Enzyme Recruitment and Its Role in Metabolic Expansion

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ABSTRACT: Although more than 10^9 years have passed since the existence of the last universal common ancestor, proteins have yet to reach the limits of divergence. As a result, metabolic complexity is ever expanding. Identifying and understanding the mechanisms that drive and limit the divergence of protein sequence space impact not only evolutionary biologists investigating molecular evolution but also synthetic biologists seeking to design useful catalysts and engineer novel metabolic pathways. Investigations over the past 50 years indicate that the recruitment of enzymes for new functions is a key event in the acquisition of new metabolic capacity. In this review, we outline the genetic mechanisms that enable recruitment and summarize the present state of knowledge regarding the functional characteristics of extant catalysts that facilitate recruitment. We also highlight recent examples of enzyme recruitment, both from the historical record provided by phylogenetics and from enzyme evolution experiments. We conclude with a look to the future, which promises fruitful consequences from the convergence of molecular evolutionary theory, laboratory-directed evolution, and synthetic biology.

Enzyme recruitment is the process whereby an extant catalyst is enlisted to perform a new function that provides a selective advantage to a host organism. The new function can be comparable to, or distinct from, the enzyme’s ancestral purpose. Recruitment requires a random genetic change that affords realization of the new function and subsequent fixation of the gene encoding the new catalyst within a given population. In many instances, enzyme recruitment represents the initial molecular genetic event upon which the acquisition of new metabolic potential relies.

Although enzyme recruitment was long proposed to be a driving force in metabolic evolution,1,2 conclusive evidence of recruitment required advances in comparative phylogenetics and high-throughput structural biology. The enormous number of protein primary structure data resulting from genomic sequencing efforts of the past 25 years provide compelling evidence that enzyme recruitment is pervasive in extant metabolic pathways. Structural and functional studies have revealed surprising evolutionary relationships between enzymes that catalyze seemingly disparate transformations.3,4 This information is leading to a more detailed appreciation of the interrelatedness within, and between, primary and secondary metabolism. Moreover, recent experimental work suggests that recruitment likely contributed to the earliest stages of evolution, including the templated synthesis of polypeptides.5–7

Investigating the molecular mechanisms of enzyme recruitment is not simply an exercise in understanding the past. Recruitment also plays a central role in important contemporary biological processes. It facilitates the appearance of drug resistance in microorganisms,8 as well as the emergence of bioremediation pathways for anthropogenic toxins.9 Our understanding of enzyme recruitment is now sufficiently advanced that protein engineers and synthetic biologists are beginning to utilize this knowledge for the discovery of new catalysts and the design of novel metabolic pathways. For these reasons, a review of enzyme recruitment is particularly timely.

I. MECHANISMS THAT FACILITATE ENZYME RECRUITMENT

All mechanisms that drive enzyme recruitment involve genetic change. The simplest of these is the accumulation of point mutations. A point mutation can facilitate enzyme recruitment by enhancing a latent activity or by installing a new function onto a preexisting catalyst (Figure 1A). Point mutations appear at an average rate of 0.0033 nucleotide change per genome per DNA replication in microorganisms.10 However, a vast majority of these mutations do not become fixed, because they are either neutral or deleterious.11,12 Most instances of recruitment are not driven by a gain-of-function point mutation but instead involve a beneficial mutation that causes a loss of regulatory control leading to overproduction of an enzyme possessing hidden activity.1,13 For example, the transcriptional level of a coding region can be elevated by mutations that inactivate a repressor protein or by promoter mutations that disrupt the binding site for a repressor (Figure 1B). Similarly, the translational level of an enzyme can be increased by mutations that alter codon usage efficiency, mRNA stability, or ribosome binding strength. Hence, enzyme recruitment can be facilitated by any mutation that amplifies the cellular concentration of a catalyst to an extent such that a latent function rises to a physiologically relevant level.
A more substantial change at the genomic level that can facilitate recruitment is gene duplication (Figure 1C). Gene duplication events occur frequently during the normal course of cell division.14,15 Under normal environmental conditions, the estimated frequency of gene duplication in *Salmonella typhimurium* is $10^{-2}$ to $10^{-4}$ per genome per DNA replication.16,17 This level increases under extreme environmental conditions, but can rapidly return to normal levels within a few generations after the selective pressure disappears.19 It is estimated that at least 10% of all prokaryotic cells contain at least one duplicated gene.20,21 Gene duplication provides another mechanism for the overproduction of a gene product with latent activity.22–26 Duplication also removes constraints associated with the retention of a gene’s ancestral function, as one copy of the gene continues to fulfill its intended metabolic task, while the second copy can be subjected to more intense diversification.27,28

