Enzyme Promiscuity: A Mechanistic and Evolutionary Perspective

Olga Khersonsky and Dan S. Tawfik

Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel; email: tawfik@weizmann.ac.il

Annu. Rev. Biochem. 2010. 79:471–505

First published online as a Review in Advance on March 17, 2010

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

This article's doi: 10.1146/annurev-biochem-030409-143718

0066-4154/10/0707-0471$20.00

Key Words

enzyme mechanism, protein evolution, superfamilies

Abstract

Many, if not most, enzymes can promiscuously catalyze reactions, or act on substrates, other than those for which they evolved. Here, we discuss the structural, mechanistic, and evolutionary implications of this manifestation of infidelity of molecular recognition. We define promiscuity and related phenomena and also address their generality and physiological implications. We discuss the mechanistic enzymology of promiscuity—how enzymes, which generally exert exquisite specificity, catalyze other, and sometimes barely related, reactions. Finally, we address the hypothesis that promiscuous enzymatic activities serve as evolutionary starting points and highlight the unique evolutionary features of promiscuous enzyme functions.

Contents

INTRODUCTION

Enzymes are traditionally referred to as remarkably specific catalysts. Yet the notion that many enzymes are capable of catalyzing other reactions and/or transforming other substrates, in addition to the ones for which they are physiologically specialized, or evolved, is definitely not new. Early examples of enzyme promiscuity include pyruvate decarboxylase (1), carbonic anhydrase (2), pepsin (3), chymotrypsin (4), and L-asparaginase (5). Nonetheless, the notion of "one enzyme—one substrate—one reaction" dominated, and still dominates the textbooks, and until recently, the wider implications of the "darker" side of enzyme promiscuity were largely ignored.

The idea of nature as an opportunistic modifier of preexisting suboptimal functions is also relatively old and has been formulated by Jacob in his classical note "Evolution and Tinkering" (6). The first direct connection between promiscuity and protein evolution was made, to our knowledge, in 1976 by Jensen (7). Jensen boldly forwarded the hypothesis that, unlike modern enzymes that tend to specialize in one substrate and reaction, the primordial, ancient enzymes possessed very broad specificities. Thus, relatively few rudimentary enzymes acted on multiple substrates to afford a wider range of metabolic capabilites. Divergence of specialized enzymes, via duplication, mutation, and selection, led to the current diversity of enzymes and to increased metabolic efficiency.

During the past decade, protein, and especially enzyme, promiscuity received considerable attention. Reviews by O'Brien & Herschlag (8) and Copley (9) were the first to highlight the potential mechanistic and evolutionary implications of promiscuity from an enzymologist's point of view. More recent reviews focused on practical implications of promiscuity (1, 10–12), on promiscuity and divergence in specific enzyme families (13–16), on mechanistic aspects of promiscuity (1, 17), and on promiscuity in the context of protein evolution and design (17, 18).

Here, we focus on the structural and mechanistic aspects of promiscuity as well as its role in the evolution of new functions. New enzymes have constantly emerged throughout the natural history of this planet. Enzymes that

degrade synthetic chemicals introduced to the biosystem during the last decades (19–24), enzymes associated with drug resistance (25–28), and enzymes in plant secondary metabolism (29–31) provide vivid examples of how fast and efficient the evolution of new enzymatic functions can be. Indeed, extensive research since Jensen's article provided ample evidence for the notion that promiscuity is a key factor in the evolution of new protein functions. Here, we attempt to summarize this accumulating knowledge and point out some open questions in this emerging field of research.

DEFINING AND QUANTIFYING PROMISCUITY

The term enzyme promiscuity (8) is loosely applied and is used to describe a wide range of fundamentally different phenomena. We, and several others (8, 9, 32), use promiscuity to only describe enzyme activities other than the activity for which an enzyme evolved and that are not part of the organism's physiology. Thus, enzymes, such as glutathione *S*-transferases (GSTs) and cytochrome P450s (33), which a priori evolved to transform a whole range of substrates, are not promiscuous; they are multispecific or broad-specificity enzymes.

Degree of promiscuity refers to the level of specificity breach, namely, how diverse are the promiscuous activities of a given enzyme (34), and how different are the native and promiscuous functions. The degree of promiscuity can be assessed by examining the type of bonds that are being formed or broken and by differences in the mechanism between the native and promiscuous reactions (10). An "index of promiscuity," which computes the degree of variability between different substrates, has also been proposed (35). However, this method assumes that the same chemical transformation occurs on all substrates. As such, it is more suitable for the analysis of multispecific enzymes such as GSTs (as originally demonstrated), rather than promiscuity. We proposed a simple, relatively objective, way of assessing the degree of promiscuity by comparing differences in the Enzyme Commission (EC) numbers (33). In enzymes exhibiting multispecificity, or substrate ambiguity, EC numbers for the various substrates should be the same, or differ only by the fourth digit (which generally distinguishes between enzymes of the same class). Catalytic promiscuity generally correlates with cases in which the EC numbers of the various substrates and reactions catalyzed by the same enzyme differ in the second, or the third, digits (which refer to different classes of substrates) or even by the first digit (which indicates a different reaction category). (For examples see **Supplemental Table 1**. Follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**.)

Magnitude of promiscuity refers to the kinetic parameters for the promiscuous activity relative to the native one. Whereas most enzymes exhibit k_{cat}/K_M values in the order of 10^5–10^8 $M^{-1}s^{-1}$ for their native substrates, the magnitude of promiscuous activities varies over more orders of magnitude, in absolute terms and also relative to the native activity. Catalytic proficiency ($k_{cat}/K_M/k_{uncat}$) and rate acceleration (k_{cat}/k_{uncat}) can provide a measure of the magnitude of catalytic effects exerted on native versus promiscuous substrates. In many cases, although the k_{cat}/K_M values for the promiscuous substrates are very low, and hence might have little physiological relevance, the rate accelerations and catalytic proficiencies are impressively high (34, 36–38).

PROMISCUITY: RULE OR EXCEPTION?

Numerous examples for enzyme promiscuity are currently known, but these are anecdotal and hardly provide an indication for the scale of this phenomenon. Systematic, high-throughput screens for promiscuous enzymatic activities are not a feasible option at present; no single detection method is available that can detect the whole range of different substrates and reactions. In contrast, high-throughput screens for binding cross-reactivities are relatively straightforward. These reveal a clear

Promiscuity: coincidental catalysis of reactions other than the reaction(s) for which an enzyme evolved

Multispecific or broad-specificity enzymes: enzymes performing the same reaction on a whole range of substrates, usually with similar efficiency

Substrate ambiguity: the activity of enzymes with substrates whose structure resembles the native substrate

trend whereby the number of identified cross-reactants (small molecules or proteins) increase exponentially with the number of tested ligands (39–41). Several theoretical models account for these observations (42–44; see also 45, 46). Future screens, using large diversities of substrates and reactions performed with enzymes, are likely to reveal that essentially every enzyme exhibits a range of promiscuous functions.

Despite the absence of systematic data, outlined below are several arguments in favor of the notion that promiscuity is a wide phenomenon and thus should be regarded as a rule, rather than an exception.

Native function(s): physiologically relevant chemical transformation(s) and substrate(s) for which an enzyme evolved and is maintained under selection

Secondary function: an additional function in secondary metabolism or signaling. Secondary functions are also defined as native

Primary function: a well-defined function, often in central metabolism, typically shared by all orthologs

Specificity Is Context Dependent

High specificity can bear a high cost in substrate-binding energies, thereby resulting in higher activation energies and lower turnover rates (k_{cat}) for the cognate substrate (47) (for an alternative mode whereby noncognate substrates exhibit low k_{cat} values due to poor positioning relative to the active site's catalytic residues, see Kinetic Parameters for Native versus Promiscuous Functions, below). Even the most specific enzymes, e.g., enzymes involved in DNA or protein synthesis, exhibit measurable substrate infidelities, often at surprisingly high rates. High fidelity is often achieved via proofreading, or proofediting, mechanisms that reverse the process and redo it (47). For example, the selectivity of aminoacyl-tRNA synthetases is under tight selection—having the wrong amino acid loaded onto a given tRNA yields a mutated protein. Because of the close similarity of certain amino acids, proofediting mechanisms have evolved whereby formation of a noncognate aminoacyl-tRNA leads to its rapid hydrolysis and resynthesis of the aminoacyl-tRNA at the cost of ATP (48). Similarly, the proofreading domain of polymerases is an exonuclease that can digest parts of the extended strand.

Specificity is shaped by natural selection. Promiscuous activities that are harmful were selected against. The adenylation domain *TycA* is highly selective for its cognate amino acid L-phenylalanine, primarily with respect to naturally occurring amino acids (e.g., L-tyrosine exhibits ~800-fold lower k_{cat}/K_M). However, an artificial substrate D-phenylalanine, to which the enzyme has probably never been exposed, is accommodated by *TycA* with k_{cat}/K_M only twofold lower than that of L-phenylalanine (49).

Many enzymes perform secondary tasks (50, 51) that are likely to have stemmed from their promiscuity. Examples include enzymes that have been under intense selection for high specificity, such as aminoacyl-tRNA synthetases. Lysyl-tRNA-synthetase, for example, mediates the synthesis of the signaling molecule Ap4A (two adenosines linked via four phosphates) (52, 53), and so do most other tRNA synthetases (54). This side reaction occurs within the same active site. In the absence of tRNA, the aminoacyl-AMP intermediate reacts with a second ATP molecule to generate the free amino acid and Ap4A. Certain aminoacyl-tRNA synthetases bind DNA or mRNA and thus regulate transcription, splicing, and translation, or they act as cofactors in RNA trafficking (51). It is likely that these oft-called secondary functions were recruited well after the primary function had emerged. Once recruited, they remained under selection and therefore became an additional native function of the enzyme.

Regulation and Masking of Promiscuity

Few of the promiscuous activities found in vitro bear a physiological or evolutionary meaning. Even those that might are not manifested in vivo (this is, by definition, what promiscuous activities are). A primary factor to consider is regulation, which prevents many of the undesirable outcomes of promiscuity. Of the entire enzyme diversity available to organisms, only a small fraction is accessible and active at a given time and cellular location. Regulation at the level of expression prevents the spending of unnecessary resources (51, 55), but the fitness costs

associated with unncessary transcription and translation are relatively low (56). However, regulation regimes are also the key in controlling enzyme activity, especially with enzymes whose specificity is broad. For example, *Escherichia coli* has 23 different haloacid dehalogenase (HAD)-like hydrolases. Most of these are phosphatases exhibiting very broad substrate specificity (57, 58), but these operate under different regulation, and specificity is achieved via regulation and not by restricting enzyme specificity (58).

Regulation occurs also at the protein level, such as allosteric regulation that prevents the wasteful conversion of costly metabolites. As expected, this regulation is mostly product controlled. But in some cases, the substrate is an allosteric regulator of its own enzyme. Why would such a regulatory mechanism evolve? In the absence of its substrate, an enzyme is supposed to remain silent. Preventing active sites from promiscuously reacting with undesirable substrates could be one of the driving forces for the evolution of substrate-dependent allosteric regulation.

Promiscuity within Living Cells

Despite the action of natural selection to increase enzyme selectivity by various means, ranging from shaping the active site itself to regulation of enzyme expression and activity, numerous cross-reactions and breaches of specificity occur, not just in vitro, but also within living cells. Such cross-reactivities are often unraveled by the analysis of auxotrophic knockout strains that lack a crucial enzyme. Such deficiencies are often complemented by other enzymes, or even other enzyme pathways, sometimes in an unexpected manner. For example, knockouts of the *phn* operon in *E. coli* that utilizes phosphite (HPO_3^{2-}) led to the identification of promiscuous phosphite-dependent hydrogenase activity in alkaline phosphatase (see Mechanistic Aspects of Promiscuity, below) (59). Glutamyl phosphate reductase (*ProA*) exhibits low promiscuous activity with *N*-acetylglutamyl phosphate, the substrate for *ArgC* (*N*-acetylglutamyl phosphate reductase). Following a single mutation in the enzyme's active site, and changes in regulation, *ProA* could complement the *ArgC* knockout from a single-copy plasmid (60).

The level of cross-reactivity between different metabolic pathways was also indicated by an in silico experiment that attempted to dock 125 common metabolites into the active sites of 120 key metabolic enzymes. Numerous potential cross-reactions were found among these 15,000 potential pairs. Although docking has obvious limitations, this study further highlights the potential for promiscuity (12, 61). Complementation of *E. coli* knockout strains by selection from a library of *E. coli*'s own genes under overexpression from a multiple-copy plasmid revealed a similar picture (32). The deleted gene and its suppressor were, in most cases, unrelated. Complementation was achieved through the promiscuous action of other enzymes, through increased transport (and not necessarily of the deficient metabolite), and, most often, by an alternative metabolic pathway. Thus, promiscuity is not necessarily limited to the single enzyme level, but often, whole pathways act promiscuously, namely, outside their ordinary functional role. Other examples of metabolic plasticity, or "underground metabolism," are reviewed in References 7, 13, and 62.

The "Flexible Metabolome"

The above observations led to new hypotheses that suggest that genetic and metabolic pathways are inherently ambiguous and stochastic. By these hypotheses, the well-defined linear pathways described in textbooks are cross wired in a variety of unexpected ways. Evolution may capitalize on these cross-wirings, as a way of adaptive plasticity (i.e., with no genetic changes to begin with), to generate new metabolic capabilities (63). Phenomena similar to underground metabolism and adaptive plasticity were also observed in genetic analyses, wherein altered phenotypes turned out to be correlated with changes in many different genes, including genes from unrelated pathways. As is the case

with enzymes and metabolic pathways, genome flexibility is an inevitable outcome of limited specificity, or promiscuity, of gene action and of intergenic interactions (64, 65). Thus, it appears that, beyond the well-studied, linear pathways, there exist flexible genomes (64), as well as flexible proteomes and flexible metabolomes, whose contribution to evolutionary adaptation is still understudied.

MECHANISTIC ASPECTS OF PROMISCUITY

How does the very same active site and catalytic machinery show exquisite specificity with respect to the native substrate but still promiscuously catalyze other, often completely unrelated, reactions? The answer to this question is complex, and different scenarios, mechanisms, and other aspects of the specificity-promiscuity dichotomy are outlined below.

Specificity and Promiscuity Coincide within the Same Active Site

Conformational diversity. The role of structural plasticity in facilitating enzyme action, promiscuity, and evolution is discussed in several reviews (67–69). In many cases, promiscuity is linked to diverse conformations, whereby the native and the promiscuous functions are mediated by different active-site configurations (**Figure 1**). For example, isopropylmalate isomerase is an enzyme with dual-substrate specificity, where a loop structure depends on the substrate present (70). In sulfotransferase SULT1A1, conformational changes enable the same enzyme to accommodate a range of different substrates (66), as is the case with glutathione-*S*-transferases GSTA1-1 and GSTA4-4 (71) and with certain P450s (72). And, in β-lactamase, an expanded spectrum of antibiotic substrates is accommodated through increased flexibility and altered dynamics (73, 74).

