Catalytic promiscuity and the evolution of new enzymatic activities

Patrick J O'Brien and Daniel Herschlag

Several contemporary enzymes catalyze alternative reactions distinct from their normal biological reactions. In some cases the alternative reaction is similar to a reaction that is efficiently catalyzed by an evolutionary related enzyme. Alternative activities could have played an important role in the diversification of enzymes by providing a duplicated gene a head start towards being captured by adaptive evolution.

Address: Department of Biochemistry, B400 Beckman Center, Stanford, CA 94305-5307, USA.

Correspondence: Daniel Herschlag
E-mail: herschla@cmgm.stanford.edu

Chemistry & Biology April 1999, **6**:R91–R105
http://biomednet.com/elecref/1074552100 6R0091

 ISSN 1074-5521

Introduction

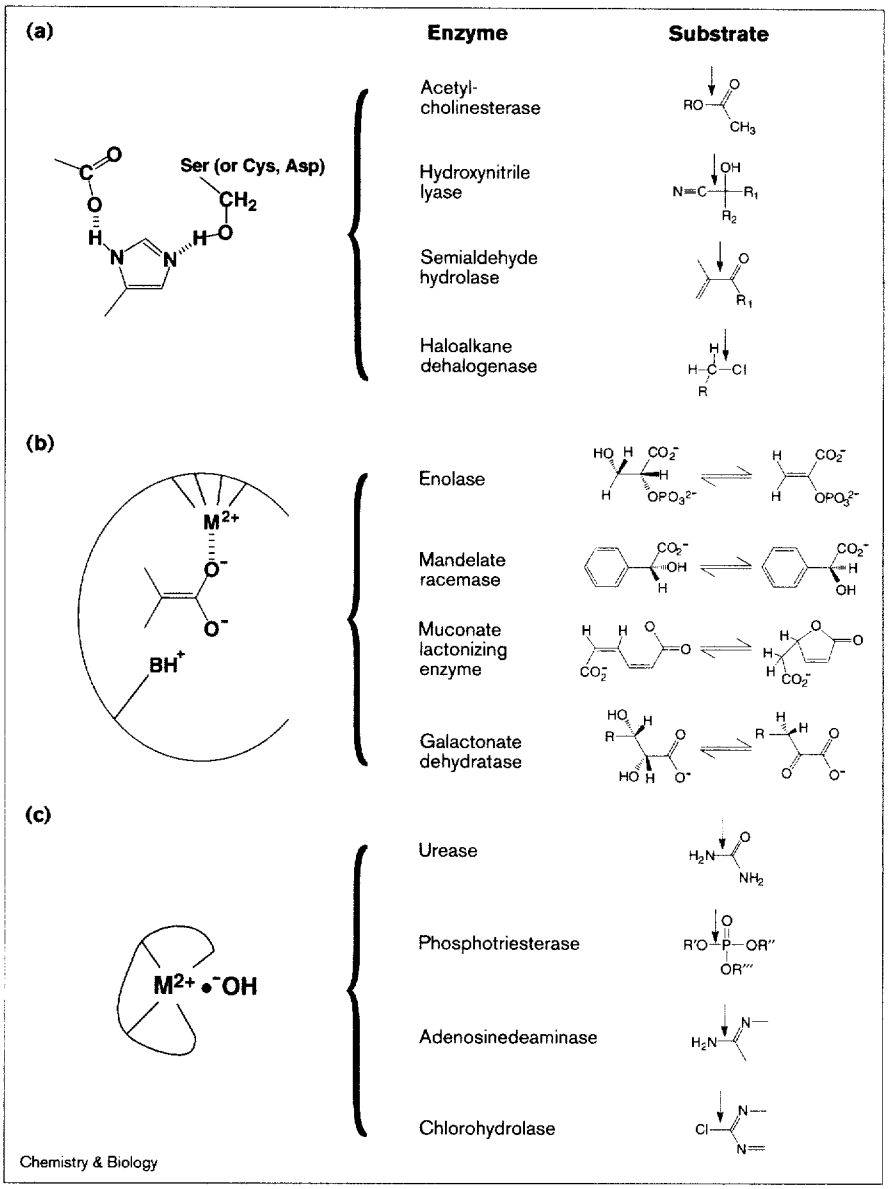
It is widely accepted that many enzymes evolved from pre-existing enzymes via gene duplication [1–3]. The results of many elegant studies suggest that nature has used common binding sites and common mechanistic features to catalyze the analogous reactions with different substrates, and, further, has used common mechanistic features to catalyze different reactions [4–13]. Numerous enzyme superfamilies have been identified by sequence and structural homologies. These superfamilies, which share structural and functional features but include enzymes that can catalyze a number of different reactions, provide strong support for the central role of divergent evolution in biology (Figure 1; e.g., [5,6,8–31]).

Enzymes with the α/β-hydrolase fold provide an example of a superfamily with conserved mechanistic features that catalyze an array of different reactions [10]. These enzymes have the same α/β-sheet architecture and superimposable catalytic triad of an aspartate or glutamate, a histidine, and a nucleophilic residue that is a serine, cysteine or aspartate (Figure 1a). The conserved histidine, positioned by the aspartate or glutamate, activates the nucleophilic residue for attack, leading to formation of an acyl enzyme intermediate. Different reactions are catalyzed, however (Figure 1a); for example, acetylcholinesterase hydrolyzes the ester bond of acetylcholine, hydroxynitrile lyase breaks a carbon–carbon bond to release hydrogen cyanide from a cyanohydrin [32], semialdehyde dehalogenase cleaves a carbon–carbon bond adjacent to a carbonyl [33], and haloalkane dehalogenase hydrolyzes carbon–halogen bonds [10].