Horizontal gene transfer is another genetic alteration that promotes enzyme recruitment by providing new genetic material to a host organism (Figure 1D). Short DNA sequences, as well as complete coding fragments, can be transferred via conjugation and plasmid exchange or directly acquired from the environment.29–31 Importantly, this exchange of genetic material can occur within or between species.32–34 Genome sizes can increase by 10–80% as a result of gene transfer or gene duplication, providing a wealth of new genetic material for subsequent diversification.35 Duplication and horizontal gene transfer also allow for shuffling of partial coding regions, which can lead to the generation of new multifunctional proteins. Although gene duplications and horizontal transfer events are less genetically stable than point mutations, their natural frequency exceeds the rate of beneficial point mutation by several orders of magnitude.17 For this reason, the duplication and transfer of genes are particularly effective in promoting enzyme recruitment.

### PROPERTIES OF ENZYMES THAT FACILITATE THEIR RECRUITMENT

Genetic variation is constantly occurring within organisms, providing ample opportunities to recruit enzymes for new biological purposes. What makes one recruitment event successful while others are only transient in nature? The answer to this question depends, in part, upon the environ-mental context in which recruitment takes place. However, specific characteristics of the catalyst appear to contribute to the long-term success of the recruitment process. In general, the more evolvable an enzyme, the more likely that it will become permanently recruited to perform a new task.35

As with other proteins, the evolvability of an enzyme is determined by its stability and its potential for functional innovation.36–38 Stability promotes mutational robustness,39–41 as newly introduced mutations are often destabilizing in nature.36,42 Thus, a highly stable enzyme can tolerate the accumulation of a larger number of destabilizing mutations. Because the acquisition and optimization of new function often require the accumulation of multiple mutations, stability is beneficial for evolvability.43,44 Stability often correlates with reduced conformational flexibility,45–47 however, and biochemists now appreciate that structural plasticity and dynamism can empower the evolution of function by providing access to different conformational states and unique arrangements of active site residues (Figure 2A).38 In nature, the apparent conflict between the benefits of stability (i.e., mutational robustness) and the benefits of flexibility (i.e., functional plasticity) is addressed by specific features of the polypeptide scaffold. For many proteins, an apparent correlation exists between the thermal stability of the folded state and the physiological temperature that provides optimal growth for the host organism.49 This correlation indicates that proteins are, in general, only marginally stable at the specific environmental conditions under which selective pressures operate.50 Marginal stability provides a balance between maintaining a well-folded structure and retaining sufficient flexibility to promote evolvability.49 Protein scaffolds are equipped with discrete structural elements that confer flexibility and stability. For example, the $(\beta/\alpha)_8$-barrel scaffold appears to be highly evolvable.51,52 It represents a common enzymatic fold that is present in more than 120 enzyme families.53 The $(\beta/\alpha)_8$-barrel fold is built from a stable core of repeating $\alpha$-helix–$\beta$-strand units, which are linked together by a series of intervening loops. The loops cap the active site, which is located near the C-terminal end of the $\beta$-strands. In this scaffold, the core provides mutational robustness while the loops provide flexibility. A similar segregation of protein structural elements is apparent in other polypeptide folds.55

The potential for future functional innovation is another contributor to enzyme evolvability.55–57 Enzymes have long been depicted as highly specialized catalysts with finely tuned functionalities.56 However, recent experiments demonstrate that such a picture is oversimplified. We now recognize that contemporary enzymes can be both promiscuous in the reactions they catalyze and ambiguous in their choice of substrates (Figure 2B).57–59 Catalytic promiscuity is defined as the ability of an enzyme to promote distinct chemical transformations that are often related by a common half-reaction or involve a common intermediate.55 Substrate ambiguity is a related but conceptually distinct attribute, which describes an enzyme’s ability to catalyze the same chemical transformation on a series of structurally distinct reactants. Although catalytic promiscuity and substrate ambiguity were most likely more pronounced in ancestral proteins, they continue to facilitate enzyme recruitment by contributing to evolvability.60 Promiscuity and ambiguity provide flexibility to modern metabolism and endow multifunctionality to catalysts. As such, finding new ways to detect and characterize promiscuous or ambiguous catalysts is likely to

