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Enzyme promiscuity: evolutionary and mechanistic aspects

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The past few years have seen significant advances in research related to the ‘latent skills’ of enzymes — namely, their capacity to promiscuously catalyze reactions other than the ones they evolved for. These advances regard (i) the mechanism of catalytic promiscuity — how enzymes, that generally exert exquisite specificity, promiscuously catalyze other, and sometimes barely related, reactions; (ii) the evolvability of promiscuous functions — namely, how latent activities evolve further, and in particular, how promiscuous activities can firstly evolve without severely compromising the original activity. These findings have interesting implications on our understanding of how new enzymes evolve. They support the key role of catalytic promiscuity in the natural history of enzymes, and suggest that today’s enzymes diverged from ancestral proteins catalyzing a whole range of activities at low levels, to create families and superfamilies of potent and highly specialized enzymes.

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Introduction

Despite their great proficiency of action and robustness, enzymes exhibit a remarkable evolutionary adaptability (evolvability). New enzymes have emerged all throughout the natural history of this planet. In fact, we now know that new enzymatic functions can evolve in a matter of few decades, or even months, as with enzymes that degrade synthetic chemicals that first appeared on this planet during the 20th century [1–3], and the alarming evolution of drug resistance.

An oft-forgotten essence of Darwinian processes is that they occur gradually, while maintaining organism fitness throughout. Consequently, a reasonable assumption is that, ever since the emergence of the primordial living forms, very little novelty has evolved at the molecular level [4]. Rather, existing genes were modified, or

‘tinkered with’, to generate new protein structures and functions that are related to those of their ancestors [4]. Unlike ‘out of the blue’ scenarios advocated by the ‘intelligent design’ school, ‘tinkering’ scenarios depend on the availability of evolutionary starting points. The hypothesis that the broad specificity, or promiscuous functions, of existing enzymes provide these starting points was first formalized by Jensen in a review that has inspired many [5]. Jensen proposed that, in contrast to modern enzymes, primitive enzymes possessed very broad specificities. This catalytic versatility enabled fewer enzymes to perform the multitude of functions that was necessary to maintain ancestral organisms. Duplication of genes and divergence led to specialized genes and increased metabolic efficiency. Since Jensen, the structures of >30 000 proteins, and the sequences of hundreds of thousands, have taught us that these processes led to the creation of enzyme families and superfamilies. The vestiges of these divergence processes are the scaffold and active site architecture shared by all family members [6].

This review addresses several aspects of enzyme evolvability related to promiscuity. Previous reviews by O’Brien and Herschlag [7], and later by Copley [8], highlighted the importance of promiscuity, and surveyed the mechanistic and structural works in this area. Other more recent reviews have focused on the practical implications of enzyme promiscuity in organic synthesis [9,10], or on promiscuity and divergence in certain enzyme families [11]. Here, we aim at providing an update of the recent literature while focusing on three key issues: first, the mechanism of promiscuous enzyme functions; second, whether and how promiscuous enzyme functions might evolve further; and third, the vestiges of functional divergence from broad-specificity progenitors seen in enzyme families and superfamilies.

Mechanistic aspects of catalytic promiscuity

Traditionally, textbooks and research articles have highlighted the truly remarkable specificity of enzyme action, and have largely ignored the ‘darker’ side of enzyme cross-reactivity, or promiscuity. (For early discussions of the catalytic versatility of enzymes, see the work of Pocker [12]). However, in the past several years, the promiscuity of proteins, including enzymes, has drawn considerable attention. Aside from being an act of post-modernism, research into promiscuity leads to interesting insights, in particular with regard to how specificity and promiscuity coincide with a single active site.

Generally, promiscuous activities share the main active site features with the native activity, and besides