The enzymes of the enolase superfamily also catalyze different overall reactions, but each has a similar α/β-barrel fold and catalyzes the formation of a carbanion intermediate via abstraction of a proton adjacent to a carboxylate, as discussed extensively by Gerlt, Babbit and colleagues [7,8,12]. Superposition of these enzyme structures aligns active-site residues, including a histidine and/or lysine that act via general acid/base catalysis and a divalent metal ion that stabilizes the development of negative charge (Figure 1b; [6,7,27,31,34–38]).

Conservation of structural and catalytic features in the α/β-hydrolase-fold superfamily, in the enolase superfamily and in other superfamilies strongly suggests that enzymes in each superfamily arose via divergent evolution from a common ancestor to accept different substrates and to catalyze different reactions [8,12]. Despite the high degree of structural homology, enzymes within superfamilies often share as little as 10% sequence identity ([32] and references therein; see also [7,22]). This suggests that once an

Figure 1

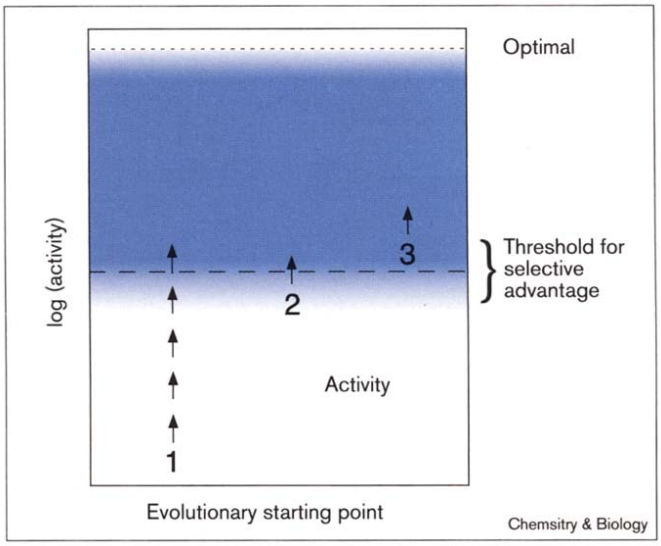


Representative enzyme superfamilies. Conserved active-site features of each superfamily are shown along with several enzymes and their substrates, illustrating the diversity of reactions catalyzed within the superfamilies. The overall reaction is shown or an arrow denotes the bond that is broken. **(a)** The α/β-hydrolase-fold superfamily [10,32,33]. **(b)** The enolase superfamily, shown with a bound enolate intermediate [6–8,27,31,34]. **(c)** The α/β-barrel-hydrolase superfamily [9].

enzyme adopts a different function (i.e. catalyzes a different chemical transformation) sequence diverges rapidly [39]. The ability of residues that do not contact the substrates to influence substrate specificity and catalytic efficiency is expected to hasten divergence [40–42], and distant evolutionary relationships are expected to be difficult to identify solely from global sequence comparisons.

Divergent evolution requires duplication to free a gene from its previous functional constraints. Random drift will cause an accumulation of mutations in duplicated genes, however, many of which will be deleterious to structure and function, thus rendering the probability of obtaining a new function extremely low, even in evolutionary terms [43]. If random drift has such low probability of generating a functional gene, how have enzymes evolved to catalyze such a remarkable diversity of reactions? Perhaps enzymes that evolved to catalyze one chemical transformation can, with some frequency, also catalyze alternative reactions at a low level. Such alternative activities might then provide the raw material for the evolution of new enzymes, as a newly duplicated gene that has an activity near the threshold level required to provide a selective advantage would have a head start towards being captured by adaptive evolution (Figure 2). Uncovering and understanding such activities could provide information about past and present evolutionary potential and pathways and could also help to guide random or directed engineering of enzymes with new activities.

Figure 2



Threshold model for evolution of a new activity. The gene product for a duplicated enzyme must provide a level of activity above a certain threshold to confer a selective advantage. The intensity of blue indicates the strength of selective pressure to improve the level of activity. The parenthesis shows that the activity level required for a selective advantage from the duplicated gene product is not discrete, as very low activities can give a slight selective advantage, whereas higher activities can give a large selective advantage. Three potential starting points, reflecting the activity of the enzyme encoded by a newly duplicated gene, are designated. A gene product with very low activity towards a desired reaction (1) requires many advantageous mutations (denoted by arrows) to achieve a level of activity sufficient to confer a selective advantage, whereas the vast majority of mutations will be neutral or detrimental. In contrast, a gene product with promiscuous activity near (2) or above (3) the threshold for selective advantage will have a higher probability of its gene being fixed in the genome and optimized for the new activity. The level of the threshold depends on the genetic background and extracellular environment. It is important to recognize the probabilistic nature of the evolutionary process. The particular pathway taken is chosen at random, but weighted by its probability relative to that of other potential pathways. A promiscuous activity near to or above the threshold for selective pressure does not therefore ensure that it will be optimized for the new activity because other pathways can exist. In addition, not every promiscuous activity can be readily optimized to efficient levels. Conversely, even an enzyme lacking significant activity for a particular reaction could, in some cases, rapidly acquire a selectable level of activity, perhaps aided by large insertions and swapping of domains between proteins.

The ideas presented in this review are related to and extend the hypothesis presented by Jensen [3] for the creation of new metabolic pathways from enzymes that were capable of accepting a wide range of related substrates [44,45]. We first describe several examples of enzymes that have been demonstrated to catalyze more than one type of reaction. These activities apparently represent the fortuitous use of active-site features to catalyze an alternative reaction, and we refer to this as catalytic promiscuity. Next we describe examples of enzymes that catalyze an alternative reaction and are evolutionarily related to modern enzymes whose physiological function is to catalyze this second reaction. This raises the possibility that a modest level of promiscuity has indeed played a role in divergent evolution. We then review several successes in protein engineering in which pre-existing enzymes have been modified to carry out new activities. These examples show that single mutations can provide substantial contributions towards the optimization of a new activity. Finally, we discuss the possible role of catalytic promiscuity in the evolutionary divergence between enzyme superfamilies.