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**Figure 1.** Genetic mechanisms that facilitate enzyme recruitment. (A) Gain-of-function point mutations (red) that endow new activity to an extant gene. (B) Beneficial point mutations (red) that afford enzyme overproduction by inactivating a repressor protein (R, yellow) or by disrupting the binding site of the repressor in the promoter (P) region. (C) Gene duplication. (D) Horizontal gene transfer by virtue of a phage or an extrachromosomal plasmid.
be advantageous to protein engineers seeking to repurpose extant enzymes.

Studies of molecular evolution have demonstrated that the trajectory of natural protein evolution is highly context-dependent. In many cases, a particular amino acid substitution that proves to be beneficial in one genetic context can be deleterious in another. Epistasis is the term used to describe such a situation, in which the positive or negative fitness impact of a mutational event is contingent upon the genetic background, and thus the past evolutionary history, of a protein. From an evolvability perspective, epistasis appears to be a key limiting factor in the ability to generate new molecular function, as it limits sampling of many distinct evolutionary trajectories (Figure 2C). Epistasis can also constrain the end point of an evolutionary trajectory, as it can lead to a rugged fitness landscape containing many local minima into which evolving species can become trapped. In some instances, epistasis stems from the multifaceted nature of a protein’s biophysical properties. This is because the impact of a mutation is not solely limited to its effect upon activity. It can also impact the stability, solubility, and interaction network of a protein. These features act in concert to determine the fitness outcome of specific amino acid substitutions. In one experimental evolution study, Hartl and co-workers demonstrated that epistasis resulting from such “biophysical pleiotropy” is so strong that the trajectory of evolution is limited to a very narrow ridge along the adaptive landscape. From a total of 120 potential evolutionary trajectories available for the evolution of a highly active β-lactamase from a progenitor catalyst, more than 100 were inaccessible because of epistasis. Identifying methods to overcome epistatic constraints upon the evolutionary process could be useful in future efforts to expand the functionality of biological catalysts.

EVIDENCE OF NATURAL ENZYME RECRUITMENT

Instances of enzyme recruitment have been detected in multiple primary metabolic pathways. In general, past recruitment events are most easily identified by searching for similar chemical transformations within unrelated branches of metabolism. Subsequent comparative sequence and structural analyses of the associated enzymes reveal potential homology. Several excellent reviews describing specific recruitment events are available. Below we highlight only a few examples for illustrative purposes.

An interesting example of recent enzyme recruitment has been discovered in the pentachlorophenol catabolism pathway of Sphingobium chlorophenolicum. Pentachlorophenol is a halogenated aromatic pollutant found in several pesticides and disinfectants. Pentachlorophenol hydroxylase (PcpB) is a flavin monooxygenase that catalyzes the first step in pentachlorophenol bioremediation. Sequence analysis reveals that PcpB was likely recruited from a pathway involving hydroxylation of natural products. The dehalogenation reaction catalyzed by PcpB results in the formation of the highly reactive intermediate tetrachlorobenzoquinone. To protect against modification of cellular constituents by tetrachlorobenzoquinone, PcpB has evolved the ability to form a transient interaction with tetrachlorobenzoquinone reductase, which catalyzes the second step of pentachlorophenol degradation. This protein–protein interaction prevents release of the PcpB reaction product. In this example, the recruited PcpB appears to have emerged from evolutionary
alteration of the progenitor’s substrate specificity, as well as optimization of an interaction surface on the protein.