Accommodating alternative substrates. In many cases, promiscuous activities share the same active-site configuration and main active-site features with the native activity. For example, guanidine-transferring enzymes utilize the same catalytic triad in their promiscuous action on various derivatives of arginine (75). In this case of substrate ambiguity, the active-site residues that bind the Cα-carboxyl and the guanidino-NH_2 of different substrates are different. Another case where the network of hydrogen bonds is the main feature that differentiates the native reaction from the promiscuous one is D-2-keto-3-deoxygluconate aldolase (**Figure 2**) (78).

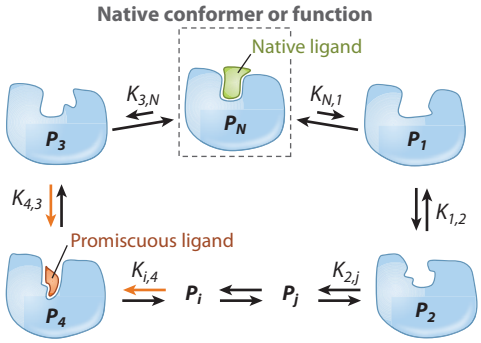

Figure 1

Protein promiscuity, evolvability, and conformational diversity. Proteins exist as an ensemble of different conformations (depicted as P_1, $P_2..P_j$) that exchange via the respective equilibrium constants (Kij). The primary conformation is the native state (P_N), which interacts with the native ligand. The alternative conformers relate to structural variations spanning from different side chain rotamers and active-site loop rearrangements to more profound fold transitions. Minor conformers (e.g., P_4) may mediate alternative functions, such as binding of a promiscuous ligand. Mutations can gradually alter this equilibrium such that scarcely populated conformers become more favorable with significant effects on the corresponding promiscuous function (e.g., an increase in occupancy of P_4 from 0.01 to 0.1 can yield a tenfold increase in the overall level of promiscuous function). The relative occupancy of the native conformer would be hardly affected (e.g., from 0.5 to ≥ 0.41, leading to <20% loss of the native function). This model also accounts for weak negative trade-offs between the existing and evolving functions as well as the evolutionary potential of neutral mutations. Adapted from Reference 69.

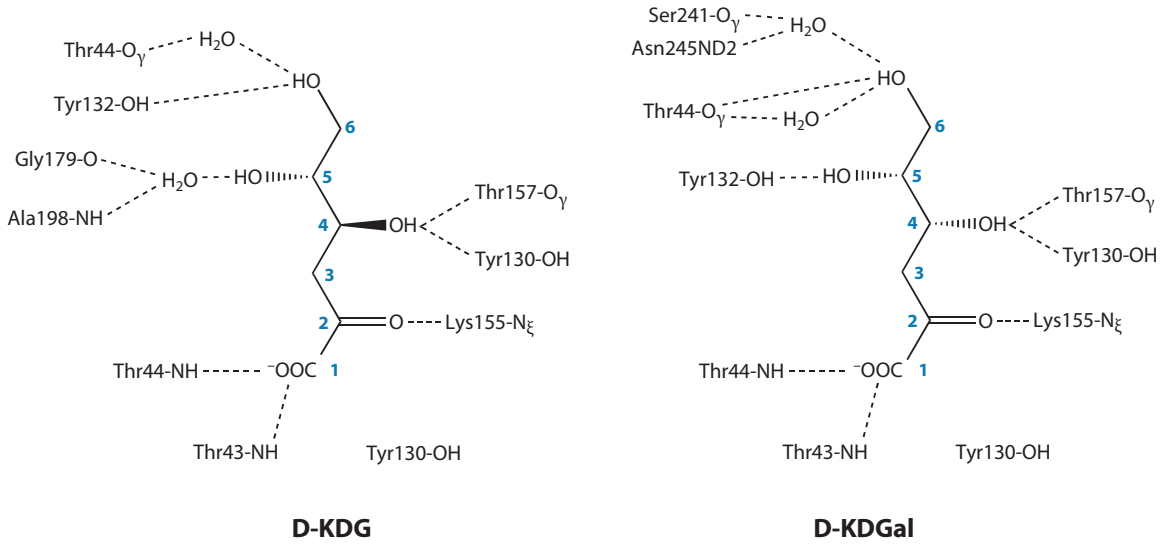

Figure 2

Schematic summary of the different interactions made in the active site of D-2-keto-3-deoxy-gluconate aldolase (KDGA) from *Sulfolobus solfataricus* (adapted from Reference 78). This enzyme transforms both D-2-keto-3-deoxy-gluconate (D-KDG) and D-2-keto-3-deoxy-galactonate (D-KDGal) with similar rates (176). The mechanism with both substrates involves Schiff base formation by Lys155 and subsequent hydration and cleavage. The differences between the gluconate and galactonate substrates are in the hydrogen bonds formed with KDGA's active site and, in particular, in the manner by which the 5′ and 6′ hydroxyl groups are bound.

Other examples include enzymes that apply nucleophilic catalysis, such as alkaline phosphatase, a highly proficient ($k_{cat}/K_M > 10^7$ M^{-1} s^{-1}) phosphate monoesterase that promiscuously hydrolyzes phosphodiesters, phosphoamides, and sulfate esters (36, 38, 79), as well as phosphite (while reducing water to release hydrogen) (**Figure 3*a***) (59). The catalytic mechanism is presumed to be similar for all these reactions and involves nucleophilic attack by Ser102 and stabilization of the negatively charged intermediate by the active site Zn^{2+} ions and Arg166 (**Figure 3*b***) (38, 59). Comparison between the activities revealed that, although these substrates all bind in a similar mode, the interactions with both Zn^{2+} ions and Arg166 are much more favorable for the native phosphate-monoester substrates than for other promiscuous substrates (36, 38). This difference accounts for the orders-of-magnitude higher rates and catalytic proficiencies of the native substrates versus the promiscuous ones.

The very same active site can therefore offer several different modes of interactions, and some of these might be utilized by promiscuous substrates. It should be noted, however, that most of the above describes cases analyzed by kinetics and site-directed mutagenesis. Because very few structures of the enzyme-substrate or enzyme-transition state analog complexes exist for both the native and promiscuous substrates, subtle changes of the active site's conformation cannot be excluded.

Different protonation states. The same catalytic residue can act in a different protonation state in the native compared to the promiscuous function. In the tautomerase superfamily, various enzymes share the catalytic Pro residue at the enzyme's amino terminus, but the mechanism of catalysis depends on its pK_a. In the 4-oxalocrotonate tautomerase (4-OT) the pK_a of Pro1 is ~6.4, and it acts as a general base. In *trans*-3-chloroacrylic acid

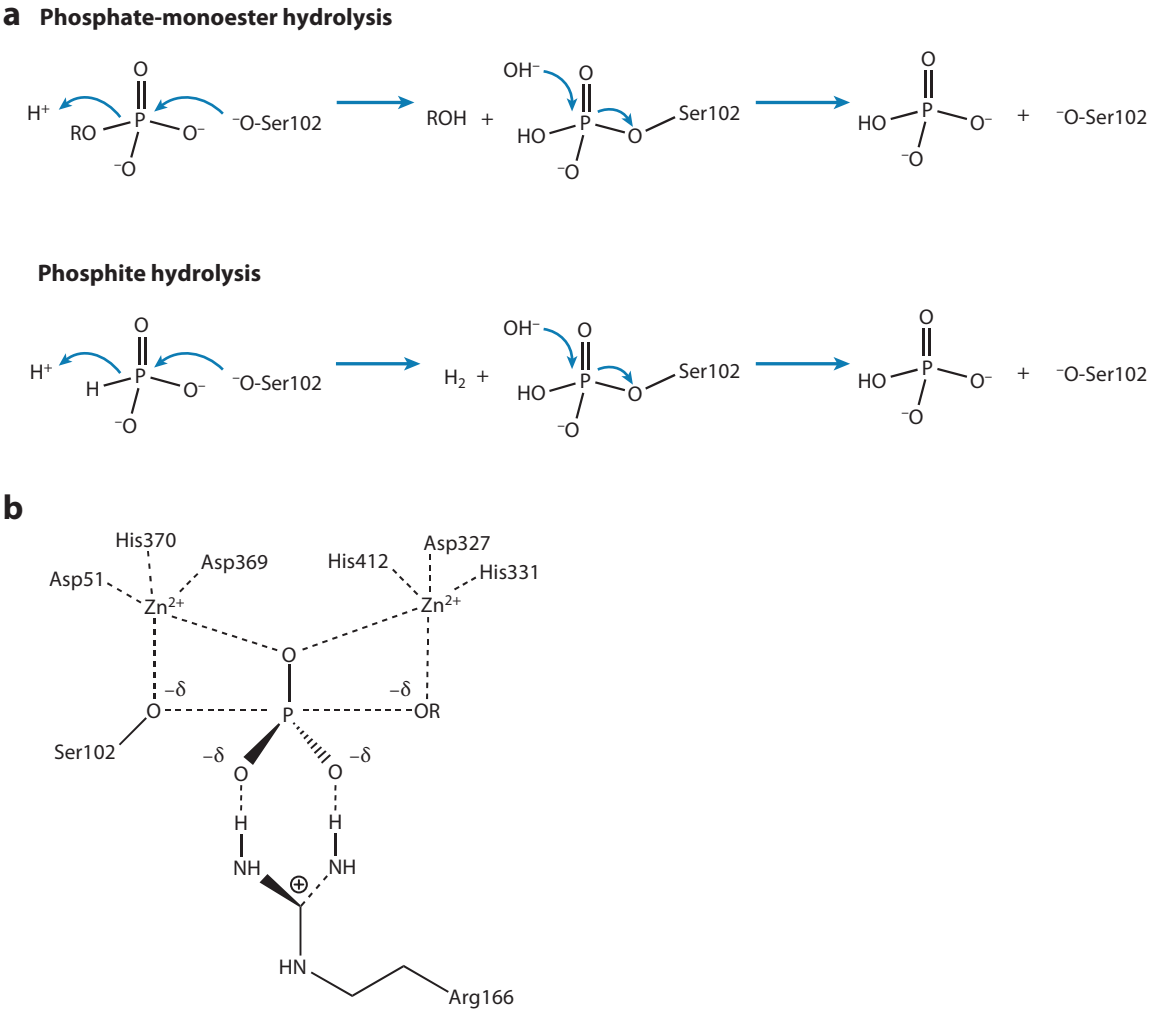

Figure 3

(*a*) The native monoester phosphatase activity and the promiscuous phosphite hydrolysis reactions catalyzed by alkaline phosphatase (adapted from Reference 59). (*b*) The active-site arrangement of alkaline phosphatase with a bound transition state model (adapted from Reference 36).

dehalogenase (CaaD), which catalyzes the hydrolytic halogenation of haloacrylates, Pro1 is protonated (pKa ~9.2) and serves as a general acid (80, 81). Because in 4-OT only a small fraction of Pro1 is present in the protonated state, it exhibits very weak promiscuous general acid catalysis of the hydratase activity. However, another family member, malonate semialdehyde decarboxylase (MSAD), exhibits a substantial promiscuous hydratase activity, primarily because Pro1 is protonated and serves as a general acid in the mechanism of the enzyme's native activity (82, 83).

Different subsites within the same active site. In several cases, although both the original and promiscuous activities reside within the same active site and rely on its major feature (e.g., an oxyanion hole), other key parts of the catalytic machinery differ. One example is serum paraoxonase (PON1), a mammalian lactonase with promiscuous esterase and

phosphotriesterase (PTE) activities (**Figure 4*a***) (84). The coordination of the phosphoryl/carbonyl oxygen to the active-site calcium is a feature shared by all the activities. However, whereas the hydrolysis of lactones and esters is mediated by a His115-His134 dyad, the promiscuous phosphotriesterase activity is mediated by another set of residues (84, 85), possibly via a nucleophilic attack of Asp269 (86).

An analogous example is *Candida antarctica* lipase B (CAL-B) whose native activity (lipids hydrolysis) is mediated by a Ser105-His224-Asp187 catalytic triad. Using its oxyanion hole, CAL-B also catalyzes various carbon-carbon bond formation reactions, such as Michael additions and aldol condensations (87–89). However, in these reactions, the nucleophilic serine takes no role, and acid-base transfer is presumably mediated by His224 in conjunction with Asp187 (**Figure 4*b***).

Promiscuity via alternative cofactors and amino acids. In a particular case of cofactor ambiguity, changes in enzyme specificity can also be induced by metal substitutions. Following work by Kaiser & Lawrence (90), the introduction of copper ions induced promiscuous oxidase activities in several hydrolytic enzymes (91, 92). In carbonic anhydrase, substitution of the native Zn^{2+} by Mn^{2+}

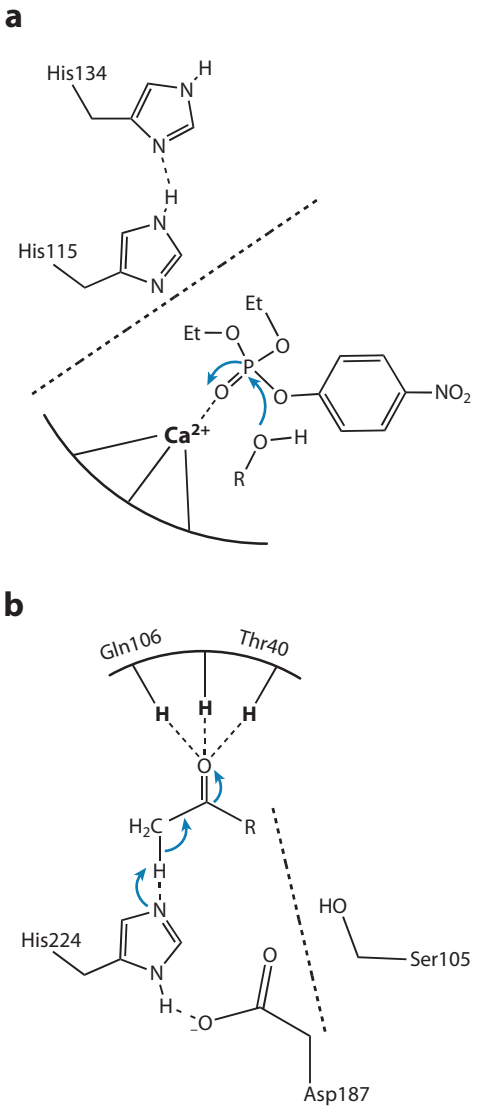

Figure 4

Different subsites within the same active site. (*a*) The main active-site feature of the serum paraoxonase (PON1) is the catalytic calcium ion, which lies at the bottom of a deep and hydrophobic active site and is thought to act as the "oxyanion hole" of PONs. The native function, hydrolysis of lactones, is mediated by a His115-His134 dyad, which deprotonates a water molecule to generate the attacking hydroxide. Although the same dyad appears to mediate the promiscuous arylesterase activity of PON1, the promiscuous phosphotriesterase activity (shown here for paraoxon as substrate) is independent and is mediated by other residues that act as base or nucleophile (84, 174). Indeed, mutations of both His residues diminish the lactonase activity but may increase the promiscuous phosphotrieterase activity by up to 300-fold with certain organophosphate substrates (85, 175). (*b*) A similar scenario has been described for *Candida antarctica* lipase B (CAL-B). Its native activity (lipid hydrolysis) is mediated by the Ser105-His224-Asp187 triad, and the acyl-enzyme intermediate is stabilized by its oxyanion hole. CAL-B also catalyzes promiscuous C-C bond formation reactions. In these promiscuous activities, the oxyanion hole is also utilized for negative charge stabilization (shown here). However, the catalytic serine takes no part, and acid-base transfer is thought to be mediated by His224 in conjunction with Asp187 (87–89).

enabled the catalysis of styrene epoxidation (93), and rhodium-substituted carbonic anhydrase acts as a hydrogen-utilizing reductase (94). Similarly, incorporating selenocysteine into the active sites of subtilisin (95), glyceraldehyde-3-phosphate dehydrogenase (96), and GST (97) endowed these enzymes with novel peroxidase activities.