Catalytic promiscuity of enzymes

It has long been recognized that most enzymes accept some alternative substrates, usually substrates that are very similar to the normal substrates [3]. Here we introduce another level of catalytic promiscuity: enzymes with an ability to catalyze multiple chemical transformations that are normally classified as different types of reactions (e.g., different bonds are broken). The examples in Table 1 indicate that some active sites can catalyze seemingly disparate reactions. We further suggest the possibility that many enzymes are able to provide a low level of activity in alternative reactions.

A simple and common type of catalytic promiscuity is exemplified by chymotrypsin, which catalyzes the hydrolysis of many different types of compounds, including amides, esters, thiol esters, acid chlorides and anhydrides [46]. Although bonds to different atoms are broken in each case, all of these substrates are thought to react via similar tetrahedral transition states or intermediates, with attack by a serine nucleophile at a carbonyl carbon in the first step of the reaction (Figure 3a shows the amide reaction). Chymotrypsin also catalyzes attack on a tetrahedral phosphoryl group, however, a reaction that proceeds via a trigonal bipyramidal species (Figure 3b). This alternative reaction, which results in covalent modification of the enzyme, involves attack on a different atom, with different geometry, and involves cleavage of a different type of bond. These adducts and reactions have been extensively characterized in several cases. The activated serine nucleophile is used in both reactions, and the oxyanion hole may be able to stabilize the buildup of charge in the transition states for both acyl transfer and phosphoryl transfer (e.g., see [47,48] and references therein). Chymotrypsin therefore exhibits catalytic promiscuity by catalyzing both amidase and phosphotriesterase reactions at its active site.

Bovine carbonic anhydrase II has been reported to have phosphotriesterase activity, in addition to its carbon esterase activity and its physiological CO_2 hydratase activity ([49]; for a recent review of carbonic anhydrase, see [50]). The Zn^{2+}-coordinated hydroxide ion that is the nucleophile for attack on a carbon ester or carbon dioxide via a tetrahedral transition state is also able to attack a phosphotriester, a reaction proceeding via a pentavalent transition state.

Table 1

Examples of catalytic promiscuity.

Enzyme	Primary activity	Promiscuous activity	Catalytic proficiency[a]
L-Aspariginase[b]	Amidohydrolase	Nitrilase	–
A-Esterase	Esterase	Phosphotriesterase	4×10^{10} [c]
Carbonic anhydrase II	CO_2 hydration/esterase	Phosphotriesterase	9×10^{6} [d]
Carbonic anhydrase III	CO_2 hydration/esterase	Phosphomonoesterase	8×10^{9} [e]
Chymotrypsin[f]	Amidase	Phosphotriesterase	1×10^{11} [g]
Cytosine methyltransferase	Cytosine methylation	Cytosine deamination	–[h]
Myoglobin	O_2 binding	Sulfoxidation	3×10^{8} [i]
Pepsin A	Amidase	Sulfite hydrolase	4×10^{11} [j]
Phosphotriesterase (bacterial)	Phosphotriesterase	Phosphodiesterase	3×10^{15} [k]
Phytase	Phosphomonoesterase	Sulfoxidation	4×10^{8} [l]
Serum albumin	–	Esterase	3×10^{12} [m]
		Kemp elimination	2×10^{4} [n]
Urease	Urease	Phosphoramidate hydrolysis	7×10^{6} [p]

[a]Catalytic proficiency [110] for the promiscuous activity is defined as the ratio of the apparent second-order enzymatic rate constant by the second-order nonenzymatic rate constant ($\{k_{cat}/K_M\}/k_2$); for hydrolysis reactions $k_2 = k_{obs}/[55\ M]$. [b]β-Cyano-L-alanine is hydrolyzed to yield aspartic acid and ammonia, but no rate enhancement was reported. Another reaction catalyzed by this enzyme is the hydrolysis of 5-diazo-4-oxo-L-norvaline, which produces nitrogen gas [111]. [c]Esterase and phosphotriesterase reactions are catalyzed, but the biological function is not known. Catalytic proficiency is shown for paraoxon (diethyl *p*-nitrophenyl phosphate); calculated from $k_{cat}/K_M = 1.4 \times 10^3\ M^{-1}\ s^{-1}$ [112] and $k_2 = 3 \times 10^{-8}\ M^{-1}\ s^{-1}$, estimated from the nonenzymatic hydrolysis of diethyl 2,4-dinitrophenyl phosphate using the leaving group dependence for attack of phenol on diethyl aryl phosphates [113,114]. [d]For hydrolysis of dimethyl 2,4-dinitrophenyl phosphate [49] relative to the nonenzymatic hydrolysis of diethyl 2,4-dinitrophenyl phosphate [114]. [e]For the enzymatic hydrolysis of *p*-nitrophenyl phosphate at pH 5.4 [51], relative to the nonenzymatic hydrolysis of *p*-nitrophenyl phosphate dianion, corrected to 25°C [115]. [f]Chymotrypsin also catalyzes acylation of His57 from *N*-carbobenzoxyphenylalanine chloromethyl ketone, with ~10^6-fold rate enhancement relative to acylation of free *N*-acetyl histidine in solution [116], and from *p*-nitrobenzene sulfonate, with ~200-fold rate enhancement relative to attack of free imidazole on this compound [117]. [g]For covalent inactivation by phenacyl methyl phosphonate relative to nonenzymatic hydrolysis [48]. [h]No value for the rate enhancement was reported [118]. [i]For sulfoxidation of thioanisole, assuming saturating peroxide ($k_{cat}/K_M = 24\ M^{-1}\ s^{-1}$; [119]), relative to the nonenzymatic rate constant under similar conditions ($k_2 = 1 \times 10^{-7}\ M^{-1}\ s^{-1}$; [120]). Other oxidation reactions are catalyzed, including the oxidation of thioethers, styrenes, and iodide, and mutants with increased oxidation activities have been engineered (e.g. [119,121–123]). [j]For phenyl sulfite hydrolysis [124]. [k]For hydrolysis of ethyl *p*-nitrophenyl phosphate in the presence of 2 M dimethylamine, which stimulates the reaction [125]. The nonenzymatic hydrolysis of ethyl *p*-nitrophenyl phosphate was estimated from the hydrolysis of *bis*-*p*-nitrophenyl phosphate; $k_2 = 2 \times 10^{-13}\ M^{-1}\ s^{-1}$, corrected from 80°C to 25°C using the reported temperature dependence [126]. [l]For vanadium-dependent sulfoxidation of thioanisole with saturating vanadate, assuming saturating peroxide and subsaturating thioanisole (k_{cat}/K_M ~40 $M^{-1}\ s^{-1}$), relative to the nonenzymatic reaction ($k_2 = 1 \times 10^{-7}\ M^{-1}\ s^{-1}$) [120]. [m]For acylation of human serum albumin by *p*-nitrophenyl acetate ($3 \times 10^4\ M^{-1}\ s^{-1}$; [55]), relative to the nonenzymatic hydrolysis reaction ($k_2 = 1 \times 10^{-8}\ M^{-1}\ s^{-1}$; [127]). [n]Catalytic proficiency relative to the nonenzymatic reaction catalyzed by alkyl amines [56,57]. [p]For hydrolysis of phosphoramidate [128].