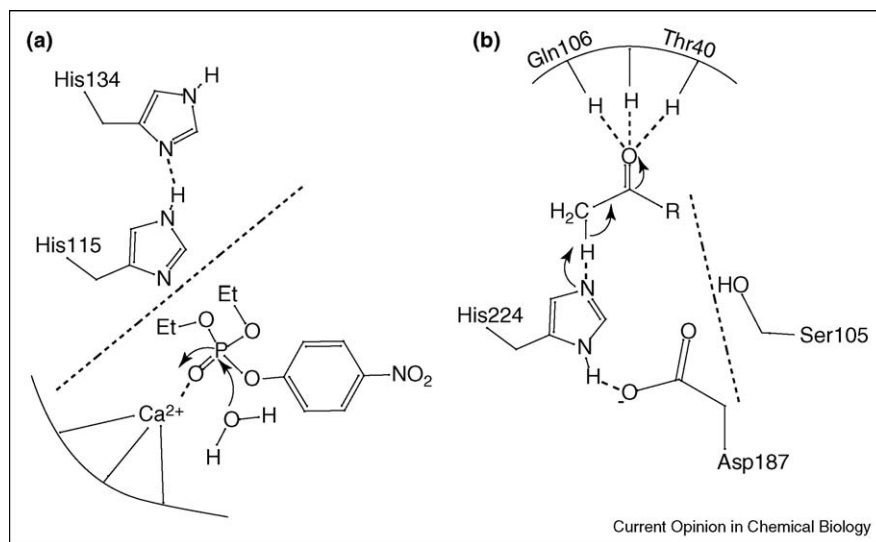
differences such as substrate positioning, their mechanism is largely the same. One example is alkaline phosphatase, which in addition to its highly proficient native phosphate monoesterase activity ($k_{\text{cat}}/K_M = 3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$), hydrolyzes phosphodiester, phosphoamides, sulfate esters, thiophosphates, as well as phosphite (while releasing hydrogen). 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 [13[•],14]. In the tautomerase superfamily, various enzymes share the catalytic Pro1 residue, but the mechanism of catalysis depends on its $\text{p}K_a$. In 4-oxalocrotonate tautomerase (4-OT) its $\text{p}K_a$ is ~ 6.4 and it acts as a general base. However, in *trans*-3-chloroacrylic acid dehalogenase (CaaD), which catalyzes the hydrolytic halogenation of 3*E*-haloacrylates, Pro1 is protonated ($\text{p}K_a \sim 9.2$) and serves as a general acid [15]. 4-OT exhibits some promiscuous CaaD activity, but because little Pro1 is present in 4-OT in the protonation state for general acid catalysis, its promiscuous CaaD activity is quite low. In the family of guanidine-transferring enzymes, three mutually inter-promiscuous enzymes arginine deiminase (PaADI), agmatine deiminase (PaAgDI), and $N^{\text{sw}}, N^{\text{sw}}$ -dimethyl-arginine dimethyl-aminohydrolase (PaDDAH)

utilize the same catalytic triad (Cys–His–Asp) in their action on various derivatives of arginine, but vary in the mode of substrate binding interactions [16[•]].

In several cases, promiscuity has been linked to conformational diversity [17]; namely, conformational changes enable the same enzyme to accommodate different substrates [18]. In particular, the mobility of active site loops appears to play a key role in mediating promiscuity [19,20^{••}].

In other and probably fewer cases, although both the original and promiscuous activities take place in the same active site, and rely on its major feature (e.g., an oxyanion hole), other parts of the catalytic machinery may differ significantly. One such example is serum paraoxonase (PON1), a mammalian lactonase with promiscuous esterase and phosphotriesterase activities. All these activities depend on a calcium ion that serves as PON1's 'oxyanion hole', but the general base that activates a water molecule differs [21] (Figure 1a). An analogous example is *Candida antarctica* lipase B (CAL-B), whose native activity (lipid hydrolysis) is mediated by a Ser–His–Asp catalytic triad. Using its oxyanion hole, CAL-B also catalyzes various carbon-carbon bond formation reactions [22,24]. However,

Figure 1



Mechanisms of promiscuous enzyme activities. **(a)** PON1 is the best-characterized member of a family of enzymes, found in all mammals and numerous other organisms. The native function of mammalian PONs was shown to be that of a lipo-lactonase [59,69]. The main active site feature 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 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) appears to be independent, and mediated by other residues, as indicated by the dashed lines [21]. Indeed, mutations of both His residues can increase the promiscuous phosphotriesterase activity [21,73] by >300-fold with certain substrates [74]. **(b)** A similar scenario has been described for the lipase CalB. Its native activity (lipid hydrolysis) is mediated by the Ser105–His224–Asp187 triad and the negative charge of the transition states and the acyl-enzyme intermediate are stabilized by its 'oxyanion hole'. CalB also catalyzes promiscuous C–C bond formation reactions, such as Michael additions and aldol condensations, with various ketone and aldehyde substrates. 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 [22,24]. Indeed, as in PON1, the Ser105Ala mutant exhibits higher promiscuous activities than wild-type CalB.

in these reactions, the nucleophilic serine — the key part of the catalytic triad — takes no role (Figure 1b).