Water-assisted promiscuity. Although the native substrate may interact directly with active-site residues, accidental hydrogen bonds mediated by water molecules may play a role in promiscuous interactions. Water molecules can buffer opposing dipoles or charges between the substrate and active-site residues, or they can act as acid, base, or nucleophile in the catalysis of promiscuous reactions. Indeed, spatially defined active-site water molecules have catalytic powers that are comparable to amino acid residues, and localized water molecules may have played a key role in primordial enzymatic active sites (47). A study of the molecular dynamics of the *Bacillus subtilis* esterase suggested that promiscuous amide hydrolysis is mediated by a network of water-mediated hydrogen bonds that are not involved in the esterase reaction (98). Further evidence for water-mediated promiscuity awaits more structures of enzymes complexed with promiscuous substrates.

Enzyme Mechanisms Analyzed by Studying Promiscuous Functions

Enzymologists have discovered that a systematic research of the hidden skills of enzymes can provide valuable insights regarding their catalytic mechanisms. For example, the promiscuous hydrolysis of phosphonate diesters by *Tetrahymena thermophila* ribozyme provided key insights regarding the relative importance of transition state geometry versus charge (99). In another study, the promiscuous chorismate mutase activity of PchB was used to derive mechanistic insights into its native activity (isochorismate pyruvate lyase) (100).

Kinetic Parameters for Native versus Promiscuous Functions

Differences between the efficiency of promiscuous and native activities can be manifested in differences in either k_{cat} or K_M. Although it is expected that promiscuous substrates that bind weakly will exhibit high K_M values, many promiscuous substrates are characterized by low k_{cat} values. Thus, specificity may result not only from substrate binding interactions per se, but also from appropriate positioning relative to the catalytic machinery. For example, analysis of substrates of PON1, the primary function of which is lipophilic lactonase, indicated that all promiscuous aryl esters and phosphotriester substrates exhibit K_M values in the mM range (0.8–5 mM), and the differences in reactivity are primarily due to k_{cat} values that vary by >1000-fold (101). For the promiscuous substrates, substrate binding is driven primarily by nonspecific hydrophobic forces within the deep and hydrophobic active site of PON1. However, promiscuous substrates are inadequately positioned relative to the catalytic machinery and therefore exhibit low k_{cat} values. Interestingly, for the lactones that comprise the native substrate of this enzyme, K_M values vary by ~200-fold (from about 0.1 up to 20 mM), whereas the variations in k_{cat} values are an order of magnitude lower (~10–200 s^{-1}). Indeed, binding of the native substrate is typically mediated by enthalpy-driven interactions, such as hydrogen bonds, whereas for the promiscuous substrates, hydrophobic and other entropy-driven interactions play a key role (12, 102).

PROMISCUITY AND DIVERGENCE OF ENZYME SUPERFAMILIES

An enzyme superfamily combines dozens to thousands of enzymes that, although distant in sequence and catalyzing different chemical transformations of many different substrates, share the same fold and a common catalytic strategy (e.g., abstraction of a proton from a position alpha to a carboxylate, and stabilization of

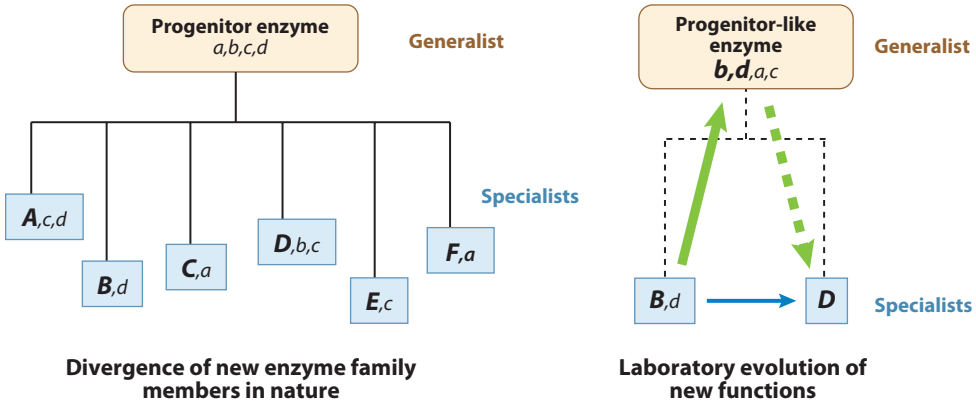

Figure 5

Divergence of a generalist progenitor enzyme to a family of specialist enzymes. (*left*) Jensen's hypothesis (7) surmises that, in nature, an ancestor protein displaying a low level of a range of activities (denoted as *a*, *b*, *c*, *d*) has been subjected to selection pressures for those activities, thus duplicating and diverging into a family of potent and highly specialized enzymes of the kind seen today (denoted *A*, *B*, etc.). In the course of divergence, new activities that were not present in the progenitor may also emerge (denoted as *E* and *F*). Today's specialists may still retain some of the functions of the common ancestor (denoted in lower case letters) as low levels of promiscuous activities. Indeed, several reports indicate a low level of shared activities within a family and, in particular, that the native activity of one member is the promiscuous activity of another, and vice versa (**Table 1**). (*right*) Additional support to the above model comes from the results of many directed evolution experiments. Direct switches of specificity, e.g., from B to D (*blue arrow*) are rare and are typically seen following a parallel selection for an increase in the target activity and elimination of the original one. Upon mutation and selection for an increase of a promiscuous activity (*green arrow*), the resulting variants usually show significant increases in the target activity and a smaller decrease in the original one, thus yielding, in effect, a generalist intermediate exhibiting both *d* and *b* at relatively high levels (the weak negative trade-off line in **Figure 6**). Such intermediates are often observed in the lab; some even gain other activities, for which they were never selected (denoted *a*, *c*), and may therefore resemble the progenitor of this enzyme family or node intermediates along past routes of its divergence. Adapted from Reference 17.

the resulting enolate intermediate, in the enolase superfamily) (14). Analysis of enzyme families and superfamilies provides ample evidence for the role of promiscuity in the evolution of new functions. Specifically, the identification of promiscuous activities, or cross-reactivities, between different members of the same enzyme family or superfamily and the ability to evolve these promiscuous activities in the laboratory provide important hints regarding evolutionary, structural, and mechanistic relationships within enzyme superfamilies (**Figure 5**). Examples of the promiscuous catalytic activities within enzyme families and superfamilies are listed in **Table 1**. Conclusions supported by these data are summarized below:

1. The primary, or native, function of one family member is often identified as a promiscuous activity in other family members (**Table 1**, entries 2 and 5–9). This overlap may reflect the common catalytic strategy that underlines these families and superfamilies as well as a common evolutionary origin (**Figure 5**). It can therefore guide the identification of the native function of new superfamily members by virtue of its similarity to the promiscuous activity of related family members. This principle was demonstrated in an attempt to trace the origins of a bacterial phosphotriesterase (PTE from *Pseudomonas diminuta*), an enzyme thought to have evolved for the degradation of paraoxon, an insecticide introduced in the twentieth century. PTE possesses a promiscuous lactonase

Table 1 Examples for promiscuous activities within enzyme families and superfamilies

Entry number	Family/superfamily	Enzymes	Native activity (substrate, k_{cat}/K_M in $M^{-1}s^{-1}$)	Promiscuous activity (substrate, k_{cat}/K_M in $M^{-1}s^{-1}$)	References
1	Mammalian paraoxonases (PONs)	PON1 (serum paraoxonase) PON2 PON3	Lipo-lactonase (γ-dodecanoic lactone, 1.2×10^5)	PON1: aryl esterase (phenyl acetate, $\sim 6 \times 10^5$) Phosphotriesterase (paraoxon, 6×10^3)	101, 109
				PON2: barely detectable aryl esterase; no phosphotriesterase	
				PON3: low aryl esterase; barely detectable phosphotriesterase	
2	Tautomerase superfamily	Malonate semialdehyde decarboxylase (MSAD)	Decarboxylation (malonate semialdehyde, 2.2×10^7)	Hydration (2-oxo-3-pentynoate, 6×10^2)	80–83
		4-oxalocrotonate tautomerase (4-OT)	Isomerization (2-oxo-4E-hexenedioate, 2.0×10^7)	Hydrolytic dehalogenation (CaaD activity) (3E-chloroacrylate, 2.6×10^{-2})	
		YwhB tautomerase (4-OT analog)	Isomerization (2-oxo-4E-hexenedioate, 2.8×10^4)	Hydrolytic dehalogenation (CaaD activity) (3E-chloroacrylate, 4.4×10^{-2})	
		trans-3-chloroacrylic acid dehalogenase (CaaD)	Hydrolytic dehalogenation (3E-chloroacrylate, 1.2×10^5)	Hydration (2-oxo-3-pentynoate, 6.4×10^3)	
3	ROK family (repressor, open reading frame, kinase)	NanK	*N*-acetyl-D-mannosamine kinase (*N*-acetyl-D-mannosamine, 2.7×10^5)	Glucose kinase (glucose, 5.1×10^2)	123, 127
		YajF	Fructose kinase (fructose, 1.1×10^4)	Glucose kinase (glucose, 2×10^2)	
		YcfX	Unknown	Glucose kinase (glucose, 2.4×10^3)	
		AlsK	Allose kinase (allose, 6.5×10^4)	Glucose kinase (glucose, 15)	
4	Enolase superfamily: MLE (muconate lactonizing enzyme) subgroup	*o*-succinylbenzoate synthase (OSBS)	Dehydration [2-succinyl-6R-hydroxy-2,4-cyclohexadiene-1R-carboxylate (SHCHC), 2.5×10^5]	*N*-acylaminoacid racemase (NAAAR) (*N*-acetyl methionine isomers, $4.9–5.9 \times 10^2$)	16, 177

5	Amido hydrolase superfamily	Phosphotriesterase (PTE)	Phosphotriesterase (paraoxon, 4×10^7)	Aryl esterase (2-naphthyl acetate, 500); lactonase (dihydrocoumarin, 6.5×10^5)	103, 104
		Phosphotriesterase homology protein	Unknown	Aryl esterase (2-naphthyl acetate, 70)	
		Dihydroorotase	Dihydroorotic acid hydrolysis (dihydroorotic acid, 1.2×10^6)	Phosphotriesterase (paraoxon, 2.8)	
		AhlA; a member of the PLL family (PTE-like lactonases)	Lactonase (*N*-3-oxooctanoyl L-homoserine lactone, 0.7×10^6)	Phosphotriesterase (paraoxon, 0.5)	
		PPH; a member of the PLL family (PTE-like lactonases)	Lactonase (*N*-3-oxooctanoyl L-homoserine lactone, 0.55×10^5)	Phosphotriesterase (paraoxon, 8.6)	
		PTE-like lactonase SsoPox	Lactonase (*N*-3-oxooctanoyl L-homoserine lactone, $>10^6$)	Aryl esterase (naphthyl acetate, 400); phosphotriesterase (paraoxon, 4000)	
6	Orotidine 5′ monophosphate decarboxylase suprafamily (OMPDC)	3′ keto L-gluconate 6-phosphate decarboxylase (KGPDS)	Decarboxylation (3′ keto L-gluconate 6-phosphate, 7.7×10^4)	HPS activity, aldol condensation (D-ribulose 5-phosphate and formaldehyde, 8.2×10^{-2})	178
		D-*arabino*-hex-3-ulose 6-phosphate synthase (HPS)	Aldol condensation (D-ribulose 5-phosphate and formaldehyde, 1.6×10^4)	KGPDS activity, decarboxylation (3′ keto L-gluconate 6-phosphate, 2.3×10^3)	
7	*N*-acetyl-neuraminate lyase (NAL) family, pyruvate-dependent aldolases	*N*-acetyl-neuraminate lyase	Cleavage of *N*-acetyl-neuraminate (3.1×10^3)	DHDPS activity, aldol condensation (pyruvate and L-aspartate-β-semialdehyde, 20)	179
		Dihydrodipicolinate synthase (DHDPS)	Aldol condensation (pyruvate and L-aspartate-β-semialdehyde	—	
8	Alkaline phosphatase superfamily	Alkaline phosphatase	Phosphomonoesters hydrolysis (p-nitrophenyl phosphate, 3.3×10^7)	Phosphodiesters hydrolysis (bis-p-nitrophenyl phosphate, 5×10^{-2})	36–38, 79
				Sulfate ester hydrolysis (p-nitrophenyl sulfate, 1×10^{-2})	
		Arylsulfatase	Sulfate ester hydrolysis (p-nitrophenyl sulfate, 5×10^7)	Phosphomonoesters hydrolysis (p-nitrophenyl phosphate, 790)	
		Nucleotide pyrophosphatase-phosphodiesterase	Phosphodiesters hydrolysis (thymidine 5′-monophosphate 4-nitrophenyl ester, 1.6×10^6)	Phosphomonoesters hydrolysis (p-nitrophenyl phosphate, 1.1)	

(*Continued*)

Table 1 (*Continued*)

Entry number	Family/superfamily	Enzymes	Native activity (substrate, k_{cat}/K_M in $M^{-1}s^{-1}$)	Promiscuous activity (substrate, k_{cat}/K_M in $M^{-1}s^{-1}$)	References
9	Guanidino-modifying enzyme superfamily, hydrolase branch	Arginine deiminase (PaADI)	Arginine hydrolysis (arginine, 4.5×10^4)	PaDDAH activity, N^w,N^w-dimethylarginine hydrolysis (N^w,N^w-dimethylarginine, 1.8×10^3)	75
		Agmantine deiminase (PaAgDI)	Agmantine hydrolysis (agmantine, 7×10^3)	None	
		N^w,N^w-dimethylarginine dimethylaminohydrolase (PaDDAH)	N^w,N^w-dimethylarginine hydrolysis (N^w,N^w-dimethylarginine, 1.8×10^3)	PaADI activity, arginine hydrolysis (arginine, 1.8)	
10	Pyridoxal 5′-phosphate-dependent transferases superfamily	Dopa decarboxylase	Decarboxylation of L-aromatic amino acids into aromatic amines [3,4-dihydroxyphenylalanine (Dopa), 6.1×10^4]	Half-transamination of D-aromatic amino acids (5-hydroxytryptophan, 1.3) Oxidative deamination of aromatic amines (5-hydroxytryptamine, 35)	76, 77
		Phenylacetaldehyde synthase (PAAS)	Coupled decarboxylation and amine oxidation (phenylalanine, 667)		
11	C-C hydrolase family (branch of α/β hydrolase superfamily)	C-C hydrolase *MhpC*	C-C bond cleavage (2-hydroxy 6-keto-nona-2,4-dienoic acid, 28 units)	Esterase (monoethyl adipate, 0.0027 units)	180–182
				Thioesterase (thioethyl adipate, 0.46 units)	
				Hydroxamic acid formation (monoethyl adipate + NH_4OH, 0.013 units)	
		Haloperoxidase/esterase *TbcF*	Haloperoxidase (monochlorodimedon, V_{max} = 0.45 nmol/min)	Esterase (p-nitrophenyl acetate, V_{max} = 2.58 nmol/min)	
		Lactonase	Lactonase (3,4-dihydrocoumarin, V_{max} = 4760 units)	Haloperoxidase (monochlorodimedon, V_{max} = 199 units)	
12	Glutathione-*S*-transferase (GST) superfamily (zeta class)	TCHQ dehalogenase	Dehalogenation [tetrachlorohydroquinone (TCHQ), 3.6×10^4]	Isomerization of double bonds (maleylacetone, 410) Dehalogenation [dichloroacetic acid (DCA), 23]	114
		MAA isomerase	Isomerization of double bonds [maleylacetoacetate (MAA), 1670]	—	
		Zeta GST	Dehalogenation [dichloroacetic acid (DCA), 8500]	—	

activity (103) that could comprise a vestige of its progenitor. Indeed, three homologs from the same superfamily (amidohydrolase) turned out to be representatives of a new group of microbial lactonases, dubbed PTE-like lactonases (PLLs) (104). These three PLLs, and some newly identified ones (105–108), proficiently hydrolyze lactones, particularly *N*-acyl homoserine quorum-sensing lactones, and exhibit weaker promiscuous PTE activities. PLLs share key sequence and active-site features with PTE and differ primarily by an insertion in one active-site loop (104, 107, 108). Given their function and phylogeny, PLLs emerged dozens of millions of years ago. The latent promiscuous phosphotriesterase activity of a yet-to-be-identified PLL served as the essential starting point for the evolution of PTE (104).