A different isozyme of carbonic anhydrase, carbonic anhydrase III, has been shown to catalyze the hydrolysis of a phosphomonoester monoanion in addition to the hydrolysis of carbon esters and the hydration of carbon dioxide [51,52]. It is possible that this phosphatase activity is physiologically important and that the enzyme is under selection for both phosphatase and carbonic anhydrase activities [53,54].

Serum albumins, although not typically classified as enzymes, illustrate the principles of catalytic promiscuity by accelerating the Kemp elimination reaction, which involves general-base-catalyzed proton abstraction, and cleavage of an ester bond, which involves nucleophilic attack and results in acylation of the albumin [55–57]. The hydrophobic pocket of albumins contains a lysine that appears to fortuitously act as a general base in the Kemp elimination and a tyrosine that acts as a nucleophile towards esters and amides [56,58].

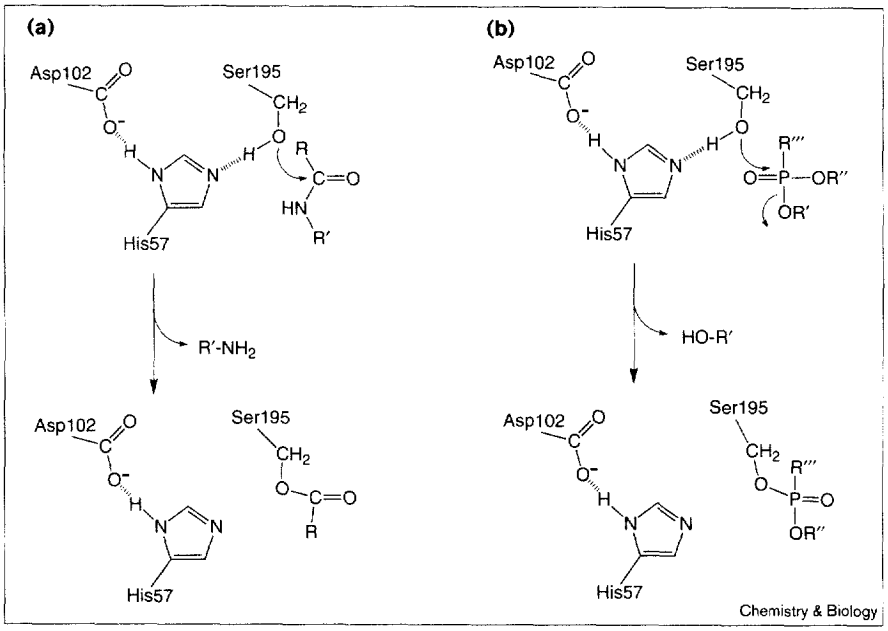
Additional examples of promiscuous enzymes that cover a broad range of physiological reactions are summarized in Table 1, and more examples are discussed below. Most generally, the widespread preferential modification of enzyme active sites by covalent modifying reagents suggests that active-site features commonly accelerate the rates of other reactions (for review, see [59]).

These observations are surprising if considered solely from the viewpoint that enzymes must have extraordinary specificity for a particular transition state relative to a ground state for the substrate, as evidenced by the large rate enhancements achieved by enzymes. Such exquisite specificity might be expected to impede the reactions of potential alternative substrates.

Nevertheless, evolutionarily related enzymes often use conserved catalytic groups and mechanistic features to catalyze

Figure 3

Catalytic promiscuity of chymotrypsin.
(a) Acylation by an amide substrate.
(b) Modification by a phosphonate diester.



different reactions (e.g., [8,12]). Furthermore, there is typically a concentration of potentially catalytic groups, such as metal ions, general acids and bases, hydrogen-bond donors and acceptors, nucleophiles, and bound cofactors, within an enzymatic active site. These functional groups could allow low levels of catalysis of alternative reactions, in which the role of these groups is the same or different as in the primary reaction.

Although the catalytic proficiencies for the alternative reactions in Table 1 are smaller than for their primary activities, substantial rate enhancements over the uncatalyzed reactions are achieved. These activities might approach or surpass the level required for a selective advantage under certain conditions. This could provide a duplicated gene that has an important head start towards being captured and optimized by adaptive evolution (Figure 2).

Examples of catalytic promiscuity in divergent evolution

Several enzymes have been found to have a low level of an alternative activity similar to the physiological activity of an evolutionarily related enzyme (Table 2). These examples, discussed below, raise the possibility that the emergence of a new enzyme might have been facilitated by catalytic promiscuity.