Enzymologists have also discovered that a systematic research of the ‘hidden skills’ of enzymes can provide valuable insights regarding their catalytic mechanism. For example, the promiscuous phosphonate diester substrates of *Tetrahymena thermophila* ribozyme provided key insights regarding the relative importance of transition state geometry *versus* charge [25[•]]. The promiscuous chorismate mutase activity of PchB was used to ascribe a 1,5-sigmatropic reaction mechanism to its native activity (isochorismate pyruvate lyase) [26]. In the tautomerase superfamily, a promiscuous hydratase activity was used to elucidate the protonation state of the catalytic Pro1 in MSAD (malonate semialdehyde decarboxylase). One family member, CaaD, possesses a promiscuous hydratase activity, using Pro1 as a general acid, whereas another family member, 4-OT, does not. Because MSAD was found to exhibit a promiscuous hydratase activity, it was deduced that its Pro1 is cationic and has a high pK_a [27[•],28].

The evolvability of promiscuous enzyme functions

To assign an unambiguous role for promiscuity in the divergence of new enzyme functions, one must assume that, once a certain latent promiscuous function becomes relevant, it can be easily improved through one, or just few, mutations, to provide a distinct selective advantage. Directed laboratory evolution has provided ample evidence in support of this hypothesis. In general, there are very few cases in which ‘something could be evolved out of nothing’, namely that a completely novel substrate and activity were incorporated into an existing enzyme. Indeed, these switches require major sequence alterations, such as the simultaneous deletion and insertion of few active site loops [29]. The vast majority of directed evolution projects aim at further evolving a promiscuous activity, typically a substrate, or a reaction, that bears significant resemblance to the original function. The conclusion from hundreds of such works is that promiscuous functions exhibit high ‘plasticity’ — a few mutations can readily increase a promiscuous activity, typically by 10–1000-fold, and 10^4 – 10^6 -fold improvements in response to a single mutation were also reported [30^{••},31^{••}]. Indeed, ‘plasticity residues’ can be identified, and targeted for mutagenesis, to obtain a series of specific enzymes, each preferentially producing a different product [32].

Negative trade-offs and the divergence of new functions

Another intriguing aspect of promiscuous functions is that, in many cases, improvements in promiscuous functions do not seem to trade-off with parallel decreases in the original function [33^{••}]. Evolvability, or evolutionary adaptability, is the capacity of biological systems, be they organisms, cells or proteins, to evolve. Evolvability

comprises two elements [34]. The first is the induction of novel phenotypic traits by a relatively low number of mutations. As discussed above, this requirement, dubbed ‘plasticity’, has been established by numerous directed evolution experiments [35]. However, this plasticity is in conflict with the fact that most mutations are deleterious, whereas beneficial mutations are very rare. Thus, organisms and proteins must constantly endure a significant number of mutations with little change in their structure and function (‘robustness’) [36]. It appears that proteins exhibit both traits, namely plasticity and robustness, and the two need not be mutually exclusive [33^{••}]. The promiscuous, accidental functions of a protein are highly plastic. They can be shaped through mutations that dramatically increase or decrease them. However, these mutations need not have a large effect on the protein’s original activity. Indeed, the results of directed evolution experiments indicate that, in clear contrast to the dramatic shifts observed with the promiscuous substrates, the native activities that take place in the very same active site show *comparatively* small changes. This conservation of the native function was observed despite the fact that only one selection criterion was applied — an increase in one of the promiscuous activities of these enzymes [33^{••}] (Table 1).

The above observation has important implications regarding our understanding of the early steps in the evolution of new protein functions [37]. There is no doubt that gene duplication is a necessary step. But the most-widely accepted model, by Ohno [38], surmises that the generation of a redundant gene copy that is relieved from the burden of selection is the first step in the evolution of a new function. This assumption is driven by the notion that negative trade-offs dominate evolutionary processes at all levels [37] — it is therefore impossible to evolve a new function without compromising the original one first. There is little doubt that, ultimately, the acquisition of a highly proficient new enzyme comes at the expense of the old function. Yet the *relative rates* by which a new function is gained, and the old one is lost, matter. The model depicted in Figure 2 assumes that trade-offs can be determined and quantified, in particular with enzymatic activities. It suggests that, in those cases where the negative trade-off is weak (red line), the divergence of new function can proceed via a ‘generalist’ intermediate that exhibits broad specificity. Gene duplication may then follow this process, rather than initiate it, and lead to divergence of a new ‘specialist’.

It therefore appears that, in the case of evolving promiscuous protein functions, negative trade-offs are weaker than generally assumed. We have recently described this trend in three enzymes subjected to a selection for the increase of six different promiscuous activities [33^{••}] (Table 1). We also explored the literature and identified this pattern in other laboratory experiments aimed at

Table 1

Examples of directed evolution of promiscuous enzyme functions and the effect on the native function^a.