2. The same promiscuous activity is often shared by more than one family member (**Table 1**; entries 2, 3, 5, and 11).
3. The magnitude of promiscuous functions varies dramatically between family members (**Table 1**, entries 1, 5, and 9). For example, in the mammalian paraoxonases family, the promiscuous PTE activity is high in one paralog (PON1; k_{cat}/K_M $\sim 10^4$ $M^{-1}s^{-1}$) and barely detectable or undetectable in the two other paralogs. Indeed, the consistency of the lactonase function in all PON paralogs and orthologs and the haphazardness of others' activities (phosphotriesterase and aryl esterase; **Table 1**, entry 1) prompted the identification of the lactonase as the native function of PONs (101, 109). This pattern is consistent with promiscuous activities not being under selection and also with the observation that promiscuous activities show large increases and decreases in response to one or a few mutations that are neutral with respect to the primary function (110, 111).
4. Laboratory evolution of one promiscuous activity often leads, indirectly, to the appearance of other promiscuous activities thus yielding "generalist" intermediates (see Evolutionary Aspects of Promiscuity, below) (112). Activities found in these generalist intermediates can be shared by other family members, as either their native or promiscuous function (103, 113).

EVOLUTIONARY ASPECTS OF PROMISCUITY

Studies of divergent evolution within enzyme families and superfamilies support the hypothesis that throughout evolution promiscuous activities served as the starting points for the divergence of new functions and that broad-specificity enzymes served as progenitors for today's specialized enzymes (7). Evidence of this, however, is largely circumstantial and provides little insight into the mechanisms and mutational paths that underlined these processes of divergence. Describing the mutational paths is a particular challenge, because in today's enzymes, even within the same superfamily, different functions imply sequence differences ranging from 30% up to 80%. In addition, most of these sequence changes relate to "drift" rather than change of function. Furthermore, paths leading from one function to another are most likely to be gradual (one mutation at a time) and smooth (via intermediates that are all folded and functional to some degree) (115). A detailed discussion of evolutionary mechanisms is beyond the scope of this review but we do outline several key points (for additional information see References 116 and 117).

That natural paths of divergence are most likely to be gradual is also supported by laboratory evolution, where it seems that one "should select what is already there" (118), i.e., evolving an existing weak, promiscuous function is the most feasible option. Indeed, to our knowledge, there exists only one example for the laboratory evolution of an enzymatic function in a noncatalytic fold (RNA ligase evolved from a zinc finger scaffold), and this evolution demanded the exploration of genetic diversity ($>10^{12}$ library variants) that exceeds natural diversities

(119). When no initial activity was present, incorporation of a new function demanded intensive sequence alterations, including deletion and insertion of active-site loops, even within an enzyme from the same superfamily (120). Generation of novel enzymes by computational design involved the simultaneous exchange of 8–20 amino acids (121, 122). Most notably, all the above noted cases involve starting points, and/or intermediates, that possess no activity, or even folding capability, whatsoever.

Evolvability of Promiscuous Functions: The Three Basic Postulates

Given the likelihood of gradual, smooth transitions, it is likely that natural evolution routinely takes advantage of promiscuous activities as starting points for the divergence of new enzymes. However, for promiscuity to lead the divergence of new enzyme functions, the following three basic prerequisites (discussed in detail in sections below) should be met.

1. The promiscuous activities provide an immediate physiological advantage and could thus become selected.
2. Once a promiscuous function becomes physiologically relevant, it can be improved through one, or just few, mutation(s), initially without abolishing the primary, native function of the enzyme.
3. The divergence path can be completed to give a newly specialized enzyme, for which the promiscuous activity became the native one.

Promiscuous Functions Can Provide an Immediate Advantage

Many reports indicate that weak promiscuous activities can provide an immediate selective advantage to an organism, typically following a deficiency created by a genetic manipulation in the laboratory. A systematic study conducted by Patrick et al. (32) is discussed in the section Promiscuity: Rule or Exception? In an *E. coli* strain deficient in glucokinase activity, several sugar kinases were found that promiscuously phosphorylate glucose (123). These promiscuous activities are notably weak (**Table 1**); the k_{cat}/K_M values of the promiscuous sugar kinase *YajF* are in the range of 10^2 $M^{-1}s^{-1}$ and are $\sim 10^4$ lower than that of the primary *E. coli* glucokinase (*Glk*). Indeed, in these cases, overexpression of the promiscuous enzyme from a multiple-copy plasmid was necessary, as low catalytic efficiency can be clearly compensated by higher enzyme levels (60, 124).

Another notable example is alkaline phosphatase, whose promiscuous phosphite oxidation complemented the growth deficiency of *E. coli phn* knockout strains (**Figure 3*a***). The ability to grow on phosphite as the sole source of inorganic phosphorous occurred via the chromosomal gene of alkaline phosphatase, owing to the extremely high expression levels of the native alkaline phosphatase under phosphate starvation (59). In other cases, changes in regulation of chromosomal genes, leading to higher expression, were observed (60). In Hall's classical experiment (125, 126) of the emergence of an alternative β-galactosidase, mutations increased a weak promiscuous β-galactosidase activity in *egb* (a glycosylase whose native function remains unknown). The first mutation dramatically increased the expression of *egb* by disabling its repressor (125). A promoter mutation in a complementing plasmid also led to ~100-fold increase in expression level of the promiscuous glucokinase *YajF* (127). Gene duplication is another abundant event, leading to increased enzyme levels (117, 128–130). Thus, if and when a new function becomes necessary, the combination of a weak promiscuous activity with an increase in enzyme levels via regulatory mutations and/or gene duplication can provide the organism an immediate advantage.

Negative Trade-offs and the Evolvability of Promiscuous Functions

The second postulate regarding the evolvability of promiscuous functions is that promiscuous functions can be readily improved through one, or just a few, mutations and that mutations leading to improvements in promiscuous

functions need not induce parallel decreases in the native function. Strong negative trade-offs between the evolving trait and existing traits are a dominant factor in evolution (131). Hence, gene duplication, and a split of the original and evolving functions between the two copies, is considered a prerequisite for adaptation. The weak trade-off hypothesis allows alternative modes for emergence of new genes carrying new functions.

Evolvability, or evolutionary adaptability, is the capacity of biological systems, whether they are organisms, cells, or proteins, to evolve. Evolvability comprises two elements: plasticity and robustness (51, 132). Plasticity is the induction of novel phenotypic traits by a relatively small number of mutations. This property of promiscuous enzyme functions has been demonstrated by numerous laboratory evolution experiments. Moreover, it seems that the more promiscuous and versatile is a metabolic pathway, the more evolvable are the enzymes within it (133). However, plasticity is in conflict with the fact that most mutations are deleterious (134–136). Organisms constantly endure mutations while maintaining fitness. They therefore maintain a certain level of resistance to the effects of mutations (robustness). These two features may appear to be conflicting: Can mutations simultaneously induce no phenotypic changes and significant changes? It appears that biological systems, including proteins, exhibit both traits, namely plasticity and robustness, and the two are not necessarily mutually exclusive (51, 137). The promiscuous, accidental functions of the protein are highly plastic and can be reshaped through a few mutations. However, these mutations need not have a large effect on the protein's native activity. Indeed, many directed evolution experiments indicate that, in contrast to the large shifts observed with the promiscuous substrates, native activities taking place in the same active site show comparatively small changes. This robustness of the native function was observed, although the only selection criterion applied in these experiments was an increase in a promiscuous activity of the target enzyme.

The weak trade-off trend was first described in three different enzymes subjected to a selection for an increase in six different promiscuous activities (138), yet it was also observed in many other laboratory experiments (**Table 2**). On average, mutations increased the promiscuous activity under selection by 10–10^6-fold, whereas the original activity decreased by 0.8–42-fold. In most cases, the ratio of increase in the evolving promiscuous function versus decrease in the original function is $\geq$10. Similar trends were seen in receptors, where mutations leading to the binding of a new ligand initially broadened the spectrum of bound ligands while retaining the original one (139, 140). In bacterial transcription factors, new effector specificities were acquired during natural or laboratory evolution based on existing promiscuous effectors, and with weak trade-offs with respect to the original effector (141–143).

The different effects of mutations on the native versus the promiscuous functions are particularly striking in view of the fact that many of these mutations are found within the active site's wall and perimeter. Structural and thermodynamic insights into the effects of these generalist mutations are needed before any definite statements can be made. Yet, it seems likely that the plasticity of the mutated residues is related to the fact that they are not part of the protein's scaffold or of the catalytic machinery of the enzyme. They are typically located on surface loops that exhibit high conformational flexibility and comprise the substrate-binding part of the active site (66, 67, 138, 144, 145). Indeed, conformational plasticity provides a straightforward explanation for weak trade-offs at the early stages of divergence (**Figure 1**). For example, in α-lytic protease, structural flexibility of the substrate-binding loops (146) enabled a single amino acid substitution to increase the activity toward promiscuous substrates by a factor of 10^5, whereas the native activity was reduced by only twofold (147). In an evolved aminoacyl-tRNA synthetase, the disruption of an α-helix introduced structural plasticity to the enzyme's active site and thus enabled it to accept a range of unnatural amino acid substrates (148).

Original, or existing, function: the native function in the evolutionary context of divergence of new functions

Table 2 Examples of directed evolution of promiscuous enzyme functions and their trade-offs with the native function[a]

Entry number	Enzyme	Native activity (catalytic efficiency of wild type)	Promiscuous activity (catalytic efficiency of wild type)	Mutations in selected variants for higher promiscuous activity	Changes in the evolved promiscuous activity ($k_{cat}/K_M^{variant}/k_{cat}/K_M^{wt}$)	Changes in native activity ($k_{cat}/K_M^{variant}/k_{cat}/K_M^{wt}$)	Comments	References
1	Aspartate amino-transferase (AATase)	Transamination of dicarboxylic substratrates (k_{cat}/K_M = 9.1$M^{-1}s^{-1}$)	Transamination of tyrosine (k_{cat}/K_M = 0.055 $M^{-1}s^{-1}$) and phenylalanine (k_{cat}/K_M = 0.012 $M^{-1}s^{-1}$)	*Pro13Thr Asn69Ser Gly72Asp Arg129Gly Thr167Ala Ala293Val Asn297Ser Asn339Ser Ala381Val Asn396Asp Ala398Val*	130- and 270-fold higher, respectively	1.2-fold higher	This is a clear example of a generalist intermediate. The in vitro–evolved enzyme exhibits wild-type-like AATase activity and TATase activity that is >10% of that of wild-type TATase	113
2	Muconate lactonizing enzyme (MLE II)	Cycloisomerization (k_{cat}/K_M = 2 × 10^4 $M^{-1}s^{-1}$)	β-elimination (*o*-succinylbenzoate synthase, OSBS activity). No detectable promiscuous activity (nondetectable) (k_{cat}/K_M < 1.5 × 10^{-3} $M^{-1}s^{-1}$)	*Glu323Gly*	>1.2 million-fold higher	15-fold lower	The corresponding mutation in a homologous enzyme decreased the native function far more significantly (see Reference 158)	183
3	Galactokinase (GalK)	Phosphorylation of D-galactose to produce α-D-galactose-1-phosphate (k_{cat}/K_M = 860 $M^{-1}s^{-1}$)	Phosphorylation of C5- or C6-substituted sugars (k_{cat}/K_M = 138 $M^{-1}s^{-1}$ for 2-deoxy D-galactose, and lower for the other substrates)	*Tyr371His*	Fivefold higher for 2-deoxy D-galactose, and higher improve-ments for the other target substrates	1.3-fold lower	This variant accommodates an expanded spectrum of substrates, including substrates that were not used in the screen	184

4	β-glucuronidase (GUS)	Hydrolysis of β-glucuronides (k_{cat}/K_M = 8.3×10^5 $M^{-1}s^{-1}$)	Hydrolysis of pNP-galactoside (k_{cat}/K_M = 2.3 $M^{-1}s^{-1}$)	*Ile12Val Phe365Ser Trp529Leu Ser557Pro Ile560Val*	16-fold higher	8.3-fold lower	Larger increases in the evolving promiscuous galactosidase function of *E. coli* GUS, with smaller changes of the native function, and acquisition of specificities, which were not selected for, were also described (112)	185
5	*SinI* DNA-methyltransferase	Methylation of the internal cytosine of the GG(A/T)CC sequence (k_{cat}/K_M = 2.9×10^5 $M^{-1}s^{-1}$)	Relaxation of sequence specificity toward GG(N)CC (k_{cat}/K_M = 2×10^3 $M^{-1}s^{-1}$)	*Leu214Ser Tyr229His*	18.5-fold higher for the GG(G/C)CC sequence	4.5-fold lower	Specificity broadening was also observed with *Hae*III methyltransferase, where the native activity increased together with the acquisition of higher promiscuous activities	186, 187
6	Phosphotriesterase (PTE)	Phosphotriesterase (paraoxon, k_{cat}/K_M = 4×10^7 $M^{-1}s^{-1}$)	Ester hydrolysis (2-naphthyl acetate, k_{cat}/K_M = 480 $M^{-1}s^{-1}$)	*His254Arg Phe306Cys Pro342Ala*	13-fold higher	Threefold lower	≤150-fold higher activities were observed with other esters, for which there was no selection	103, 138
7	Human carbonic anhydrase (hCAII)	Bicarbonate dehydration (k_{cat}/K_M = 3×10^7 $M^{-1}s^{-1}$)	Esterase (p-nitrophenyl acetate, k_{cat}/K_M = 2×10^3 $M^{-1}s^{-1}$)	*Ala65Val Asp110Asn Thr200Ala*	40-fold higher	Twofold lower	Mutations in conserved regions of the active site did not affect the highly proficient native activity	138, 157