Alkaline phosphatase

Alkaline phosphatases share a high degree of structural similarity with arylsulfatases, despite their low sequence similarity. Superposition of the central β sheets of *Escherichia coli* alkaline phosphatase and arylsulfatase B results in a root mean square deviation of 1.9 Å for 169 Cα atoms (Figure 4a; [60]). This structural superposition aligns the nucleophilic residues, the phosphoryl/sulfuryl moieties, and divalent metal ions at the active sites, strongly suggesting that these two families of enzymes are distantly related by divergent evolution [60,61]. The recent observation that *E. coli* alkaline phosphatase has a low level of sulfatase activity raises the possibility that this activity played a role in the divergence of arylsulfatases and alkaline phosphatases ([62]; Table 2). Alkaline phosphatases and arylsulfatases have been grouped into a superfamily on the basis of conserved metal-binding ligands [21], and one member of this superfamily, autotaxin, was suggested to have both phosphatase (i.e. phosphomonoesterase) and phosphodiesterase activity [63,64]. Alkaline phosphatase and arylsulfatase A also show phosphodiesterase activity, further extending the functional inter-relationship between members of the alkaline phosphatase superfamily (Table 2).

An ancestor of alkaline phosphatase might have been duplicated at times when there were selective advantages for hydrolysis of sulfate esters or phosphate diesters. Natural selection could then have improved these promiscuous activities, ultimately resulting in the evolution of the efficient sulfatases and phosphodiesterases that are the current members of the alkaline phosphatase superfamily.

Adenylate kinase

Recently it was recognized that estrogen sulfotransferase, a sulfuryl transfer enzyme, is structurally homologous to a family of kinases that includes adenylate kinase, a phosphoryl transfer enzyme (Figure 5a; [65]), suggesting that these

Table 2

Enzymes with promiscuous catalysis of a reaction that is also catalyzed by an evolutionarily related enzyme.

Enzyme	Primary activity	Promiscuous activity	Catalytic proficiency[a]
Adenylate kinase	Phosphoryl transfer	Sulfuryl transfer	1×10^{7} [b]
Alkaline phosphatase	Phosphomonoesterase	Sulfatase	1×10^{9} [c]
		phosphodiesterase	1×10^{11} [d]
Arylsulfatase A	Sulfatase	Cyclic phosphodiesterase	1×10^{16} [e]
Aspartate aminotransferase	Aminotransferase	β-Elimination	7×10^{4} [f]
		β-Decarboxylation[g]	–
Autotaxin/ PC1[h]	Phosphomonoesterase/phosphodiesterase	Phosphomonoesterase/phosphodiesterase	–
D-glucarate dehydratase[i]	Dehydratase	Epimerase	–
OSBS[i]	*O*-succinylbenzoate synthase	*N*-Acylamino acid racemase	–
Pyruvate oxidase[k]	Pyruvate oxidase	Acetohydroxy acid synthase	–
Threonine synthase[l]	γ-Elimination of phosphate	Dehydratase	–
V-CPO[m]	Chloroperoxidase	Phosphomonoesterase	1×10^{13} [n]

[a]For the promiscuous activity, as defined in Table 1. [b]Catalytic proficiency calculated relative to the rate of nonenzymatic hydrolysis. Nucleotide diphosphate kinase and pyruvate kinase also catalyze sulfuryl transfer from adenosine 5′diphosphosulfate [66,129]. [c]For the hydrolysis of *p*-nitrophenyl sulfate [62]. [d]For hydrolysis of bis-*p*-nitrophenyl phosphate by *E. coli* alkaline phosphatase (P.J.O. and D.H., unpublished observations). Alkaline phosphatases from other organisms have also been reported to have phosphodiesterase activity [130,131]. [e]For hydrolysis of adenosine 3′,5′-monophosphate (cAMP) at 37°C, pH 4.3 [132], relative to the estimated nonenzymatic hydrolysis at 50°C, pH 7.0 [133]. [f]For β-elimination of sulfate from L-serine *O*-sulfate relative to a nonenzymatic model system. β-Elimination of chloride from β-chloro-L-alanine is also catalyzed [134,135]. [g]Wild-type catalyzes a low level of β-decarboxylation, but mutants with increased activity have also been identified (see Table 3; [79,80]). [h]No reported value for the rate enhancements. The physiological relevance of these reactions are not known [63,64]. There are also a number of examples of phosphodiesterases with phosphomonoesterase activity [136–139], although this alternative activity could arise from the protonated phosphomonoester monoanionic species acting as a mimic of a phosphodiester. [i]No rate enhancement for the intraconversion of D-glucarate and L-idarate has been reported, but this reversible side reaction, which has no known physiological consequence, is nearly as efficient as the dehydration reaction [36,140]. [j]*o*-Succinylbenzoate synthase (OSBS) from *Amycolaptosis* was identified and cloned for its *N*-acylamino acid racemase activity [141], however, it has recently been shown to be a proficient *o*-succinylbenzoate hydrolase (D. Palmer, J. Garrett, V. Sharma, R. Meganathan, P. Babbit and J. Gerlt, personal communication). [k]Pyruvate oxidase, which has sequence homology to a family of acetohydroxy acid synthases, has been shown to also have a low level of acetohydroxy acid synthase activity, producing α-acetolactate from pyruvate; no rate enhancement has been reported [142]. [l]No value has been reported for the rate enhancement of L-serine and L-threonine dehydration and deamination (see Figure 7a; [75, 77]). [m]Phytase, an acid phosphatase with a different fold than the vanadium-dependent chloroperoxidase superfamily, has been suggested to have the converse promiscuous activity, catalyzing a low level of a different vanadate-dependent peroxidation reaction, sulfoxidation (Table 1;[120]). [n]Vanadium-dependent chloroperoxidase (V-CPO) catalyzed hydrolysis of *p*-nitrophenyl phosphate [67]; catalytic proficiency is relative to the nonenzymatic hydrolysis of *p*-nitrophenyl phosphate dianion, corrected to 25°C [115].