Enzyme	Native activity ^c	Promiscuous activity under selection ^c	Mutations in selected variants	Effect on native activity ^d	Effect on the evolved promiscuous activity ^d	Comments	Refs
1 Aspartate aminotransferase (AATase) from <i>E. coli</i>	Transamination of dicarboxylic substrates (9.1)	Transamination of tyrosine (0.055) and phenylalanine (0.012) (TATase activity)	Pro13Thr Asn69Ser Gly72Asp Arg129Gly Thr167Ala Ala293Val Asn297Ser Asn339Ser Ala381Val Asn396Asp Ala398Val	1.2-fold higher	130- and 270-fold higher, respectively	This work provides a clear example of a generalist' intermediate. The <i>in vitro</i> evolved enzyme exhibits wild-type-like AATase activity, and TATase activity that is >10% of that of wild-type TATase.	[45**]
2 Muconate lactonizing enzyme (MLE II) from <i>Pseudomonas sp.</i> P51	Cycloisomer-ization (2×10^4)	β -Elimination (<i>o</i> -succinylbenzoate synthase, OSBS activity). No detectable promiscuous activity ($<1.5 \times 10^{-3}$)	Glu323Gly	15-fold lower	>1.2 million-fold higher	The corresponding mutation when engineered in an homologous enzyme (Asp297Gly, in AEE) decreased the native function far more significantly [52*].	[30**]
3 Galactokinase (GalK) from <i>E. coli</i>	Phosphorylation of β -galactose to produce α - β -galactose-1-phosphate (860)	Phosphorylation of C5- or C6-substituted sugars (9.8 for β -fucose, non-detectable for the other substrates)	Tyr371His	1.3-fold lower	21-fold higher for β -fucose, and higher improvements for other target substrates	This variant strikingly expanded the spectrum of substrates to others that were not used in the screen. Although the Y317H mutation retains the stringent requirement for the C-4 galactose architecture, it has enhanced substrate flexibility at all other positions.	[63]
4 β -Glucuronidase (GUS) from <i>E. coli</i>	Hydrolysis of β -glucuronides (8.3×10^5)	Hydrolysis of pNP-galactoside (2.3)	Ile12Val Phe365Ser Trp529Leu Ser557Pro Ile560Val	8.3-fold lower	16-fold higher	Larger increases in the evolving promiscuous galactosidase function of <i>E. coli</i> GUS, with smaller changes of the native function, and acquisition of specificities not selected for, have been previously described [44].	[64]
5 Sini DNA-methyltransferase from bacteriophage	Methylation of the internal cytosine of the GG(A/T)CC sequence (2.9×10^5)	Relaxation of sequence specificity towards GG(N)CC (2×10^3)	Leu214Ser Tyr229His	4.5-fold lower	18.5-fold higher for the GG(G/C)CC sequence	Similar trends of specificity broadening were observed with <i>HaeIII</i> methyltransferase [47].	[65]
6 Phosphotriesterase from <i>P. diminuta</i> (PTE)	Phosphotriesterase (e.g. paraoxon, 4×10^7)	Ester hydrolysis (e.g. 2-naphthyl acetate, 480)	His254Arg Phe306Cys Pro342Ala	3-fold lower	13-fold higher	Up to 150-fold higher activity was observed with esters not selected for.	[33**] [62*]
7 Human carbonic anhydrase (hCAII)	Bicarbonate dehydration (3×10^7)	Esterase (e.g. <i>p</i> -nitrophenyl acetate, 2×10^3)	Ala65Val, Asp110Asn Thr200Ala	2-fold lower	40-fold higher	Mutations in conserved regions of the protein did not affect the highly proficient native activity despite the absence of a purifying selection for bicarbonate dehydration.	[33**] [51]

Table 1 (Continued)