Melamine deaminase and atrazine chlorohydrolase are two enzymes that have nearly identical structures and perform similar chemistry, but have been recruited into distinct degradative pathways.85 Atrazine (2-chloro-4-N-ethylamino-6-N-isopropylamino-triazine) and melamine (2,4,6-triamino-triazine) are intensively used in the industrial synthesis of herbicides and pesticides.86 Microbial degradation of both compounds begins with the hydrolytic removal of the C2 substitutent (Figure 3A). Melamine deaminase and atrazine chlorohydrolase have 98% identical sequences.85 Despite the fact that these two enzymes differ by only nine amino acid residues that are distributed throughout the scaffold, atrazine chlorohydrolase does not catalyze the deamination of melamine and melamine deaminase possess exceedingly low chlorohydrolase activity. The lack of substantial cross-reactivity within these two highly homologous enzymes provides strong support for epistasis, leading to a very narrow evolutionary trajectory from the progenitor catalyst to the present-day enzymes.

A similar situation is observed by comparing the isochorismate pyruvate lyase (PchB) and chorismate mutase (CM). PchB catalyzes the chorismate mutase reaction at a low level (red), but CM is incapable of performing the PchB transformation.

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A similar situation is observed by comparing the isochorismate pyruvate lyase (PchB) from Pseudomonas aeruginosa and Escherichia coli chorismate mutase (CM). These enzymes have 20% identical sequences, adopt similar tertiary structures, and catalyze comparable pericyclic reactions.87–89 PchB catalyzes the conversion of isochorismate to salicylate and pyruvate in bacterial siderophore biosynthesis (Figure 3B). PchB can also complement a chorismate mutase deficient bacterial strain,87 by catalyzing the conversion of chorismate to prephenate with respectable efficiency \( k_{\text{cat}}/K_m = 2200 \text{ M}^{-1} \text{ s}^{-1} \).89 In contrast, chorismate mutase from E. coli displays no detectable isochorismate pyruvate lyase activity.91 These findings suggest that an ancestral protein was recruited to perform both transformations, but that the subsequent evolutionary trajectory of the chorismate mutase was incompatible with retention of PchB activity. In contrast, the epistatic constraints encountered during PchB divergence allowed for the persistence of a promiscuous chorismate mutase function. Whether the ancestral polypeptide possessed both activities remains unknown; however, ancestral protein reconstruction could shed light on this issue.

In some cases, only single enzymes are recruited, whereas in other instances, entire metabolic pathways appear to have been co-opted for new function. Sequence comparisons reveal an evolutionary relationship between enzymes in the microbial arginine biosynthetic pathway and the enzymes that constitute the mammalian urea cycle.92 Four of the first five enzymes in the urea cycle catalyze reactions identical to those involved in arginine biosynthesis. The fifth enzyme, arginase, transforms arginine into ornithine and urea. Arginase is homologous to two enzymes, agmatine ureohydrolase and formiminoglutamate hydrodrolase, which participate in arginine and histidine degradation, respectively.93 Thus, it appears that the urea cycle of terrestrial animals was assembled from a combination of anabolic and catabolic pathways of amino acid metabolism after the transition from ocean- to land-dwelling organisms. Presumably, this recruitment process was advantageous, as it provided a mechanism for detoxifying ammonia produced by amino acid recycling. A similar example of pathway recruitment can be found in the Krebs cycle, which is postulated to have evolved from a combination of glutamate and aspartate biosynthetic enzymes.71

**EXPERIMENTALLY FACILITATED ENZYME RECRUITMENT**

Several experimental approaches have been developed to foster recruitment of enzymes for altered metabolic function. These studies highlight the scope of latent activities harbored within existing genomes and provide insight into the flexibility of modern metabolism. A powerful tool for experimental enzyme recruitment is the ASKA library, a collection of more than 4000 plasmid-borne open reading frames from E. coli.94 The ASKA collection allows controlled overproduction of each protein encoded within the E. coli genome via the powerful, IPTG-inducible T5 promoter. This collection can be used to identify proteins with latent activities capable of altering normal cellular metabolism. For example, the ASKA library was used to detect proteins whose overproduction allowed resistance to bromoacetate, a compound that mimics electrophilic toxins.95 Nine genes were identified whose overexpression resulted in bromoacetate resistance. Eight of the recruited genes encode transporters, while the ninth gene encodes UDP-N-acetylgalactosamine enolpyruvoyl transferase (MurA), an essential cell wall biosynthetic enzyme. MurA was found to be the primary target of bromoacetate and overproduction restored growth by outcompeting the toxic effects of this halogenated compound.95 In a related set of experiments, bacteria harboring the ASKA collection were challenged for growth in the presence of 237 toxic compounds, including many antibacterial agents.96 In total, 61 open reading frames were identified that increased the fitness in 86 of the 237 toxic environments. The encoded proteins possessed a variety of defined and putative functions, with many postulated to possess latent enzymatic activity arising from catalytic promiscuity or substrate ambiguity. These genome-wide overproduction studies demonstrate a surprising
degree of metabolic flexibility that can result from enzyme recruitment as driven by multiplicity suppression.