(*Continued*)

Table 2 (*Continued*)

Entry number	Enzyme	Native activity (catalytic efficiency of wild type)	Promiscuous activity (catalytic efficiency of wild type)	Mutations in selected variants for higher promiscuous activity	Changes in the evolved promiscuous activity ($k_{cat}/K_M^{variant}/k_{cat}/K_M^{wt}$)	Changes in native activity ($k_{cat}/K_M^{variant}/k_{cat}/K_M^{wt}$)	Comments	References
8	Mammalian serum paraoxonase (PON1)	Lipo-lactonase[b] (δ-valerolactone, k_{cat}/K_M = 1.3×10^5 $M^{-1}s^{-1}$; and γ-heptanolide, k_{cat}/K_M = 2×10^4)	Thiolactonase (γ-butyryl thiolactone, k_{cat}/K_M = 75 $M^{-1}s^{-1}$)	*Ile291Leu Thr332Ala*	80-fold higher	No change[b]	The selected mutations are located on surface loops that contain the substrate-binding pocket	138, 188
			Esterase (2-naphthyl octanoate, k_{cat}/K_M = 1.5×10^3 $M^{-1}s^{-1}$)	*Phe292Val Tyr293Asp*	31-fold higher	No change		
			Esterase (7-acetoxy coumarin, k_{cat}/K_M = 1.2×10^5 $M^{-1}s^{-1}$)	*Phe292Ser Val346Met*	62-fold higher	~22-fold lower		
			Phosphotri-esterase (7-diethyl-phosphoro 4-cyano-7-hydroxycoumarin, k_{cat}/K_M = 9×10^3 $M^{-1}s^{-1}$)	*Leu69Val Ser138Leu Ser193Pro Asn287Asp*	155-fold higher	2.6-fold lower		

9	Deacetoxy cephalosporin C synthase (DAOCS)	Ring expansion of penicillin N into deacetoxy-cephalosporin C (k_{cat}/K_M = 2.2×10^4 $M^{-1}s^{-1}$)	Ring expansion of penicillin G into phenylacetyl-7-aminodeacetoxy cephalosporanic acid (k_{cat}/K_M = 18 $M^{-1}s^{-1}$)	*Val275Ile Ile305Met*	32-fold higher	1.1-fold higher		189
				Cys155Tyr Tyr184His Val275Ile Cys281Tyr	41-fold higher	42-fold lower		
10	β-lactamase TEM-1	Ampicillin hydrolysis (k_{cat}/K_M = 4.18×10^7 $M^{-1}s^{-1}$)	Cefotaxime hydrolysis (k_{cat}/K_M = 2.07×10^3 $M^{-1}s^{-1}$)	*Gly238Ser*	86-fold higher	6.2-fold lower		27
			Ceftazidime hydrolysis (k_{cat}/K_M = 32.1 $M^{-1}s^{-1}$)	*Gly238Ser*	19-fold higher			
			Cefotaxime hydrolysis (k_{cat}/K_M = 2.07×10^3 $M^{-1}s^{-1}$)	*Gly238Ser Glu104Lys*	806-fold higher	29-fold lower		
			Ceftazidime hydrolysis (k_{cat}/K_M = 32.1 $M^{-1}s^{-1}$)	*Gly238Ser Glu104Lys*	284-fold higher			
11	Extended-spectrum β-lactamase CTX-M	Hydrolysis of cephalothin and cefotaxime (4×10^6–2×10^7)	Hydrolysis of ceftazidime (k_{cat}/K_M = 3.3×10^3 $M^{-1}s^{-1}$)	*Gln87Leu His112Tyr Thr230Ile Ala231Val Asp240Gly Arg276His*	24-fold higher	1.5-fold higher, and 1.4-fold lower for cephalothin and cefotaxime, respectively		190

(*Continued*)

Table 2 (*Continued*)

Entry number	Enzyme	Native activity (catalytic efficiency of wild type)	Promiscuous activity (catalytic efficiency of wild type)	Mutations in selected variants for higher promiscuous activity	Changes in the evolved promiscuous activity ($k_{cat}/K_M^{variant}/k_{cat}/K_M^{wt}$)	Changes in native activity ($k_{cat}/K_M^{variant}/k_{cat}/K_M^{wt}$)	Comments	References
12	*Not*I endonuclease	Recognition and cleavage of GCGGCCGC DNA sequence (5×10^5 units/mg enzyme)	Recognition and cleavage of altered 8-bp sequence (no detectable star activity)	*Met91Val Glu156Gly*	>32-fold higher than the Glu156Gly intermediate with GCTGCCGC sequence	23-fold lower	Although a considerable reduction in the rate of cleavage of the original sequence is reported, the cleavage specificity of the *Met91Val/Glu156Gly* mutant appears to be relaxed toward a whole set of 8-bp sequence targets, with a distinct preference for the original target	191
13	D-allose kinase (AlsK)	Phosphorylation of D-allose (k_{cat}/K_M = 2.5×10^5 $M^{-1}s^{-1}$)	Phosphorylation of D-glucose (k_{cat}/K_M = 3.4×10^2 $M^{-1}s^{-1}$)	*Ala73Gly*	62-fold higher	1.25-fold lower		145
				Phe145Leu	11.4-fold higher	1.28-fold higher		
	N-acetyl D-mannosamine kinase (NanK)	Phosphorylation of *N*-acetyl D-mannosamine (k_{cat}/K_M = 1.5×10^5 $M^{-1}s^{-1}$)	Phosphorylation of D-glucose (k_{cat}/K_M = 3.4×10^3 $M^{-1}s^{-1}$)	*Leu84Pro*	11.8-fold higher	Twofold lower		
				Val138Met	6.4-fold higher	1.25-fold lower		

14	ProFAR isomerase (HisA)	Isomerization of *N*′-[(5′-phosphoribosyl) formimino]-5-aminoimidazole-4-carboxamide ribonucleotide ($k_{cat}/K_M = 1.2 \times 10^6\ M^{-1}s^{-1}$)	Isomerization of phosphoribosyl-anthranilate: TrpF activity is below detection limits	*Asp127Val*	Wild-type activity is below detection limits	$\sim 10^4$-fold lower	Almost all the original HisA activity was lost	192

[a]These are examples from the past few years for which kinetic parameters are available for both the promiscuous activity under selection and the original activity, which was not subjected to selection. For additional examples, see supplementary Table 8 in Reference 138. Because the above analysis aims at providing insights to the evolution of new enzyme functions in nature, the examples selected involve selection for only one parameter, an increase in a promiscuous activity, and make use of gene libraries prepared by mutagenesis in a completely random manner (point mutations or shuffling) and throughout the gene length.

[b]Since the publication of Reference 138, it has been established that serum paraoxonase (PON1) is a lipo-lactonase, and its preferred substrates are 5- and 6-membered ring lactones, typically with aliphatic side chains (101, 109, 193). In the original article (138), data for trade-offs with the native activity were presented with both the aromatic lactone dihydrocoumarin and aliphatic lactones. However, more recent works indicated that dihydrocoumarin is not binding PON1's active site in the same mode as aliphatic lactones (84, 101). Thus, the trade-offs presented here are the average values of two aliphatic lactones (δ-valerolactone and γ-heptanolide).

In addition, as discussed above, there seem to be fundamental differences between the mode of binding of the native substrate versus the promiscuous substrates, and it is therefore likely that the same mutation could differently affect the native and the promiscuous substrates. Better understanding of the effect of mutations awaits a sufficient number of structures of both the wild-type enzyme and its evolved mutants in complex with analogs of both the native and promiscuous substrates.

Something for Nothing: For How Long?

Ultimately, the acquisition of a proficient new activity must come at the expense of the old one. Yet, the relative rate by which a new function is gained, and the old one is lost, matters (**Figure 6**). In those cases, where the negative trade-off is initially weak (convex route), the divergence of new function proceeds via a generalist intermediate exhibiting broad specificity. This route suggests that, under selection for increasingly higher proficiency, specialists might evolve spontaneously (i.e., without an explicit selection against the original function) because at a certain point increases in the new function will be accompanied by large losses in the original one. At present, however, laboratory evolution experiments demonstrate that generalist intermediates re-specialize primarily upon dual selection for an increase in the newly evolving activity and a decrease in the original activity (149, 150–153). However, in a living cell, the toll of a generalist on fitness might be too high, and the driving force for specialization is likely to be stronger than observed in vitro (150).

Altogether, the above observations support the hypothesis of evolutionary progenitors and intermediates being of broad specificity or high promiscuity (7) and that a frequent (but not exclusive) evolutionary route leads from a specialist to a generalist and, in turn, to a new specialist (**Figures 5** and **6**). The reconstruction of evolutionary ancestors of both enzymes and receptors (154, 155), as well as laboratory evolution of protein-protein interfaces (149),

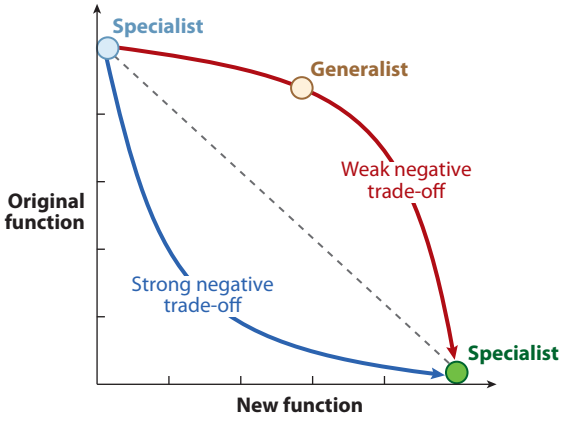

Figure 6

Possible routes to the divergence of a new function. Under selection, a weak, promiscuous activity of a protein with a given primary function (*blue circle*) gradually evolves. By the end of this process, which typically requires many generations of mutation and selection, a new protein emerges with a new function that replaced the original one (*green circle*). The dynamics of this divergence process may vary. The gain-loss of the new versus old function and the conversion of one specialist protein into another may trade off linearly (*dotted line*) or follow either the concave or the convex route. The convex route (weak negative trade-off) is supported by the observation that large increases in the promiscuous function under selection for a new function are often accompanied by significantly smaller decreases in the original function (**Table 2**). By virtue of gaining a new function without losing the original one (and often gaining other new functions that were not selected for), the intermediates of these routes are generalists, and their evolution can therefore proceed prior to gene duplication. In contrast, the concave route implies that gene duplication (or other means of significantly increasing enzyme levels) is a necessary prerequisite because acquisition of even low levels of the new function is accompanied by large losses of the original one. Adapted from Reference 17.

and transcription factors (142, 143) supports the idea of generalist progenitors.

Exceptions to Weak Negative Trade-offs

Although weak negative trade-offs are common, this generalization has notable exceptions. In few cases, a single amino acid exchange can completely switch the specificity of an enzyme (152). For example, the *His89Phe* mutation in the active site of tyrosine ammonia lyase switched its substrate selectivity from tyrosine to phenylalanine, with kinetic parameters and selectivity comparable to phenylalanine ammonia lyase (156). In another example, a mutant (*Glu383Ala*) of *proA* that exhibits higher promiscuous activity with *N*-acetylglutamyl phosphate (*argC* activity) traded off strongly with the original *proA* function (glutamyl phosphate as substrate). In this case, upregulation of the mutated *proA*'s facilitated growth despite the overall low rates with both acetylglutamyl phosphate (the new activity) as well as a significance decrease in the original one (60).

Size and charge considerations. The magnitude of trade-offs may depend on differences in size and charge between the native and promiscuous substrates (157, 158). Most reported studies involve promiscuous substrates that are larger than the native one and cases in which both the native and the promiscuous substrates are uncharged. In these cases, a mutation that makes the active site larger may increase activity toward the promiscuous substrate with no drastic effect on the native substrate. However, in cases where promiscuous substrates are smaller than the native ones, mutations that reshape the active site to increase contacts with the smaller substrate can reduce the activity with the larger native substrate. Still, examples for weak trade-offs at the early steps of directed evolution for smaller substrates exist (159). Other cases in which the native and promiscuous activities might trade off strongly involve differences in charge, e.g., a charged native substrate and a neutral, hydrophobic promiscuous substrate. Mutations that favor the charged substrate are likely to restrict binding of the hydrophobic one, and vice versa (60).

Targeted versus random mutagenesis. Mutations incorporated through rational design (and probably by computational design) show larger trade-offs relative to mutations obtained by selection from random repertoires, **Supplemental Table 2**. Follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org.** This difference can be largely ascribed to the location of the function-altering mutations. Evolutionary processes, in the laboratory or in

nature, usually involve mutations in the active-site periphery (second and third shell mutations) with more subtle effects. However, rational design aims at the replacement of key active-site residues (first shell), and such exchanges yield more drastic changes of specificity. An interesting exception is the introduction of glutathione transferase activity in a glutathione-dependent peroxidase via a single point mutation, which may relate to the evolutionary history of these enzymes (160).

Stability Trade-offs

An important facet of the trade-off that is not reflected in the kinetic parameters per se is the effect of mutations on stability. Most mutations are destabilizing, and mutations that affect function often exhibit even higher destabilizing effects (136). Destabilization usually results in reduced cellular enzyme levels, owing to misfolding and aggregation, proteolytic digestion, or clearance. This phenomenon was first highlighted through the analysis of mutations found in clinical isolates of TEM-1 β-lactamase (27). Thus, although in terms of k_{cat} and K_M the trade-off between the native and the promiscuous functions of TEM-1 mutants is weak (**Table 2**, entry 10), the function-altering mutations are destabilizing, leading to much reduced enzyme levels and slow bacterial growth. For the evolutionary process to continue (in nature or in the laboratory), this loss of stability must be compensated. Indeed, many mutations that appear in directed evolution variants with no obvious role in the new function exert compensatory stabilizing effects (136, 161). A recent review addresses in detail the stability effects of mutations on protein evolution (162).

Promiscuity and Mechanisms for Divergence of New Gene Functions

The mechanisms governing the divergence of new gene-protein functions are a central part of evolutionary theory, a discipline that is unfamiliar to most biochemists. However, the notions of protein promiscuity, and the unique evolutionary features of promiscuous functions, have fundamental implications for this theory. These are briefly mentioned below.

The textbook paradigm, Ohno's model (163), assumes that duplication is a frequent event which is largely neutral, i.e., initially, duplication provides no fitness advantage, or disadvantage, and occurs under no selection (164). The duplicated copy is redundant and free from the burden of selection, and it can therefore accumulate mutations, including deleterious ones. If and when the need arises, variants carrying duplicated genes with mutations that endow a new function become under positive, adaptive selection, thus leading to the divergence of the new gene, protein, and function.