Enzyme	Native activity ^c	Promiscuous activity under selection ^c	Mutations in selected variants	Effect on native activity ^d	Effect on the evolved promiscuous activity ^d	Comments	Refs
8 Mammalian serum paraoxonase (PON1)	Lipo-lactonase ^b (e.g. δ -valerolactone, 1.3×10^5 ; and γ -heptanolid, 2×10^4)	Thiolactonase (e.g. γ -butyryl thiolactone, 75)	Ile291Leu Thr332Ala	~No change ^b	80-fold higher	The selected mutations are all located on surface loops that comprise the substrate-binding pocket.	[33**]
		Esterase (e.g. 2-naphthyl octanoate, 1.5×10^3)	Phe292Val Tyr293Asp	~No change	31-fold higher		
		Esterase (e.g. 7-acetoxy coumarin, 1.2×10^5)	Phe292Ser Val346Met	~22-fold lower	62-fold higher		
		Phosphotriesterase (7-diethylphosphoro 4-cyano-7-hydroxycoumarin, 9×10^3)	Leu69Val Ser138Leu Ser193Pro Asn287Asp	2.6-fold lower	155-fold higher		
9 Deacetoxycephalosporin C synthase (DAOCS) from <i>S. clavoligerus</i>	Ring expansion of penicillin N into deacetoxycephalosporin C (2.2×10^4)	Ring expansion of penicillin G into phenylacetyl-7-aminodeacetoxycephalosporanic acid (18)	Val275Ile Ile305Met Cys155Tyr Tyr184His Val275Ile Cys281Tyr	1.1-fold higher 42-fold lower	32-fold higher 41-fold higher		[66]
10 Extended-spectrum β -lactamase CTX-M	Hydrolysis of cephalothin and cefotaxime (4×10^6 – 2×10^7)	Hydrolysis of ceftazidime (3.3×10^3)	Gln87Leu His112Tyr Thr230Ile, Ala231Val Asp240Gly Arg276His	1.4-fold higher, and 1.4-fold lower, for cephalothin and cefotaxime, respectively	24-fold higher		[67]
11 NotI from <i>N. caviarum</i>	Recognition and cleavage of GCGGCCGC DNA sequence (5×10^5 μ /mg enzyme)	Recognition and cleavage of altered 8-bp sequence (no detectable star activity)	Met91Val Glu156Gly	23-fold lower	>32-fold higher than the Glu156Gly intermediate with GCTGCCGC sequence	Although a considerable reduction in the rate of cleavage of the original sequence is reported, the cleavage specificity of M91V/E156 appears to be relaxed towards a whole set of 8 bp sequence targets, with a distinct preference for the original target.	[46]

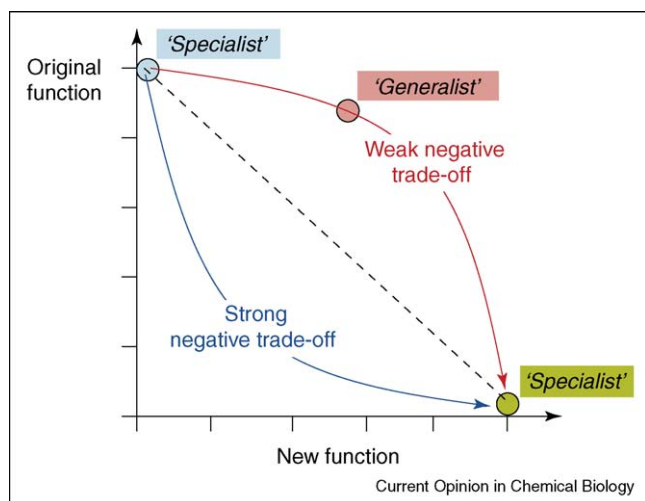
^a Shown are examples from the past few years for which kinetic parameters are available for both the promiscuous activity under selection and the original activity (for more examples see Supplementary Table 8 in [33**]). Because the above analysis aims at providing insights on the evolution of new enzyme functions in nature, the examples selected involve selection for only one parameter — increase in a promiscuous activity, and make use of gene libraries prepared by mutagenesis in a completely random manner (point mutations or shuffling) throughout the genes. Various other examples exist in which selected amino acid residues were mutated (rationale design) or randomized (semi-rational design) — these show a similar trend although decreases in the native function tend to be higher than observed with random mutagenesis (for examples, see [52*,54*,55,56*]).

^b Since the publication of [33**], 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 [59,68,69]. In the original article [33**], data for trade-offs with the native activity were presented with both the aromatic lactone dihydrocoumarin, and aliphatic lactones. However, our most recent work indicates that dihydrocoumarin is not binding PON1's active site in the same mode as aliphatic lactones [21,59,68,69]. Thus, the trade-offs presented here are the average values of two aliphatic lactones (δ -valerolactone and γ -heptanolide).

^c (k_{cat}/K_M of wild-type, in $M^{-1} s^{-1}$).

^d ($k_{cat}/K_M^{variant}/k_{cat}/K_M^{wt}$).

Figure 2



Possible routes to new function acquisition. Under selection, a weak, promiscuous activity of a protein with an existing function (blue circle) gradually evolves. By the end of this process, which typically requires many generations of mutation and selection, the 'new' function has traded off with the original one (green circle). However, the dynamics of this 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 (dashed line), or follow either concave or convex routes. Results of numerous directed evolution experiments indicate that the convex route ('weak negative trade-offs') is the more likely one — large increases in the promiscuous function under selection ('new function') are accompanied by significantly smaller decreases in the original function (Table 1). By virtue of gaining a 'new' function without losing the original one (and often gaining other new functions not selected for), the intermediates of these routes are 'generalists', and their evolution can therefore proceed *prior* to gene duplication. By contrast, the concave route implies that gene duplication is a necessary prerequisite, because acquisition of even low levels of the 'new' function is accompanied by large losses of the original one. This route is observed in the laboratory, in particular under a dual selection, for gain of a new function and loss of the old one.