Another genomic tool that has proven to be beneficial in experimental investigations of enzyme recruitment is the Keio collection, which constitutes ~4000 nonessential single-gene knockout strains of E. coli K-12.73 This collection has been used to analyze the consequences associated with the loss of a single gene under different environmental conditions.98–100 Screening of the Keio collection identified 63 strains that were hypersensitive to growth in media supplemented with bromoacetate.95 The hypersensitive strains contained deletions in a variety of gene functions, one of which encoded a previously uncharacterized glutathione transferase, GstB. Biochemical investigations revealed that GstB functions as a reasonably efficient bromoacetate dehalogenase \((k_{cat}/K_m = 5000 \text{ M}^{-1} \text{ s}^{-1})\). On the basis of these findings, one can predict that GstB represents a future candidate for recruitment to detoxify electrophilic small molecules. Merging the Keio collection with the ASKA library can identify intracellular enzymes with promiscuous functions. Patrick and co-workers found that growth of 20% of all Keio knockout strains can be rescued for growth on glucose minimal medium by overexpression, substrate ambiguity, reaction promiscuity, and metabolic bypasses, which could yield alternate sources of downstream intermediates.

The use of single-gene knockout strains in combination with the ASKA collection has also provided new insights into the plasticity of modern metabolism. In an attempt to uncover enzymes with latent triosephosphate isomerase (TIM) activity, Desai and Miller provided a TIM knockout strain with the ASKA collection and selected for genes whose overproduction restored growth on glycerol minimal medium.102 Rather than identifying a promiscuous isomerase, the investigators discovered a putative aldo-keto reductase gene that provided conditional growth. Characterization of the gene product revealed that the enzyme catalyzes the efficient, stereospecific reduction of 1-glyceraldehyde 3-phosphate, the enantiomer of the natural TIM substrate. The reaction provides an alternate route to the formation of dihydroxyacetone, thereby allowing a metabolic bypass of the disrupted pathway. In similar work, Copley and co-workers utilized an E. coli strain lacking 4-phosphoerythronate hydroxylase, which catalyzes the second step in pyridoxal phosphate (PLP) biosynthesis, to uncover three latent pathways for PLP production.103 Overexpression of the ASKA library in a 4-phosphoerythronate hydroxylase knockout strain identified seven genes that complemented the metabolic deficiency. Only two of these seven genes appeared to recapitulate 4-phosphoerythronate hydroxylase activity. The other genes encode putative or established dehydrogenases, kinases, or hydrolases. Detailed investigation of these unexpected results demonstrated that the selected enzymes are capable of generating functional intermediates of PLP biosynthesis that lie downstream of the disrupted gene. Increasing the cellular concentration of these inefficient catalysts allows improved metabolic flux, which is sufficient for survival under the selective conditions. These experiments demonstrate that enzyme recruitment is a powerful tool for adapting to new environmental challenges and for generating altered metabolism.

### APPLYING ENZYME RECRUITMENT

The catalytic repertoire of contemporary enzymes is enormous. Multiple combinations of different enzymatic functions have produced complex metabolic pathways for the synthesis or degradation of structurally diverse compounds. In principle, it should be possible to design new metabolic pathways by recruiting functionally distinct enzymes to build new molecules. Indeed, modifying or extending existing pathways for the production of value-added compounds is already possible (Figure 4). Choi and co-workers have engineered a bacterium that produces short chain alkanes for gasoline production (Figure 4A).104 This was achieved by first altering the endogenous fatty acid metabolism of E. coli and then recruiting the fatty acyl CoA reductase from Clostridium acetobutylicum and the fatty aldehyde decarboxylase from Arabidopsis thaliana. The strain was further modified to produce high yields of free fatty acids and short chain fatty esters by recruiting the wax ester synthase from Acetinobacter sp. ADP1. In related work, enzyme recruitment played a key role in engineering a Pseudomonas putida strain to produce high yields of phenol (Figure 4B), an important starting material for pharmaceutical production.105 Phenol production was achieved by recruiting the tyrosine phenol lyase from Pantoea agglomerans in combination with overproducing the endogenous P. putida enzyme that catalyzes the first step of tyrosine biosynthesis.