The prerequisites of duplication and relief from selection stem from the negative trade-off assumption: Selection for the existing function purges mutations with adaptive potential, and such mutations can only accumulate in a redundant copy. However, as described above, many promiscuous functions further evolve with little effect on the original function. Ohno's hypothesis—that gene duplication and the subsequent mutational drift occur under no selection—is being questioned for additional reasons. First, most duplicated genes found in existing genomes appear to be under functional selection that purges deleterious mutations (128, 165, 166). Second, expression of redundant mRNA and protein copies carries substantial energetic costs (56, 167), and there exists a selection pressure to inactivate their expression (55, 168). Third, gene duplication is often not a neutral event but is rather positively selected under demands for higher protein doses (129, 130). Last, over a third of the random mutations in a given protein are deleterious (134, 135, 169), whereas beneficial mutations promoting new functions are scarce (estimated frequency of $\sim 10^{-3}$). Thus, when drifting in the absence of any selection, loss of all functions (nonfunctionalization) because of mutations that undermine folding and stability (162, 170) is orders of magnitude more likely than neofunctionalization (171).

Moonlighting: utilization of protein parts outside the active site for other functions, mostly regulatory and structural

The above observations prompted a number of alternative scenarios, which include the following:

1. Gene sharing model—a gene with a given function is recruited for a different, moonlighting function without any changes in the coding region (50, 172).
2. Divergence prior to duplication model—this model (131, 138), the parallel innovation-amplification-divergence model (130), the escape from adaptive conflict model (173), and to a degree the Hughes' model (165) assume that the very first step toward divergence is the selection of a mutant protein with sufficiently high secondary, promiscuous activity, while retaining the original, primary function. Duplication follows and enables the complete re-specialization of the diverging function at the expense of the original function (**Figure 6**).
3. Duplication is a positively selected event, leading to increased variability. When divergence is capitalizing on a weak promiscuous activity in an existing protein, immediate selective advantage can be provided by increasing protein doses. Thus, duplication and the resulting higher protein levels have key roles in enabling promiscuous functions to become physiologically relevant and in enabling a wider variety of function altering mutations to accumulate. Despite the generally weak trade-offs, at the end of the day, mutations that endow new enzymatic functions often have a measurable effect on the existing enzymatic function and/or on the enzyme's stability and expression levels. The acquisition of potentially beneficial mutations can only continue as long as the existing function is reduced to an extent that does not severely compromise organismic fitness. By virtue of two genes carrying the same level of function, duplication offers a margin that allows a larger variety of potentially beneficial mutations to accumulate provided that, contrary to Ohno's model, the two genes remain under selection (117, 128, 171).

SUMMARY POINTS

1. Promiscuity regards reactions that an enzyme performs, although it never evolved to do so (as opposed to its original, native activity).
2. Promiscuous activities are not rare exceptions but are rather widely spread, inherent features of enzymes, and proteins in general.
3. Specificity and promiscuity can reside within the same active site. Promiscuous enzymatic functions may utilize different active-site conformers, and their mechanisms can overlap, partly overlap, or differ altogether from the mechanism by which an enzyme performs its native function.
4. Promiscuous enzyme functions provide immediately accessible starting points for the evolution of new functions via a gradual mutational path that eventually converts a weak, promiscuous function into the primary, native function.
5. A promiscuous function of an enzyme can be a vestige of the function of its ancestor. Promiscuous activities shared by members of same enzyme family and/or superfamily correlate with their divergence from a common ancestor.
6. Mutations that increase a promiscuous activity and have little effect on the primary, native function (weak trade-off) underlie the divergence of a new enzymatic function via a generalist intermediate.

7. The notion of promiscuity as the seed of new gene functions has significant implications for evolutionary theory. Although gene duplication is the key to divergence of new gene functions, when and how duplication occurs and how a new enzyme diverges from an existing one are still a matter of debate.

FUTURE ISSUES

1. Rigorous and quantitative measures of promiscuity are needed, including ways of systematically measuring the magnitude and degree of promiscuity in a wide range of proteins.
2. Are promiscuous functions executed in modes (structural, thermodynamic, kinetic, and/or mechanistic) that fundamentally differ from the modes of primary, native function?
3. Better physicochemical understanding of the effects of mutations on native versus promiscuous activities, and of the origins of the weak trade-offs between the evolving promiscuous activity and the original activity, is needed.
4. Clear cut cases of natural enzymes that diverged from other natural enzymes by virtue of a latent promiscuous activity might be identified from inferred ancestors, recently evolved bacterial enzymes that degrade anthropogenic or xenobiotic chemicals, or secondary metabolism of plants.
5. It remains unclear whether there are fundamental structural and mechanistic differences between generalists and specialists and whether the evolutionary history of an enzyme dictates its future. Are highly specialized enzymes of the central metabolism (enzymes that experienced little change) less promiscuous and less evolvable than secondary metabolism enzymes that have constantly evolved under changing environments?
6. Complete evolutionary trajectories from one specialist to another specialist, whereby the promiscuous activity becomes primary, and vice versa, need to be reproduced in the laboratory (**Figure 6**). Such experiments can unravel the molecular basis of conversion and possible reversion of traits such as robustness and evolvability.
7. The roles of promiscuity of individual enzymes (flexible proteomes) and of cross-wiring of metabolic pathways (flexible metabolomes), in both physiological and evolutionary adaptations, need to be examined.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Financial support by the Sasson and Marjorie Peress Philanthropic Fund, the Israel Science Foundation, the European Union via the MiFEM network, and the Adams Fellowship to O.K. is gratefully acknowledged. We thank Shelley Copley for reviewing the manuscript and for her

constructive criticism and thank John Gerlt, Patsy Babbitt, and our lab members for insightful discussions.

LITERATURE CITED

1. Hult K, Berglund P. 2007. Enzyme promiscuity: mechanism and applications. *Trends Biotechnol.* 25:231–38
2. Pocker Y, Stone JT. 1965. The catalytic versatility of erythrocyte carbonic anhydrase. The enzyme-catalyzed hydrolysis of rho-nitrophenyl acetate. *J. Am. Chem. Soc.* 87:5497–98
3. Reid TW, Fahrney D. 1967. The pepsin-catalyzed hydrolysis of sulfite esters. *J. Am. Chem. Soc.* 89:3941–43
4. Nakagawa Y, Bender ML. 1969. Modification of alpha-chymotrypsin by methyl p-nitrobenzenesulfonate. *J. Am. Chem. Soc.* 91:1566–67
5. Jackson RC, Handschumacher RE. 1970. *Escherichia coli* L-asparaginase. Catalytic activity and subunit nature. *Biochemistry* 9:3585–90
6. Jacob F. 1977. Evolution and tinkering. *Science* 196:1161–66
7. Jensen RA. 1976. Enzyme recruitment in evolution of new function. *Annu. Rev. Microbiol.* 30:409–25
8. O'Brien PJ, Herschlag D. 1999. Catalytic promiscuity and the evolution of new enzymatic activities. *Chem. Biol.* 6:R91–105
9. Copley SD. 2003. Enzymes with extra talents: moonlighting functions and catalytic promiscuity. *Curr. Opin. Chem. Biol.* 7:265–72
10. Bornscheuer UT, Kazlauskas RJ. 2004. Catalytic promiscuity in biocatalysis: using old enzymes to form new bonds and follow new pathways. *Angew. Chem. Int. Ed. Engl.* 43:6032–40
11. Kazlauskas RJ. 2005. Enhancing catalytic promiscuity for biocatalysis. *Curr. Opin. Chem. Biol.* 9:195–201
12. Nobeli I, Favia AD, Thornton JM. 2009. Protein promiscuity and its implications for biotechnology. *Nat. Biotechnol.* 27:157–67
13. Bonner CA, Disz T, Hwang K, Song J, Vonstein V, et al. 2008. Cohesion group approach for evolutionary analysis of TyrA, a protein family with wide-ranging substrate specificities. *Microbiol. Mol. Biol. Rev.* 72:13–53
14. Glasner ME, Gerlt JA, Babbitt PC. 2006. Evolution of enzyme superfamilies. *Curr. Opin. Chem. Biol.* 10:492–97
15. O'Brien PJ. 2006. Catalytic promiscuity and the divergent evolution of DNA repair enzymes. *Chem. Rev.* 106:720–52
16. Palmer DR, Garrett JB, Sharma V, Meganathan R, Babbitt PC, Gerlt JA. 1999. Unexpected divergence of enzyme function and sequence: "*N*-acylamino acid racemase" is *o*-succinylbenzoate synthase. *Biochemistry* 38:4252–58
17. Khersonsky O, Roodveldt C, Tawfik DS. 2006. Enzyme promiscuity: evolutionary and mechanistic aspects. *Curr. Opin. Chem. Biol.* 10:498–508
18. Toscano MD, Woycechowsky KJ, Hilvert D. 2007. Minimalist active-site redesign: teaching old enzymes new tricks. *Angew. Chem. Int. Ed. Engl.* 46:3212–36
19. Hartley CJ, Newcomb RD, Russell RJ, Yong CG, Stevens JR, et al. 2006. Amplification of DNA from preserved specimens shows blowflies were preadapted for the rapid evolution of insecticide resistance. *Proc. Natl. Acad. Sci. USA* 103:8757–62
20. Janssen DB, Dinkla IJ, Poelarends GJ, Terpstra P. 2005. Bacterial degradation of xenobiotic compounds: evolution and distribution of novel enzyme activities. *Environ. Microbiol.* 7:1868–82
21. Newcomb RD, Campbell PM, Ollis DL, Cheah E, Russell RJ, Oakeshott JG. 1997. A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proc. Natl. Acad. Sci. USA* 94:7464–68
22. Raushel FM, Holden HM. 2000. Phosphotriesterase: an enzyme in search of its natural substrate. *Adv. Enzymol. Relat. Areas Mol. Biol.* 74:51–93
23. Wackett LP. 2009. Questioning our perceptions about evolution of biodegradative enzymes. *Curr. Opin. Microbiol.* 12:244–51

24. Copley SD. 2009. Evolution of efficient pathways for degradation of anthropogenic chemicals. *Nat. Chem. Biol.* 5:559–66
25. Barlow M, Hall BG. 2002. Phylogenetic analysis shows that the OXA beta-lactamase genes have been on plasmids for millions of years. *J. Mol. Evol.* 55:314–21
26. Hall BG. 2004. Predicting the evolution of antibiotic resistance genes. *Nat. Rev. Microbiol.* 2:430–35
27. Wang X, Minasov G, Shoichet BK. 2002. Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. *J. Mol. Biol.* 320:85–95
28. Weinreich DM, Delaney NF, Depristo MA, Hartl DL. 2006. Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* 312:111–14
29. O'Maille PE, Malone A, Dellas N, Andes Hess B Jr, Smentek L, et al. 2008. Quantitative exploration of the catalytic landscape separating divergent plant sesquiterpene synthases. *Nat. Chem. Biol.* 4:617–23
30. Austin MB, O'Maille PE, Noel JP. 2008. Evolving biosynthetic tangos negotiate mechanistic landscapes. *Nat. Chem. Biol.* 4:217–22
31. Field B, Osbourn AE. 2008. Metabolic diversification—independent assembly of operon-like gene clusters in different plants. *Science* 320:543–47
32. Patrick WM, Quandt EM, Swartzlander DB, Matsumura I. 2007. Multicopy suppression underpins metabolic evolvability. *Mol. Biol. Evol.* 24:2716–22
33. Khersonsky O, Tawfik DS. 2010. Enzyme promiscuity—evolutionary and mechanistic aspects. In *Comprehensive Natural Products Chemistry*, Vol. 8, ed. D. Barton, O. Meth-Cohn. Oxford: Elsevier. In press
34. van Loo B, Jonas S, Babtie AC, Benjdia A, Berteau O, et al. 2010. An efficient, multiply promiscuous hydrolase in the alkaline phosphatase superfamily. *Proc. Natl. Acad. Sci. USA* 107:2740–45
35. Nath A, Atkins WM. 2008. A quantitative index of substrate promiscuity. *Biochemistry* 47:157–66
36. Catrina I, O'Brien PJ, Purcell J, Nikolic-Hughes I, Zalatan JG, et al. 2007. Probing the origin of the compromised catalysis of *E. coli* alkaline phosphatase in its promiscuous sulfatase reaction. *J. Am. Chem. Soc.* 129:5760–65
37. Olguin LF, Askew SE, O'Donoghue AC, Hollfelder F. 2008. Efficient catalytic promiscuity in an enzyme superfamily: an arylsulfatase shows a rate acceleration of 10^{13} for phosphate monoester hydrolysis. *J. Am. Chem. Soc.* 130:16547–55
38. O'Brien PJ, Herschlag D. 2001. Functional interrelationships in the alkaline phosphatase superfamily: phosphodiesterase activity of *Escherichia coli* alkaline phosphatase. *Biochemistry* 40:5691–99
39. Michaud GA, Salcius M, Zhou F, Bangham R, Bonin J, et al. 2003. Analyzing antibody specificity with whole proteome microarrays. *Nat. Biotechnol.* 21:1509–12
40. Varga JM, Kalchschmid G, Klein GF, Fritsch P. 1991. Mechanism of allergic cross-reactions. II. Cross-stimulation, by chemically unrelated ligands, of rat basophilic leukemia cells sensitized with an anti-DNP IgE antibody. *Mol. Immunol.* 28:655–59
41. Varga JM, Kalchschmid G, Klein GF, Fritsch P. 1991. Mechanism of allergic cross-reactions. I. Multi-specific binding of ligands to a mouse monoclonal anti-DNP IgE antibody. *Mol. Immunol.* 28:641–54
42. Inman J. 1978. The antibody combining region: Speculation on the hypothesis of general multispecificity. In *Theoretical Immunology*, ed. GI Bell, AS Perelson, Pimbley GH Jr, pp. 243–78. New York: Dekker
43. Lancet D, Sadovsky E, Seidemann E. 1993. Probability model for molecular recognition in biological receptor repertoires: significance to the olfactory system. *Proc. Natl. Acad. Sci. USA* 90:3715–19
44. Perelson AS, Oster GF. 1979. Theoretical studies of clonal selection: minimal antibody repertoire size and reliability of self-non-self discrimination. *J. Theor. Biol.* 81:645–70
45. Griffiths AD, Tawfik DS. 2000. Man-made enzymes–from design to in vitro compartmentalisation. *Curr. Opin. Biotechnol.* 11:338–53
46. James LC, Tawfik DS. 2003. The specificity of cross-reactivity: Promiscuous antibody binding involves specific hydrogen bonds rather than nonspecific hydrophobic stickiness. *Protein Sci.* 12:2183–93
47. Fersht A. 1999. *Structure and Mechanism in Protein Science*. New York: Freeman
48. Kotik-Kogan O, Moor N, Tworowski D, Safro M. 2005. Structural basis for discrimination of L-phenylalanine from L-tyrosine by phenylalanyl-tRNA synthetase. *Structure* 13:1799–807
49. Villiers BR, Hollfelder F. 2009. Mapping the limits of substrate specificity of the adenylation domain of TycA. *ChemBioChem* 10:671–82