enzymes evolved from a common ancestor [65]. The biological role of adenylate kinase is to transfer a phosphoryl group from adenosine 5′-triphosphate to adenosine 5′-monophosphate, but adenylate kinase also has a modest ability to transfer a sulfuryl group to adenosine 5′-monophosphate (Table 2; [66]). Estrogen sulfotransferase catalyzes a reaction analogous to this promiscuous activity of adenylate kinase—the transfer of a sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate to estrogen (Figure 5b). Both enzymes have a binding pocket for an adenine nucleotide and several of the residues responsible for binding are conserved [65]. These results suggest an evolutionary diversification of enzymatic function facilitated by catalytic promiscuity, as outlined above for the alkaline phosphatase superfamily.

Vanadium-dependent chloroperoxidase

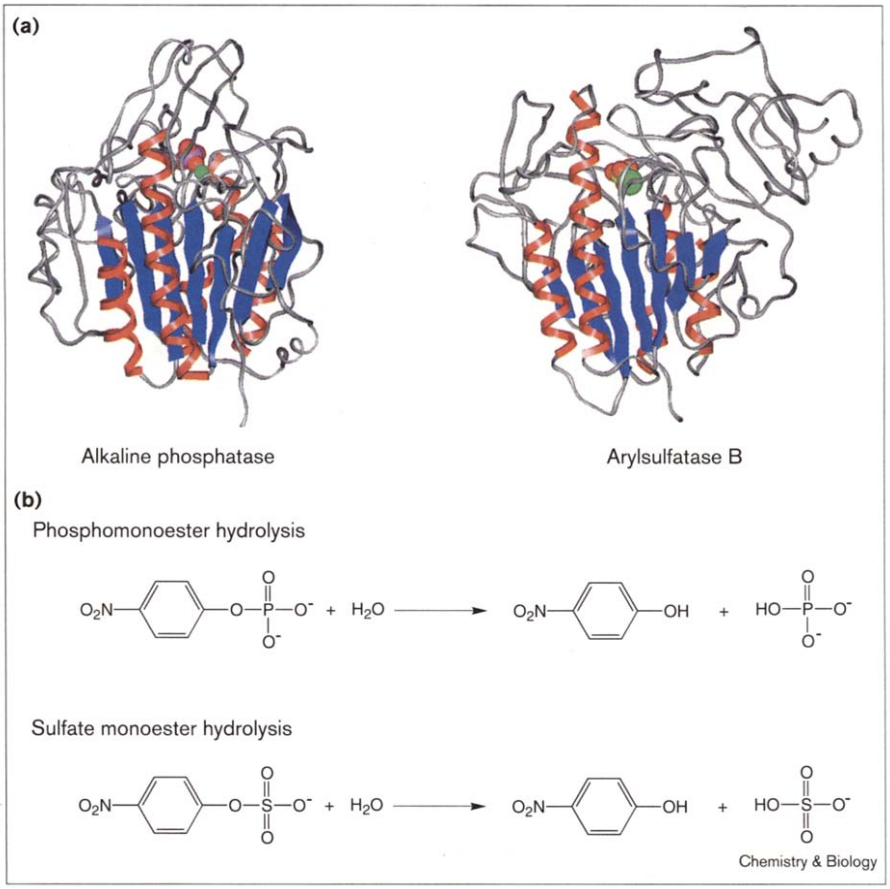
The vanadium-dependent chloroperoxidase from *Curvularia inaequalis* has sequence homology to the PAP2 family of acid phosphatases, including conservation of active-site residues [67–69]. This chloroperoxidase has been shown to also have phosphatase activity when vanadate is not present (Table 2; [67]). Although these activities constitute very different overall reactions, they involve enzyme-bound species that are structurally related. Vanadate, unlike phosphate, readily forms stable pentacoordinate species and can therefore act as a transition-state analog for phosphoryl transfer (Figure 6; [70,71]). Indeed, vanadate binds to the modern-day chloroperoxidase as a pentavalent species, with trigonal bipyramidal geometry, and this bound vanadate participates directly in the haloperoxidase reaction ([72,73]; for a recent review, see [74]). Apparently, nature was first to discover and utilize a transition-state analog.

Threonine synthase

There is considerable evidence that enzymes within the same metabolic pathway and within parallel metabolic pathways are often related to one another by divergent evolution [3,5,27,44]. For example, threonine synthase and threonine dehydratase catalyze consecutive steps in threonine metabolism (Figure 7a). Threonine synthase from