Many FDA-approved pharmaceutical agents contain fluorine substituents.106 Recently, the natural fluorooacetate pathway of Streptomyces cattleya was exploited to allow synthesis of structurally diverse fluorinated compounds in vitro.107 By engineering the specificity of endogenous enzymes and recruiting the acetocetyl CoA synthase from Streptomyces, the authors developed a new metabolic pathway for the incorporation of monomeric fluorooacetate building blocks into diverse polyketide scaffolds. The authors also succeeded in producing fluorinated triketide lactones (Figure 4C) in vivo from fluoromalonate precursor, using a designed pathway built from a recruited ketosynthase and a modified 6-deoxyerythronolide B synthase–thioesterase module.

![Figure 4](https://dx.doi.org/10.1021/bi401667f)
More extensive enzyme recruitment has been utilized to convert carbon dioxide into useful compounds. The 3-hydroxypropionate/4-hydroxybutyrate pathway allows multiple thermoacidophilic archaea to assimilate carbon dioxide, in the form of bicarbonate, to generate acetyl-CoA. The recruitment of five consecutive enzymes from this pathway into Pyrococcus furiosus, a hyperthermophile that cannot use carbon dioxide naturally, allowed the production of 3-hydroxypropionate in high yields (Figure 4D). Together, these successes demonstrate the power of using distinct recruitment strategies for the production of non-natural or non-native compounds.

Enzyme recruitment also facilitates the expansion of the degradative potential of existing pathways. Several classes of microorganisms have the ability to combat environmental contaminants such as oil spills, because they can metabolize unbranched alkanes. Although it is difficult to degrade β-methyl-branched alkanes via the β-oxidation pathway, multiple Pseudomonas strains can degrade short chain alkanes via the citronellol pathway. To expand the chain length specificity of the citronellol pathway of Pseudomonas citronellolis, this microorganism was provided with a P. putida plasmid harboring a gene cluster encoding enzymes that oxidize C₆–C₁₀ n-alkanes. The resulting strain was able to grow on multiple n-alkane substrates. After whole genome mutagenesis of P. citronellolis and selection on n-decane medium, this pathway was further expanded to allow growth on the multi-branched alkane 2,6-dimethyl-2-octene.

The construction of simple de novo pathways is now possible using enzyme recruitment. For example, the production of the common industrial monomeric building block styrene (Figure 4E) has been achieved in an engineered strain of E. coli. This required a phenylalanine ammonia lyase for the conversion of L-phenylalanine into trans-cinnamate, and a cinnamate decarboxylase to catalyze the subsequent decarboxylation of trans-cinnamate to styrene. Multiple isoenzymes from bacteria, yeast, and plants were tested for potential recruitment. The largest yield of styrene was produced by combining the phenylalanine ammonia lyase from A. thaliana with the phenylacrylate decarboxylase from Saccharomycyes cerevisae in a strain of E. coli that overproduces phenylalanine. A more complex de novo pathway has been designed with the assistance of computational methods that identify suitable candidates for recruitment based on the nature of the endogenous function of the catalyst. Computational approaches predict more than 10000 potential metabolic pathways for the production of 1,4-butanediol, a useful polymer building block (Figure 4F). A single pathway was chosen from this pool on the basis of minimizing the length of the pathway, minimizing the number of unknown enzymatic steps, and predicting the thermodynamic likelihood of each reaction. The resulting pathway combines five heterologous enzymatic conversions integrated into natural E. coli metabolic pathways and results in the production of 18 g/L 1,4-butanediol.