50. Piatigorsky J. 2007. *Gene Sharing and Evolution: The Diversity of Protein Functions*. Cambridge, MA/London: Harvard Univ. Press. 320 pp.
51. Wagner A. 2005. Robustness, evolvability, and neutrality. *FEBS Lett.* 579:1772–78
52. Brevet A, Plateau P, Cirakoglu B, Pailliez JP, Blanquet S. 1982. Zinc-dependent synthesis of 5′,5′-diadenosine tetraphosphate by sheep liver lysyl- and phenylalanyl-tRNA synthetases. *J. Biol. Chem.* 257:14613–15
53. Lee YN, Nechushtan H, Figov N, Razin E. 2004. The function of lysyl-tRNA synthetase and Ap4A as signaling regulators of MITF activity in FcεRI-activated mast cells. *Immunity* 20:145–51
54. Guo RT, Chong YE, Guo M, Yang XL. 2009. Crystal structures and biochemical analyses suggest a unique mechanism and role for human glycyl-tRNA synthetase in Ap4A homeostasis. *J. Biol. Chem.* 284:28968–76
55. Dekel E, Alon U. 2005. Optimality and evolutionary tuning of the expression level of a protein. *Nature* 436:588–92
56. Stoebel DM, Dean AM, Dykhuizen DE. 2008. The cost of expression of *Escherichia coli* lac operon proteins is in the process, not in the products. *Genetics* 178:1653–60
57. Kuznetsova E, Proudfoot M, Gonzalez CF, Brown G, Omelchenko MV, et al. 2006. Genome-wide analysis of substrate specificities of the *Escherichia coli* haloacid dehalogenase-like phosphatase family. *J. Biol. Chem.* 281:36149–61
58. Tremblay LW, Dunaway-Mariano D, Allen KN. 2006. Structure and activity analyses of *Escherichia coli* K-12 NagD provide insight into the evolution of biochemical function in the haloalkanoic acid dehalogenase superfamily. *Biochemistry* 45:1183–93
59. Yang K, Metcalf WW. 2004. A new activity for an old enzyme: *Escherichia coli* bacterial alkaline phosphatase is a phosphite-dependent hydrogenase. *Proc. Natl. Acad. Sci. USA* 101:7919–24
60. McLoughlin SY, Copley SD. 2008. A compromise required by gene sharing enables survival: implications for evolution of new enzyme activities. *Proc. Natl. Acad. Sci. USA* 105:13497–502
61. Macchiarulo A, Nobeli I, Thornton JM. 2004. Ligand selectivity and competition between enzymes in silico. *Nat. Biotechnol.* 22:1039–45
62. D'Ari R, Casadesus J. 1998. Underground metabolism. *BioEssays* 20:181–86
63. Kurakin A. 2007. Self-organization versus Watchmaker: ambiguity of molecular recognition and design charts of cellular circuitry. *J. Mol. Recognit.* 20:205–14
64. Greenspan RJ. 2001. The flexible genome. *Nat. Rev. Genet.* 2:383–87
65. van Swinderen B, Greenspan RJ. 2005. Flexibility in a gene network affecting a simple behavior in *Drosophila melanogaster*. *Genetics* 169:2151–63
66. Gamage NU, Tsvetanov S, Duggleby RG, McManus ME, Martin JL. 2005. The structure of human SULT1A1 crystallized with estradiol. An insight into active site plasticity and substrate inhibition with multi-ring substrates. *J. Biol. Chem.* 280:41482–86
67. James LC, Tawfik DS. 2003. Conformational diversity and protein evolution—a 60-year-old hypothesis revisited. *Trends Biochem. Sci.* 28:361–68
68. Meier S, Ozbek S. 2007. A biological cosmos of parallel universes: Does protein structural plasticity facilitate evolution? *BioEssays* 29:1095–104
69. Tokuriki N, Tawfik DS. 2009. Protein dynamism and evolvability. *Science* 324:203–7
70. Yasutake Y, Yao M, Sakai N, Kirita T, Tanaka I. 2004. Crystal structure of the *Pyrococcus horikoshii* isopropylmalate isomerase small subunit provides insight into the dual substrate specificity of the enzyme. *J. Mol. Biol.* 344:325–33
71. Hou L, Honaker MT, Shireman LM, Balogh LM, Roberts AG, et al. 2007. Functional promiscuity correlates with conformational heterogeneity in A-class glutathione *S*-transferases. *J. Biol. Chem.* 282:23264–74
72. Zhao Y, Sun L, Muralidhara BK, Kumar S, White MA, et al. 2007. Structural and thermodynamic consequences of 1-(4-chlorophenyl)imidazole binding to cytochrome P450 2B4. *Biochemistry* 46:11559–67
73. Tomatis PE, Fabiane SM, Simona F, Carloni P, Sutton BJ, Vila AJ. 2008. Adaptive protein evolution grants organismal fitness by improving catalysis and flexibility. *Proc. Natl. Acad. Sci. USA* 105:20605–10

74. Delmas J, Chen Y, Prati F, Robin F, Shoichet BK, Bonnet R. 2008. Structure and dynamics of CTX-M enzymes reveal insights into substrate accommodation by extended-spectrum beta-lactamases. *J. Mol. Biol.* 375:192–201
75. Lu X, Li L, Wu R, Feng X, Li Z, et al. 2006. Kinetic analysis of *Pseudomonas aeruginosa* arginine deiminase mutants and alternate substrates provides insight into structural determinants of function. *Biochemistry* 45:1162–72
76. Bertoldi M, Gonsalvi M, Contestabile R, Voltattorni CB. 2002. Mutation of tyrosine 332 to phenylalanine converts Dopa decarboxylase into a decarboxylation-dependent oxidative deaminase. *J. Biol. Chem.* 277:36357–62
77. Kaminaga Y, Schnepp J, Peel G, Kish CM, Ben-Nissan G, et al. 2006. Plant phenylacetaldehyde synthase is a bifunctional homotetrameric enzyme that catalyzes phenylalanine decarboxylation and oxidation. *J. Biol. Chem.* 281:23357–66
78. Theodossis A, Walden H, Westwick EJ, Connaris H, Lamble HJ, et al. 2004. The structural basis for substrate promiscuity in 2-keto-3-deoxygluconate aldolase from the Entner-Doudoroff pathway in *Sulfolobus solfataricus*. *J. Biol. Chem.* 279:43886–92
79. O'Brien PJ, Herschlag D. 1998. Sulfatase activity of *E. coli* alkaline phosphatase demonstrates a functional link to arylsulfatases, an evolutionarily related enzyme family. *J. Am. Chem. Soc.* 120:12369–70
80. Wang SC, Johnson WH Jr, Whitman CP. 2003. The 4-oxalocrotonate tautomerase- and YwhB-catalyzed hydration of 3E-haloacrylates: implications for the evolution of new enzymatic activities. *J. Am. Chem. Soc.* 125:14282–83
81. Wang SC, Person MD, Johnson WH Jr, Whitman CP. 2003. Reactions of trans-3-chloroacrylic acid dehalogenase with acetylene substrates: consequences of and evidence for a hydration reaction. *Biochemistry* 42:8762–73
82. Poelarends GJ, Serrano H, Johnson WH Jr, Hoffman DW, Whitman CP. 2004. The hydratase activity of malonate semialdehyde decarboxylase: mechanistic and evolutionary implications. *J. Am. Chem. Soc.* 126:15658–59
83. Poelarends GJ, Serrano H, Johnson WH Jr, Whitman CP. 2005. Inactivation of malonate semialdehyde decarboxylase by 3-halopropiolates: evidence for hydratase activity. *Biochemistry* 44:9375–81
84. Khersonsky O, Tawfik DS. 2006. The histidine 115-histidine 134 dyad mediates the lactonase activity of mammalian serum paraoxonases. *J. Biol. Chem.* 281:7649–56
85. Yeung DT, Lenz DE, Cerasoli DM. 2005. Analysis of active-site amino-acid residues of human serum paraoxonase using competitive substrates. *FEBS J.* 272:2225–30
86. Blum MM, Timperley CM, Williams GR, Thiermann H, Worek F. 2008. Inhibitory potency against human acetylcholinesterase and enzymatic hydrolysis of fluorogenic nerve agent mimics by human paraoxonase 1 and squid diisopropyl fluorophosphatase. *Biochemistry* 47:5216–24
87. Branneby C, Carlqvist P, Magnusson A, Hult K, Brinck T, Berglund P. 2003. Carbon-carbon bonds by hydrolytic enzymes. *J. Am. Chem. Soc.* 125:874–75
88. Carlqvist P, Svedendahl M, Branneby C, Hult K, Brinck T, Berglund P. 2005. Exploring the active-site of a rationally redesigned lipase for catalysis of Michael-type additions. *ChemBioChem* 6:331–36
89. Torre O, Alfonso I, Gotor V. 2004. Lipase catalysed Michael addition of secondary amines to acrylonitrile. *Chem. Commun.* 15:1724–25
90. Kaiser ET, Lawrence DS. 1984. Chemical mutation of enzyme active sites. *Science* 226:505–11
91. Bakker M, van Rantwijk F, Sheldon RA. 2002. Metal substitution in thermolysin: catalytic properties of tungstate thermolysin in sulfoxidation with H_2O_2. *Can. J. Chem. Rev. Can. Chim.* 80:622–25
92. da Silva GF, Ming LJ. 2005. Catechol oxidase activity of di-Cu^{2+}-substituted aminopeptidase from *Streptomyces griseus*. *J. Am. Chem. Soc.* 127:16380–81
93. Fernandez-Gacio A, Codina A, Fastrez J, Riant O, Soumillion P. 2006. Transforming carbonic anhydrase into epoxide synthase by metal exchange. *ChemBioChem* 7:1013–16
94. Jing Q, Okrasa K, Kazlauskas RJ. 2009. Stereoselective hydrogenation of olefins using rhodium-substituted carbonic anhydrase—a new reductase. *Chemistry* 15:1370–76
95. Wu ZP, Hilvert D. 1990. Selenosubtilisin as a glutathione-peroxidase mimic. *J. Am. Chem. Soc.* 112:5647–48

96. Boschi-Muller S, Muller S, Van Dorsselaer A, Bock A, Branlant G. 1998. Substituting selenocysteine for active site cysteine 149 of phosphorylating glyceraldehyde 3-phosphate dehydrogenase reveals a peroxidase activity. *FEBS Lett.* 439:241–45
97. Yu HJ, Liu JQ, Bock A, Li J, Luo GM, Shen JC. 2005. Engineering glutathione transferase to a novel glutathione peroxidase mimic with high catalytic efficiency. Incorporation of selenocysteine into a glutathione-binding scaffold using an auxotrophic expression system. *J. Biol. Chem.* 280:11930–35
98. Kourist R, Bartsch S, Fransson L, Hult K, Bornscheuer UT. 2008. Understanding promiscuous amidase activity of an esterase from *Bacillus subtilis*. *ChemBioChem* 9:67–69
99. Forconi M, Herschlag D. 2005. Promiscuous catalysis by the tetrahymena group I ribozyme. *J. Am. Chem. Soc.* 127:6160–61
100. Kunzler DE, Sasso S, Gamper M, Hilvert D, Kast P. 2005. Mechanistic insights into the isochorismate pyruvate lyase activity of the catalytically promiscuous PchB from combinatorial mutagenesis and selection. *J. Biol. Chem.* 280:32827–34
101. Khersonsky O, Tawfik DS. 2005. Structure-reactivity studies of serum paraoxonase PON1 suggest that its native activity is lactonase. *Biochemistry* 44:6371–82
102. James LC, Tawfik DS. 2005. Structure and kinetics of a transient antibody binding intermediate reveal a kinetic discrimination mechanism in antigen recognition. *Proc. Natl. Acad. Sci. USA* 102:12730–35
103. Roodveldt C, Tawfik DS. 2005. Shared promiscuous activities and evolutionary features in various members of the amidohydrolase superfamily. *Biochemistry* 44:12728–36
104. Afriat L, Roodveldt C, Manco G, Tawfik DS. 2006. The latent promiscuity of newly identified microbial lactonases is linked to a recently diverged phosphotriesterase. *Biochemistry* 45:13677–86
105. Chow JY, Wu L, Yew WS. 2009. Directed evolution of a quorum-quenching lactonase from *Mycobacterium avium* subsp. *paratuberculosis* K-10 in the amidohydrolase superfamily. *Biochemistry* 48:4344–53
106. Hawwa R, Aikens J, Turner RJ, Santarsiero BD, Mesecar AD. 2009. Structural basis for thermostability revealed through the identification and characterization of a highly thermostable phosphotriesterase-like lactonase from *Geobacillus stearothermophilus*. *Arch. Biochem. Biophys.* 488:109–20
107. Hawwa R, Larsen SD, Ratia K, Mesecar AD. 2009. Structure-based and random mutagenesis approaches increase the organophosphate-degrading activity of a phosphotriesterase homologue from *Deinococcus radiodurans*. *J. Mol. Biol.* 393:36–57
108. Xiang DF, Kolb P, Fedorov AA, Meier MM, Federov LV, et al. 2009. Functional annotation and three-dimensional structure of Dr0930 from *Deinococcus radiodurans*: a close relative of phosphotriesterase in the amidohydrolase superfamily. *Biochemistry* 48:2237–47
109. Draganov DI, Teiber JF, Speelman A, Osawa Y, Sunahara R, La Du BN. 2005. Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J. Lipid Res.* 46:1239–47
110. Amitai G, Devi-Gupta R, Tawfik DS. 2007. Latent evolutionary potentials under the neutral mutational drift of an enzyme. *HFSP J.* 1:67–78
111. Bloom JD, Romero PA, Lu Z, Arnold FH. 2007. Neutral genetic drift can alter promiscuous protein functions, potentially aiding functional evolution. *Biol. Direct.* 2:17
112. Matsumura I, Ellington AD. 2001. In vitro evolution of beta-glucuronidase into a beta-galactosidase proceeds through non-specific intermediates. *J. Mol. Biol.* 305:331–39
113. Rothman SC, Kirsch JF. 2003. How does an enzyme evolved in vitro compare to naturally occurring homologs possessing the targeted function? Tyrosine aminotransferase from aspartate aminotransferase. *J. Mol. Biol.* 327:593–608
114. Anandarajah K, Kiefer PM Jr, Donohoe BS, Copley SD. 2000. Recruitment of a double bond isomerase to serve as a reductive dehalogenase during biodegradation of pentachlorophenol. *Biochemistry* 39:5303–11
115. Smith JM. 1970. Natural selection and concept of a protein space. *Nature* 225:563–64
116. Copley SD. 2010. Evolution and the enzyme. In *Comprehensive Natural Products Chemistry*, Vol. 8, ed. D Barton, O Meth-Cohn. Oxford: Elsevier. In press
117. Conant GC, Wolfe KH. 2008. Turning a hobby into a job: How duplicated genes find new functions. *Nat. Rev. Genet.* 9:938–50
118. Peisajovich SG, Tawfik DS. 2007. Protein engineers turned evolutionists. *Nat. Methods* 4:991–94