Figure 4

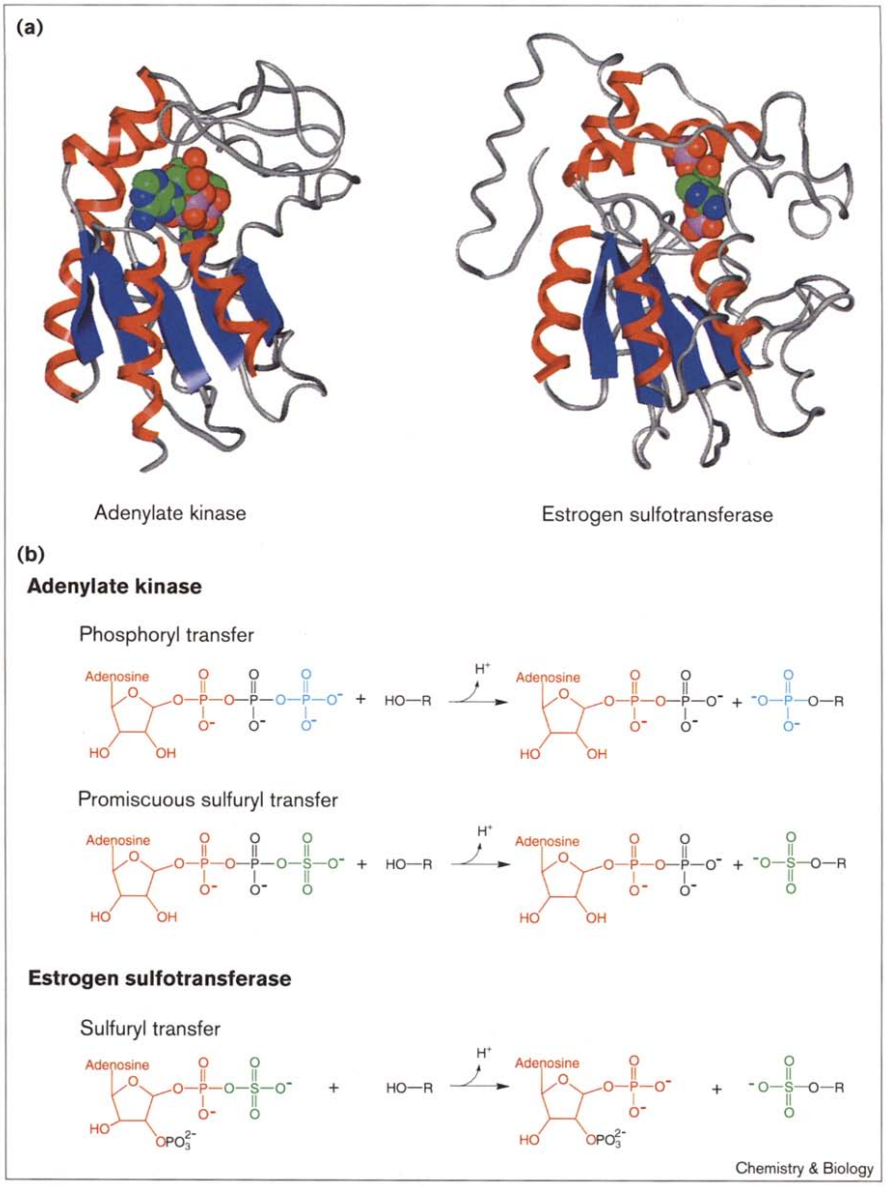
Evolutionary relationship between alkaline phosphatases and arylsulfatases [60,61]. **(a)** Structures of *E. coli* alkaline phosphatase ([143]; 1ALK) and human arylsulfatase B ([61]; 1FSU) were rendered with Insight. Structurally homologous α helices (red) and β strands (blue) are shown and the C_α backbone is traced in gray. The active site Zn_{II}^{2+} and bound phosphate group are shown for alkaline phosphatase and the active site Ca^{2+} and sulfuryl group are shown for arylsulfatase B [60,61]. Only one monomer of the alkaline phosphatase homodimer is shown. The smaller, carboxy-terminal domain of arylsulfatase B (top right) has no homology to alkaline phosphatase. **(b)** Reactions catalyzed by alkaline phosphatase.



Bacillus subtilis has both serine and threonine dehydratase activities in addition to its physiological threonine synthase activity [75]. This led Skarstedt and Greer [75] to propose an evolutionary relationship between threonine synthase and threonine dehydratase. Subsequent cloning and sequencing revealed significant overall sequence identity between these two enzymes, providing strong support for the proposed evolutionary relationship [76]. This evolutionary relationship also extends to enzymes in different metabolic pathways. The β subunit of tryptophan synthase, which catalyzes a promiscuous dehydratase reaction with L-serine in addition to its physiological condensation of L-serine and indole to yield L-tryptophan, and D-serine dehydratase, which catalyzes the physiological dehydratase reaction of D-serine, both have sequence homology to threonine synthase and threonine dehydratase [44,76]. The threonine synthase reaction, involving γ-elimination of the phosphate of homoserine phosphate, and the dehydratase reactions, involving β-elimination of ammonia, branch from a common Schiff base intermediate with a bound pyridoxal 5′-phosphate cofactor [44,77].

Analysis of sequence conservation and structural homology between these and other pyridoxal-phosphate-dependent enzymes has identified larger superfamilies of related enzymes that have apparently diverged to catalyze different types of reactions, such as transamination, racemization and α-decarboxylation, in addition to the γ-elimination and β-elimination reactions discussed above (for reviews, see [14,45,78]). Many pyridoxal-phosphate-dependent enzymes catalyze side reactions that correspond to the main reaction of other pyridoxal-phosphate-dependent enzymes [14]. Aspartate aminotransferase provides a particularly well-studied example of a promiscuous pyridoxal-phosphate-dependent enzyme. Normally it transfers the amino group of aspartate or glutamate to 2-oxoglutarate or oxaloacetate (Figure 7b), but aspartate aminotransferase also has low levels of α-carbon racemization, β-decarboxylation and β-elimination activities (e.g., [79,80]). As shown in Figure 7b, these different reaction pathways branch after formation of a common quinonoid intermediate [78,80]. Furthermore, extensive mutagenesis has shown that substrate specificity and reaction specificity can be altered by a single mutation in aspartate aminotransferase (e.g., [42,79–81]; see also [82–87]). These observations suggest that the catalytic promiscuity of pyridoxal-phosphate-dependent enzymes could have facilitated the emergence of the current diversity of these enzymes [45].

Figure 5



Evolutionary relationship between adenylate kinase and estrogen sulfotransferase [65]. **(a)** Structures of adenylate kinase ([144]; 1ZIN) and estrogen sulfotransferase ([65];1AQU) were rendered with Insight. Structurally homologous α helices (red) and β strands (blue) are shown, the Cα backbone is traced in gray, and bound ligands are in a space-filling representation. A product, 3′-phosphoadenosine-5′-phosphate, is bound to estrogen sulfotransferase and an inhibitor, P1,P5-di-(adenosine-5′-)pentaphosphate, is bound to adenylate kinase. **(b)** Comparison of the reactions catalyzed by adenylate kinase and estrogen sulfotransferase [129]. Adenylate kinase and estrogen sulfotransferase both act upon adenine nucleotide substrates (red), and the strongest structural conservation is in the nucleotide binding domain and the P-loop [65].