A de novo-designed remediation pathway for paraoxon, a powerful insecticide and an efficient acetylcholinesterase inhibitor, has been developed via enzyme recruitment. A synthetic operon assembled from genes of multiple organisms in combination with a natural operon from Pseudomonas sp. allows P. putida to efficiently degrade large amounts of paraoxon. The synthetic operon encodes the organophosphate hydrolase gene from Flavobacterium sp., which hydrolyzes paraoxon into p-nitrophenol and diethyl phosphate. Diethyl phosphate is then converted to ethyl phosphate and subsequently to orthophosphate by the sequential action of two additional operon components, the phosphodiesterase from Delftia acidovorans and the alkaline phosphatase from P. aeruginosa. p-Nitrophenol is processed by the natural pnp operon from Pseudomonas sp., which encodes five enzymes catalyzing the transformation of p-nitrophenol to β-ketoadipate. This intermediate is further processed to succinyl-CoA and acetyl-CoA by the natural tricarbonic acid cycle of the host strain P. putida.

### FUTURE PERSPECTIVES

The study of enzyme recruitment has illuminated our understanding of the natural processes that drive the evolution of molecular function. It has also empowered the application of such knowledge to engineer new catalysts and new metabolic pathways. Where does the future lie? A major stumbling block in applying enzyme recruitment for useful new purposes remains the initial identification of the desired catalytic activity. Several computational approaches currently under development may help overcome this limitation. One promising area is computational protein design, which offers the possibility of creating enzymes for both natural and non-native chemical transformations. Although the enzymes designed to date generally possess activities that are low compared to those of natural enzymes, these scaffolds serve as attractive starting points for further directed evolution that could lead to optimized performance in vitro and in vivo. Bioinformatics methods that facilitate the organization of enzymes into superfamilies based on common tertiary structure and a fully or partially shared mechanism have also emerged. While this information has been largely used to assign activities to genes of unknown function, it also provides a database of homologous proteins that can be searched for candidate enzymes with the potential to be recruited for a specific type of chemical transformation.

The computational approaches described above are complemented by experimental methods to identify potential candidates for enzyme recruitment. The use of the aforementioned E. coli ASKA collection, in a high-throughput format, provides one source for discovering latent enzyme activities. An extension of the ASKA library can also be envisioned. Developing new open reading frame libraries that include genes from the more than 1000 organisms whose genomes have been sequenced would substantially expand the search landscape. A further expansion of the search zone could be achieved by creating robust environmental DNA libraries using methods established in the past decade. These approaches, while potentially powerful, require important advances in developing universal plasmid systems and host organisms to afford efficient heterologous production of library-encoded gene products in vivo.

Loosening the epistatic constraints inherent in modern enzymes as a result of their evolutionary history offers another approach that may facilitate the identification of latent activities. Ancestral proteins are thought to have possessed greater functional plasticity than their modern counterparts. Ancestral sequence reconstruction, using weighted parsimony and maximum likelihood methods, allows one to resurrect plausible models of extinct protein sequences in the laboratory. The extent to which ancestral reconstruction of ancient proteins increases the likelihood of revealing latent functions within a protein superfamily remains to be determined. At the very least, however, such a procedure...
promises to restore evolvability within a scaffold. Targeted ancestral sequence reconstructions within a specific protein superfamily could yield polypeptides that share potential mechanistic capabilities found within the modern superfamily. To widen the catalytic repertoire, ancestrally reconstructed sequences from multiple, mechanistically distinct protein superfamilies could be pooled to generate a more complex library. The strategies outlined above could yield a variety of low-activity enzymes that may be assembled into a new pathway (Figure 5A). To date, successes in novel metabolic pathway development have largely centered on short pathways and/or the recruitment of enzymes that promote transformations currently found in nature. To generate more complex pathways, however, the recruitment of enzymes that catalyze non-natural chemical reactions will likely be necessary. Examples of such reactions include metal-catalyzed cross coupling transformations and olefin metathesis, reactions for which efficient protein catalysts are not presently available. The identification of enzymes for these reactions, which are heavily used in pharmaceutical development, could facilitate the design of metabolic pathways for the microbial production of drugs. Two potentially attractive candidates are Zytiga and Gilenya (Figure 5B), both of which bear structural resemblance to natural metabolites and are reasonable to expect impressive advances over the next decade culminating in the emergence of new organisms with genomes tailored for specific metabolic purposes.

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