increasing promiscuous enzymatic and binding activities of various proteins (see Supplementary Table 8 in [33]). Averaging 18 cases in which data were provided for the effect of the selected mutations on both the evolving promiscuous activity and the original function (of both binding and enzymatic functions) indicated that 1–4 mutations increased the promiscuous activity that was under selection by >1000-fold, on average, whereas the original activity of these proteins decreased by 3.2-fold. More recent examples are listed in Table 1 (for enzymatic functions only). They show a similar trend: 1–11 mutations increased the promiscuous activity under selection by 10–10⁶-fold, whereas the original activity of these proteins decreased, by 0.8–42 fold. In the majority of cases, the ratio of increase in the selected promiscuous function to decrease in the original one is ≥ 10 , and ratios ≥ 100 are observed in $\sim 25\%$ of cases. In only a few cases, changes in the evolving and original activities are comparable, yet these seem to be non-exclusive — namely,

other variants from the same selection show a weak trade-off (e.g., Table 1, entry 9), or involve the generation of broad-specificity generalist (entry 11).

Although out of the scope of this review, it is notable that similar trends can be clearly seen in various receptors [39], where the acquisition of specificity for a new effector exploits the promiscuity of existing receptors [40]. New specificities can then be acquired by natural, or laboratory, rounds of mutagenesis and selection, often with weak negative trade-offs with respect to the original effector [41,42]. The very same phenomenon has been observed with the lac transcriptional promoter — most mutations seem to add new regulation regimes without diminishing the original one [43].

There exists, therefore, evidence in favor of the notion that the divergence of new functions through the promiscuous functions of existing proteins can follow, in many if not most cases, the convex route of 'weak negative trade-off' (Figure 2). Results of several directed evolution experiments also convincingly demonstrate that the concave route, the 'strong negative trade-off', is also applicable, in particular when a *dual* selection pressure applies — namely, when a parallel selection for an increase of a promiscuous activity and decrease in the native activity is applied [41]. Thus, in the face of selection for specialization, proteins could be rerouted from their tendency to evolve via 'generalists' [44,45,46,47], and directly yield new 'specialists', sometimes with a surprisingly abrupt shift in selectivity [31]. Indeed, in certain cases, the toll of a 'generalist' on fitness might be too high, and the driving force for specialization might be stronger than under *in vitro* selection [36,48].

The different effect of mutations on the native *versus* the promiscuous functions is particularly striking in view of the fact that these mutations are usually found at the wall and perimeter of active sites. 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 these residues lies in the fact that they are not part of the protein's scaffold, or of the catalytic machinery of the enzyme. These mutated residues are typically located on surface loops that are part of the substrate-binding pocket, and exhibit high conformational flexibility [17,19,20]. There could also be fundamental differences between the mode of binding of the native substrate — that is, typically mediated by several independent, enthalpy-driven interactions such as hydrogen bonds — versus the promiscuous substrates where hydrophobic and other entropy-driven interactions play a chief role [49,50].

There are cases, however, in which mutations narrow substrate specificity, or show larger negative trade-offs with respect to the original function. The magnitude of

trade-off may obviously vary depending on the structural differences such as size and charge between the original and promiscuous substrates [51,52^{*}]. The location of these mutations may affect the trade-off as well: it seems that mutations of residues that directly contact the substrate usually increase the magnitude of negative trade-offs [52^{*},53]. In addition, mutations incorporated through rational design show stronger trade-offs relative to mutations selected in an homologous enzyme from random repertoires (e.g., Table 1; entry 2; [52^{*},54^{*},55,56^{*}]). Likewise, when selection was applied simultaneously for more than one promiscuous activity, a significant loss of native activity was observed [57]. Future research may reveal the structure and thermodynamic factors that govern the magnitude of trade-offs.