119. Seelig B, Szostak JW. 2007. Selection and evolution of enzymes from a partially randomized non-catalytic scaffold. *Nature* 448:828–31
120. Park HS, Nam SH, Lee JK, Yoon CN, Mannervik B, et al. 2006. Design and evolution of new catalytic activity with an existing protein scaffold. *Science* 311:535–38
121. Jiang L, Althoff EA, Clemente FR, Doyle L, Röthlisberger D, et al. 2008. De novo computational design of retro-aldol enzymes. *Science* 319:1387–91
122. Röthlisberger D, Khersonsky O, Wollacott AM, Jiang L, DeChancie J, et al. 2008. Kemp elimination catalysts by computational enzyme design. *Nature* 453:190–95
123. Miller BG, Raines RT. 2004. Identifying latent enzyme activities: substrate ambiguity within modern bacterial sugar kinases. *Biochemistry* 43:6387–92
124. James LC, Tawfik DS. 2001. Catalytic and binding poly-reactivities shared by two unrelated proteins: the potential role of promiscuity in enzyme evolution. *Protein Sci.* 10:2600–7
125. Hall BG. 1999. Experimental evolution of Ebg enzyme provides clues about the evolution of catalysis and to evolutionary potential. *FEMS Microbiol. Lett.* 174:1–8
126. Hall BG. 1982. Evolution on a petri dish: the evolved B-galactosidase system as a model for studying acquisitive evolution in the laboratory. *Evol. Biol.* 15:85–150
127. Miller BG, Raines RT. 2005. Reconstitution of a defunct glycolytic pathway via recruitment of ambiguous sugar kinases. *Biochemistry* 44:10776–83
128. Scannell DR, Wolfe KH. 2008. A burst of protein sequence evolution and a prolonged period of asymmetric evolution follow gene duplication in yeast. *Genome Res.* 18:137–47
129. McLoughlin SY, Ollis DL. 2004. The role of inhibition in enzyme evolution. *Chem. Biol.* 11:735–37
130. Bergthorsson U, Andersson DI, Roth JR. 2007. Ohno's dilemma: evolution of new genes under continuous selection. *Proc. Natl. Acad. Sci. USA* 104:17004–9
131. Kondrashov FA. 2005. In search of the limits of evolution. *Nat. Genet.* 37:9–10
132. Kirschner M, Gerhart J. 1998. Evolvability. *Proc. Nat. Acad. Sci. USA* 95:8420–27
133. Umeno D, Tobias AV, Arnold FH. 2005. Diversifying carotenoid biosynthetic pathways by directed evolution. *Microbiol. Mol. Biol. Rev.* 69:51–78
134. Bershtein S, Segal M, Bekerman R, Tokuriki N, Tawfik DS. 2006. Robustness-epistasis link shapes the fitness landscape of a randomly drifting protein. *Nature* 444:929–32
135. Camps M, Herman A, Loh E, Loeb LA. 2007. Genetic constraints on protein evolution. *Crit. Rev. Biochem. Mol. Biol.* 42:313–26
136. Tokuriki N, Stritcher F, Serrano L, Tawfik DS. 2008. How protein stability and new functions trade off. *PloS Comput. Biol.* 4:e1000002
137. Wagner A. 2008. Robustness and evolvability: a paradox resolved. *Proc. Biol. Sci.* 275:91–100
138. Aharoni A, Gaidukov L, Khersonsky O, McQ GS, Roodveldt C, Tawfik DS. 2005. The 'evolvability' of promiscuous protein functions. *Nat. Genet.* 37:73–76
139. Adami C. 2006. Evolution: reducible complexity. *Science* 312:61–63
140. Bridgham JT, Carroll SM, Thornton JW. 2006. Evolution of hormone-receptor complexity by molecular exploitation. *Science* 312:97–101
141. Galvao TC, de Lorenzo V. 2006. Transcriptional regulators a la carte: engineering new effector specificities in bacterial regulatory proteins. *Curr. Opin. Biotechnol.* 17:34–42
142. Ju KS, Parales JV, Parales RE. 2009. Reconstructing the evolutionary history of nitrotoluene detection in the transcriptional regulator NtdR. *Mol. Microbiol.* 74:826–43
143. Kivisaar M. 2009. Degradation of nitroaromatic compounds: a model to study evolution of metabolic pathways. *Mol. Microbiol.* 74:777–81
144. Bertini I, Calderone V, Cosenza M, Fragai M, Lee YM, et al. 2005. Conformational variability of matrix metalloproteinases: beyond a single 3D structure. *Proc. Natl. Acad. Sci. USA* 102:5334–39
145. Larion M, Moore LB, Thompson SM, Miller BG. 2007. Divergent evolution of function in the ROK sugar kinase superfamily: role of enzyme loops in substrate specificity. *Biochemistry* 46:13564–72
146. Bone R, Frank D, Kettner CA, Agard DA. 1989. Structural analysis of specificity: alpha-lytic protease complexes with analogues of reaction intermediates. *Biochemistry* 28:7600–9
147. Bone R, Silen JL, Agard DA. 1989. Structural plasticity broadens the specificity of an engineered protease. *Nature* 339:191–95

148. Turner JM, Graziano J, Spraggon G, Schultz PG. 2006. Structural plasticity of an aminoacyl-tRNA synthetase active site. *Proc. Natl. Acad. Sci. USA* 103:6483–88
149. Bernath Levin K, Dym O, Albeck S, Magdassi S, Keeble AH, et al. 2009. Following evolutionary paths to protein-protein interactions with high affinity and selectivity. *Nat. Struct. Mol. Biol.* 16:1049–55
150. O'Loughlin TL, Greene DN, Matsumura I. 2006. Diversification and specialization of HIV protease function during in vitro evolution. *Mol. Biol. Evol.* 23:764–72
151. Ran N, Draths KM, Frost JW. 2004. Creation of a shikimate pathway variant. *J. Am. Chem. Soc.* 126:6856–57
152. Varadarajan N, Gam J, Olsen MJ, Georgiou G, Iverson BL. 2005. Engineering of protease variants exhibiting high catalytic activity and exquisite substrate selectivity. *Proc. Natl. Acad. Sci. USA* 102:6855–60
153. Varadarajan N, Rodriguez S, Hwang BY, Georgiou G, Iverson BL. 2008. Highly active and selective endopeptidases with programmed substrate specificities. *Nat. Chem. Biol.* 4:290–94
154. Thornton JW, Need E, Crews D. 2003. Resurrecting the ancestral steroid receptor: ancient origin of estrogen signaling. *Science* 301:1714–17
155. Wouters MA, Liu K, Riek P, Husain A. 2003. A despecialization step underlying evolution of a family of serine proteases. *Mol. Cell* 12:343–54
156. Watts KT, Mijts BN, Lee PC, Manning AJ, Schmidt-Dannert C. 2006. Discovery of a substrate selectivity switch in tyrosine ammonia-lyase, a member of the aromatic amino acid lyase family. *Chem. Biol.* 13:1317–26
157. Gould SM, Tawfik DS. 2005. Directed evolution of the promiscuous esterase activity of carbonic anhydrase II. *Biochemistry* 44:5444–52
158. Vick JE, Schmidt DM, Gerlt JA. 2005. Evolutionary potential of (beta/alpha)8-barrels: in vitro enhancement of a "new" reaction in the enolase superfamily. *Biochemistry* 44:11722–29
159. Fasan R, Meharenna YT, Snow CD, Poulos TL, Arnold FH. 2008. Evolutionary history of a specialized p450 propane monooxygenase. *J. Mol. Biol.* 383:1069–80
160. Zhang ZR, Perrett S. 2009. Novel glutaredoxin activity of the yeast prion protein Ure2 reveals a native-like dimer within fibrils. *J. Biol. Chem.* 284:14058–67
161. Berstein S, Goldin K, Tawfik DS. 2008. Intense neutral drifts yield robust and evolvable consensus proteins. *J. Mol. Biol.* 379:1029–44
162. Tokuriki N, Tawfik DS. 2009. Stability effects of mutations and protein evolvability. *Curr. Opin. Struct. Biol.* 19:596–604
163. Ohno S. 1970. *Evolution by Gene Duplication*. London-New York: Allen & Unwin/Springer-Verlag. 160 pp.
164. Kimura M, Ota T. 1974. On some principles governing molecular evolution. *Proc. Natl. Acad. Sci. USA* 71:2848–52
165. Hughes AL. 2002. Adaptive evolution after gene duplication. *Trends Genet.* 18:433–34
166. Lynch M, Katju V. 2004. The altered evolutionary trajectories of gene duplicates. *Trends Genet.* 20:544–49
167. Wagner A. 2005. Energy constraints on the evolution of gene expression. *Mol. Biol. Evol.* 22:1365–74
168. Cooper VS, Lenski RE. 2000. The population genetics of ecological specialization in evolving *Escherichia coli* populations. *Nature* 407:736–39
169. Tokuriki N, Stricher F, Schymkowitz J, Serrano L, Tawfik DS. 2007. The stability effects of protein mutations appear to be universally distributed. *J. Mol. Biol.* 369:1318–32
170. Yue P, Li Z, Moult J. 2005. Loss of protein structure stability as a major causative factor in monogenic disease. *J. Mol. Biol.* 353:459–73
171. Bershtein S, Tawfik DS. 2008. Ohno's model revisited: measuring the frequency of potentially adaptive mutations under various mutational drifts. *Mol. Biol. Evol.* 25:2311–18
172. Piatigorsky J, O'Brien WE, Norman BL, Kalumuck K, Wistow GJ, et al. 1988. Gene sharing by delta-crystallin and argininosuccinate lyase. *Proc. Natl. Acad. Sci. USA* 85:3479–83
173. Des Marais DL, Rausher MD. 2008. Escape from adaptive conflict after duplication in an anthocyanin pathway gene. *Nature* 454:762–65

174. Blum MM, Lohr F, Richardt A, Ruterjans H, Chen JC. 2006. Binding of a designed substrate analogue to diisopropyl fluorophosphatase: implications for the phosphotriesterase mechanism. *J. Am. Chem. Soc.* 128:12750–57
175. Amitai G, Gaidukov L, Adani R, Yishay S, Yacov G, et al. 2006. Enhanced stereoselective hydrolysis of toxic organophosphates by directly evolved variants of mammalian serum paraoxonase. *FEBS J.* 273:1906–19
176. Lamble HJ, Heyer NI, Bull SD, Hough DW, Danson MJ. 2003. Metabolic pathway promiscuity in the archaeon *Sulfolobus solfataricus* revealed by studies on glucose dehydrogenase and 2-keto-3-deoxygluconate aldolase. *J. Biol. Chem.* 278:34066–72
177. Taylor Ringia EA, Garrett JB, Thoden JB, Holden HM, Rayment I, Gerlt JA. 2004. Evolution of enzymatic activity in the enolase superfamily: functional studies of the promiscuous *o*-succinylbenzoate synthase from *Amycolatopsis*. *Biochemistry* 43:224–29
178. Yew WS, Akana J, Wise EL, Rayment I, Gerlt JA. 2005. Evolution of enzymatic activities in the orotidine 5′-monophosphate decarboxylase suprafamily: enhancing the promiscuous D-arabino-hex-3-ulose 6-phosphate synthase reaction catalyzed by 3-keto-L-gulonate 6-phosphate decarboxylase. *Biochemistry* 44:1807–15
179. Joerger AC, Mayer S, Fersht AR. 2003. Mimicking natural evolution in vitro: an *N*-acetylneuraminate lyase mutant with an increased dihydrodipicolinate synthase activity. *Proc. Natl. Acad. Sci. USA* 100:5694–99
180. De Mot R, De Schrijver A, Schoofs G, Parret AHA. 2003. The thiocarbamate-inducible *Rhodococcus* enzyme ThcF as a member of the family of alpha/beta hydrolases with haloperoxidative side activity. *FEMS Microbiol. Lett.* 224:197–203
181. Kataoka M, Honda K, Shimizu S. 2000. 3,4-Dihydrocoumarin hydrolase with haloperoxidase activity from *Acinetobacter calcoaceticus* F46. *Eur. J. Biochem.* 267:3–10
182. Li C, Hassler M, Bugg TDH. 2008. Catalytic promiscuity in the alpha/beta-hydrolase superfamily: hydroxamic acid formation, C-C bond formation, ester and thioester hydrolysis in the C-C hydrolase family. *ChemBioChem* 9:71–76
183. Schmidt DM, Mundorff EC, Dojka M, Bermudez E, Ness JE, et al. 2003. Evolutionary potential of (beta/alpha)8-barrels: functional promiscuity produced by single substitutions in the enolase superfamily. *Biochemistry* 42:8387–93
184. Hoffmeister D, Yang J, Liu L, Thorson JS. 2003. Creation of the first anomeric D/L-sugar kinase by means of directed evolution. *Proc. Natl. Acad. Sci. USA* 100:13184–89
185. Rowe LA, Geddie ML, Alexander OB, Matsumura I. 2003. A comparison of directed evolution approaches using the beta-glucuronidase model system. *J. Mol. Biol.* 332:851–60
186. Cohen HM, Tawfik DS, Griffiths AD. 2004. Altering the sequence specificity of *Hae*III methyltransferase by directed evolution using in vitro compartmentalization. *Protein Eng. Des. Sel.* 17:3–11
187. Timar E, Groma G, Kiss A, Venetianer P. 2004. Changing the recognition specificity of a DNA-methyltransferase by in vitro evolution. *Nucleic Acids Res.* 32:3898–903
188. Aharoni A, Gaidukov L, Yagur S, Toker L, Silman I, Tawfik DS. 2004. Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. *Proc. Natl. Acad. Sci. USA* 101:482–87
189. Wei CL, Yang YB, Deng CH, Liu WC, Hsu JS, et al. 2005. Directed evolution of *Streptomyces clavuligerus* deacetoxycephalosporin C synthase for enhancement of penicillin G expansion. *Appl. Environ. Microbiol.* 71:8873–80
190. Delmas J, Robin F, Carvalho F, Mongaret C, Bonnet R. 2006. Prediction of the evolution of ceftazidime resistance in extended-spectrum beta-lactamase CTX-M-9. *Antimicrob. Agents Chemother.* 50:731–38
191. Samuelson JC, Morgan RD, Benner JS, Claus TE, Packard SL, Xu SY. 2006. Engineering a rare-cutting restriction enzyme: genetic screening and selection of NotI variants. *Nucleic Acids Res.* 34:796–805
192. Jurgens C, Strom A, Wegener D, Hettwer S, Wilmanns M, Sterner R. 2000. Directed evolution of a $(\beta\alpha)_8$-barrel enzyme to catalyze related reactions in two different metabolic pathways. *Proc. Natl. Acad. Sci. USA* 97:9925–30
193. Gaidukov L, Tawfik DS. 2005. The high affinity, stability and lactonase activity of serum paraoxonase (PON1) anchored on HDL with ApoA-I. *Biochemsitry* 44:11843–54

Annual Review of Biochemistry

Volume 79, 2010

Contents

Indexes

Errata

An online log of corrections to *Annual Review of Biochemistry* articles may be found at http://biochem.annualreviews.org