554–3559.
- Khanal A, Yu McLoughlin S, Kershner JP, Copley SD (2015) Differential effects of a mutation on the normal and promiscuous activities of orthologs: Implications for natural and directed evolution. *Mol Biol Evol* 32:100–108.
- Söderholm A, et al. (2015) Two-step ligand binding in a (β $\alpha$ )<sub>8</sub> barrel enzyme: Substrate-bound structures shed new light on the catalytic cycle of HisA. *J Biol Chem* 290:24657–24668.
- Henn-Sax M, et al. (2002) Two (β $\alpha$ )<sub>8</sub>-barrel enzymes of histidine and tryptophan biosynthesis have similar reaction mechanisms and common strategies for protecting their labile substrates. *Biochemistry* 41:12032–12042.
- Neidhardt FC, Umbarger HE (1996) *Chemical Composition of Escherichia coli. Escherichia coli and Salmonella*, ed Neidhardt FC (ASM Press, Washington, D.C.), 2nd Ed, pp 13–16.
- Campbell E, et al. (2016) The role of protein dynamics in the evolution of new enzyme function. *Nat Chem Biol* 12:944–950.
- Kaltenbach M, Jackson CJ, Campbell EC, Hollfelder F, Tokuriki N (2015) Reverse evolution leads to genotypic incompatibility despite functional and active site convergence. *eLife* 4:e06492.
- Tokuriki N, et al. (2012) Diminishing returns and tradeoffs constrain the laboratory optimization of an enzyme. *Nat Commun* 3:1257.
- Salverda ML, et al. (2011) Initial mutations direct alternative pathways of protein evolution. *PLoS Genet* 7:e1001321.
- Lang GI, Desai MM (2014) The spectrum of adaptive mutations in experimental evolution. *Genomics* 104:412–416.
- Miton CM, Tokuriki N (2016) How mutational epistasis impairs predictability in protein evolution and design. *Protein Sci* 25:1260–1272.
- Kim J, Copley SD (2012) Inhibitory cross-talk upon introduction of a new metabolic pathway into an existing metabolic network. *Proc Natl Acad Sci USA* 109:E2856–E2864.
- Dykhuizen DE, Dean AM, Hartl DL (1987) Metabolic flux and fitness. *Genetics* 115:25–31.
- Lunzer M, Miller SP, Felsheim R, Dean AM (2005) The biochemical architecture of an ancient adaptive landscape. *Science* 310:499–501.
- Hommel U, Eberhard M, Kirschner K (1995) Phosphoribosyl anthranilate isomerase catalyzes a reversible amadori reaction. *Biochemistry* 34:5429–5439.
- Li GW, Burkhardt D, Gross C, Weissman JS (2014) Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. *Cell* 157:624–635.
- Newton MS, Arcus VL, Patrick WM (2015) Rapid bursts and slow declines: On the possible evolutionary trajectories of enzymes. *J R Soc Interface* 12:20150036.
- Sterner R, Höcker B (2005) Catalytic versatility, stability, and evolution of the (β $\alpha$ )<sub>8</sub>-barrel enzyme fold. *Chem Rev* 105:4038–4055.
- Babbitt PC, Gerlt JA (1997) Understanding enzyme superfamilies: Chemistry as the fundamental determinant in the evolution of new catalytic activities. *J Biol Chem* 272:30591–30594.
- Tóth-Petrőczy A, Tawfik DS (2014) The robustness and innovability of protein folds. *Curr Opin Struct Biol* 26:131–138.
- Patrick WM, Matsumura I (2008) A study in molecular contingency: Glutamine phosphoribosylpyrophosphate amidotransferase is a promiscuous and evolvable phosphoribosylanthranilate isomerase. *J Mol Biol* 377:323–336.
- Knöppel A, Näsvall J, Andersson DI (2016) Compensating the fitness costs of synonymous mutations. *Mol Biol Evol* 33:1461–1477.