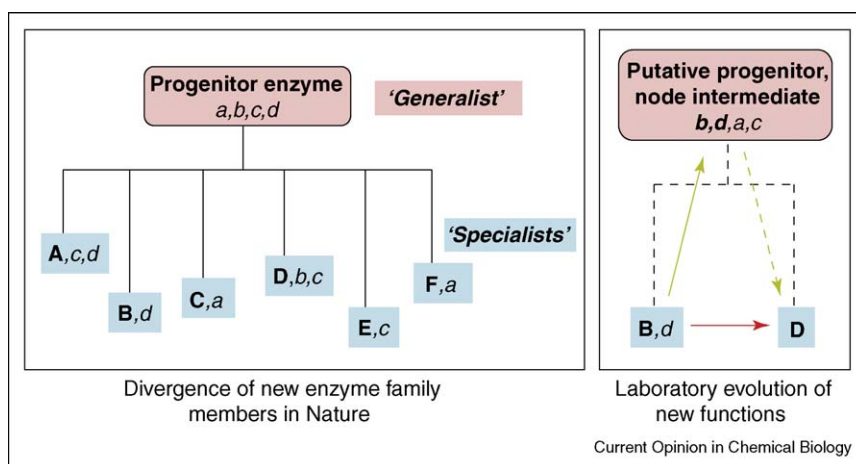
Promiscuity and the divergence of enzyme families

A growing body of evidence has been accumulating indicating that the natural divergence of enzyme families and superfamilies had proceeded through multi-specific, or highly promiscuous, progenitors, or ‘node intermediates’. Specifically, the identification of promiscuous activities, or cross-reactivities, between different members of the same enzyme family or superfamily, and the directed evolution of these activities, provide important hints regarding evolutionary and mechanistic relationships within enzyme families (Figure 3). The different

observations are summarized below, and recent examples are listed in Table 2.

1. Promiscuous activities are often shared between more than one family member (Table 2; entries 2,3,5). At the same time, promiscuous functions often appear in one family member but not the others (Table 2, entries 1,5,7). Indeed, the consistency of one function (e.g., the lactonase function shared by all PON family members), and the haphazardness of others (e.g., the paraoxonase and aryl esterase observed only in some family members; Table 2, entry 1) [58,59] may assist the distinction of the native *versus* the promiscuous functions.
2. The magnitude of promiscuous activities varies over several orders of magnitude, both in absolute terms, and relative to the native activity. In many cases, the promiscuous activities are relatively high and fall within just an order-of-magnitude or two of the native function (e.g., Table 2, entries 1, 2 (CaaD), 3(YajF), 5(PTE), 6(KGPDS)). Such activities are likely to provide a distinct selective advantage, certainly under high expression levels [60,61^{**}].
3. The primary, or native, function of one family member is often identified as a promiscuous activity in other family members (Table 2, entries 2,6,7).
4. Evolution in the laboratory of one promiscuous activity often leads, indirectly, to the appearance of other

Figure 3



Experimental evidence favours the model of divergence of a ‘generalist’ progenitor enzyme to a family of ‘specialist’ enzymes. *Left panel:* Jensen’s hypothesis [5] surmises that, in nature, an ancestor protein displaying a low level of a range of activities (denoted as *a, b, c, d*) had 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.*). Today’s ‘specialists’ may still retain some of the functions of the common ancestor (denoted in lower case), 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 2). *Right panel:* 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*; red 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 (e.g. *d*), the resulting variants usually show significant increases in the target activity, and a smaller decrease in the original one (green arrow) thus yielding, in effect, a ‘generalist’ intermediate exhibiting both *d* and *b* at relatively high levels (the ‘weak negative trade-off’ line in Figure 2). Such intermediates are often observed in the laboratory (Table 1); some even gain other activities, never selected for (denoted *a, c*), and may therefore resemble the progenitor of this enzyme family, or node intermediates along past routes of its divergence.

Table 2

Examples for promiscuous activities within enzyme families and superfamilies.

Family/superfamily	Enzymes	Enzyme: native activity ^a	Enzyme: promiscuous activity ^a	Refs
1 Mammalian paraoxonases	PON1 (serum paraoxonase) PON2 PON3	PON1, PON2, PON3: lipophilic lactonase – hydrolysis of aliphatic 5-, 6-membered ring lactones with lipophilic side-chains (e.g. PON1 activity with γ -dodecanoic lactone, 1.2×10^5)	PON1: aryl esterase (e.g. phenyl acetate, $\sim 6 \times 10^5$) Phosphotriesterase (e.g. paraoxon, 6×10^3) PON2: barely detectable aryl esterase; no phosphotriesterase PON3: low aryl esterase; barely detectable phosphotriesterase	[58,59,69]
2 Tautomerase superfamily	Malonate semialdehyde decarboxylase 4-Oxalocrotonate tautomerase YwhB tautomerase (4-OT analogue) <i>trans</i> -3-Chloroacrylic acid dehalogenase	MSAD: decarboxylation of malonate semialdehyde (2.2×10^7) 4-OT: isomerization of 2-oxo-4E-hexenedioate to 2-oxo-3E-hexenedioate through 2-hydroxy-2,4E-hexadienedioate (2.0×10^7 , 2nd reaction) YwhB: analogous to 4-OT (2.8×10^4) CaaD: hydrolytic dehalogenation (hydratase) of 3E-haloacrylates (1.2×10^5 , 3E-chloroacrylate)	MSAD: hydration of 2-oxo-3-pentynoate (6×10^2) 4-OT: CaaD activity, hydration of 3E-chloroacrylate (2.6×10^{-2}) YwhB: CaaD activity, hydration of 3E-chloroacrylate (4.4×10^{-2}) CaaD: hydration of 2-oxo-3-pentynoate (6.4×10^3)	[27*,28]
3 ROK family (repressor, open reading frame, kinase)	NanK YajF YcfX AlsK	NanK: N-acetyl-D-mannosamine kinase (2.7×10^5) YajF: fructose kinase (1.1×10^4) YcfX: unknown AlsK: allose kinase, (6.5×10^4)	NanK: glucose kinase (5.1×10^2) YajF: glucose kinase (2×10^2) YcfX: glucose kinase (2.4×10^3) AlsK: glucose kinase (15)	[60,61**]
4 Enolase superfamily MLE (muconate lactonizing enzyme) subgroup	<i>o</i> -Succinylbenzoate synthase	OSBS: dehydration of SHCHC (2-succinyl-6R-hydroxy-2,4-cyclohexadiene-1R-carboxylate, 2.5×10^5)	ODBS: <i>N</i> -acylaminoacid racemase reaction with <i>N</i> -acetyl methionine isomers ($4.9\text{--}5.9 \times 10^2$)	[70,71]
5 Amidohydrolase superfamily	Phosphotriesterase Phosphotriesterase homology protein Dihydroorotase	PTE: paraoxon hydrolysis (4×10^7) PHP: unknown DHO: dihydroorotic acid hydrolysis (1.2×10^6)	PTE: esterase (e.g. 2-naphthyl acetate, 500); lactonase (e.g. dihydrocoumarin, 6.5×10^5) PHP: esterase (e.g. 2-naphthyl acetate, 70) DHO: phosphotriesterase (e.g. paraoxon, 2.8)	[62*]
6 Orotidine 5' monophosphate decarboxylase suprafamily	3' Keto L-gluconate 6-phosphate decarboxylase D- <i>arabino</i> -hex-3-ulose 6-phosphate synthase	KGPDS: decarboxylation of 3' keto L-gluconate 6-phosphate (7.7×10^4) HPS: aldol condensation of D-ribulose 5-phosphate and formaldehyde (1.6×10^4)	HPS: KGPDS activity (2.3×10^3) KGPDS: HPS activity (8.2×10^{-2})	[72]
7 Guanidino-modifying enzyme superfamily, hydrolase branch	Arginine deiminase Agmatine deiminase <i>N</i> ^w , <i>N</i> ^w -dimethyl-arginine dimethyl-aminohydrolase	PaADI: agrinine hydrolysis (4.5×10^4) PaAgDI: agmatine hydrolysis (7×10^3) PaDDAH: <i>N</i> ^w , <i>N</i> ^w -dimethylarginine hydrolysis (1.8×10^3)	PaADI: <i>N</i> ^w , <i>N</i> ^w -dimethylarginine hydrolysis (1.8×10^3) None PaDDAH: arginine hydrolysis (1.8)	[16*]

^a (k_{cat}/K_M in $M^{-1} s^{-1}$).

promiscuous activities (e.g., Table 1, entries 3,8,11), thus yielding 'generalist' intermediates [44]. Some of the latter might appear in other family members, as either their native or promiscuous function [45**,53,62*].

Conclusions

The above observations support the hypothesis of evolutionary progenitors and intermediates being of broad-specificity or high promiscuity [5], and that a frequent, but not certainly exclusive, evolutionary route leads from

a 'specialist' to a 'generalist' and, in turn, to a new 'specialist' (Figures 2 and 3). They also provide important hints regarding the mechanism of catalysis and evolutionary relationships within enzyme families. It thus seems that 'tinkering' is indeed a most common evolutionary route [4]. When a need for new enzymatic functions arises, nature recruits existing enzymes that promiscuously bind the new substrate, or catalyze the new reaction, and then tinkers with their active sites to fit the new substrate and reaction. In doing so, new family members have diverged from existing ones, thus yielding the large and functionally diverse enzyme families and superfamilies we see today.

Update

A recent review paper by *Glasner et al.* [53] discusses the relevance of lessons learned from protein evolution, to protein engineering (see also [32,36]). Issues such as promiscuity, active site plasticity, and possible mechanisms of divergent evolution within enzyme families and superfamilies are presented. Also presented is a critical discussion of trade-offs between the new and original activities in evolving enzymes.

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