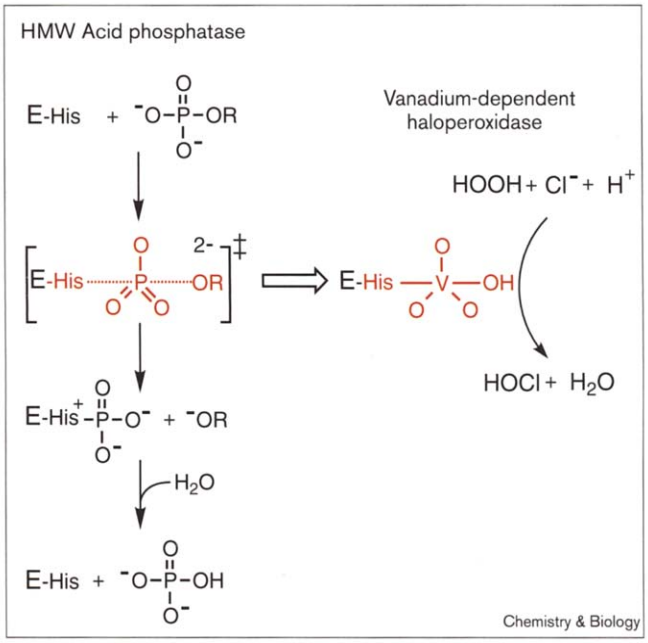
Adaption of modern enzymes to catalyze new reactions: examples from protein engineering

The potential role of catalytic promiscuity in the evolution of new enzymatic activities is underscored by recent successes in protein engineering, demonstrating that single point mutations can substantially improve the ability of enzymes to carry out new reactions. Selected examples are described below (see [88–92] for additional examples).

Steroid metabolism: reductase→dehydrogenase

Two members of the aldo-keto reductase superfamily, Δ_4-3-ketosteroid-5β-reductase (5β-reductase) and 3α-hydroxysteroid dehydrogenase (3α-HSD), catalyze consecutive steps in steroid hormone metabolism. These enzymes have a high degree of sequence identity and most of the postulated active-site residues are conserved. Each enzyme catalyzes the transfer of a hydride from NADPH and both use the same stereochemistry. 5β-Reductase catalyzes the reduction of a carbon–carbon double bond in Δ_4-3-ketosteroids, whereas 3α-HSD reduces a carbonyl of its substrate to the corresponding alcohol (Table 3; [93] and references therein). Jez and Penning [93] recently converted 5β-reductase into a 3α-HSD with a single mutation, His117→Glu. This mutation introduces an active-site glutamic acid that is conserved as a histidine in 5β-reductase and conserved as a glutamic acid in 3α-HSD. The ability of a point mutant of 5β-reductase to catalyze the 3α-HSD reaction provides strong functional evidence that these

Figure 6



Nature's transition-state analog. The PAP2 family of high molecular weight (HMW) acid phosphatases catalyze a phosphoryl transfer reaction that involves a pentavalent transition state. The structurally homologous vanadium-dependent chloroperoxidase covalently binds a pentavalent vanadate cofactor that is used in the peroxidation of halides [67–69].

two enzymes diverged from a common ancestor to catalyze consecutive reactions in a metabolic pathway [93].

Cholinesterases: esterases→phosphotriesterases

Cholinesterases have long been known to be inhibited by phosphotriesters and related compounds. These active sites greatly accelerate the rate of attack of the serine nucleophile on the phosphoryl center of phosphotriesters. Subsequent turnover of the phosphorylated intermediate is slow, however, resulting in covalent inactivation of the enzyme. Mutating Gly117 in the active site of human butyrylcholinesterase to a histidine residue greatly enhances hydrolysis of this covalent adduct, thereby allowing multiple turnover (Table 3; [94]). It has been proposed that the introduced histidine is involved in activating a water molecule for attack on the phosphorylated intermediate [95]. Although this mutant is a more efficient phosphotriesterase, it is still susceptible to an aging reaction that inactivates the enzyme when phosphorylated intermediates are formed from certain phosphotriester-like compounds, such as soman [2-(3,3-dimethylbutyl) methylphosphonofluoridate]. Glu197, when mutated to glutamine (Glu197→Gln), decreases the aging reaction and, in conjunction with the Gly117→His mutation, facilitates multiple turnover of soman [96].

Nature appears to have carried out a similar experiment on a homologous enzyme, acetylcholinesterase. Genetic analysis of insecticide resistance in blowflies identified an acetylcholinesterase allele associated with increased resistance to phosphotriester-like insecticides ([97] and references therein). One of these mutations, Gly137→Asp, is sufficient to substantially increase phosphotriesterase activity, presumably accelerating the rate of hydrolysis of the covalent intermediate in a manner analogous to the Gly117→His mutation in human butylrylcholinesterase [98].

Papain and asparagine synthetase B: amidase/amidotransferase→nitrile hydratases

Papain and asparagine synthetase B, an amidase and an amidotransferase, exhibit promiscuity by catalyzing nucleophilic attack on nitriles. Their nitrile hydratase activities have been greatly improved by addition of a general acid residue (Table 3; [99,100]). Single point mutations, Gln19→Glu in papain and Asn74→Asp in asparagine synthetase B, increase the rate of multiple turnover by more than 10^4-fold. It has been suggested that the introduced residues act as general acids that facilitate the successive additions of water required to convert the nitrile to its carboxylic acid and ammonia products [99,100].

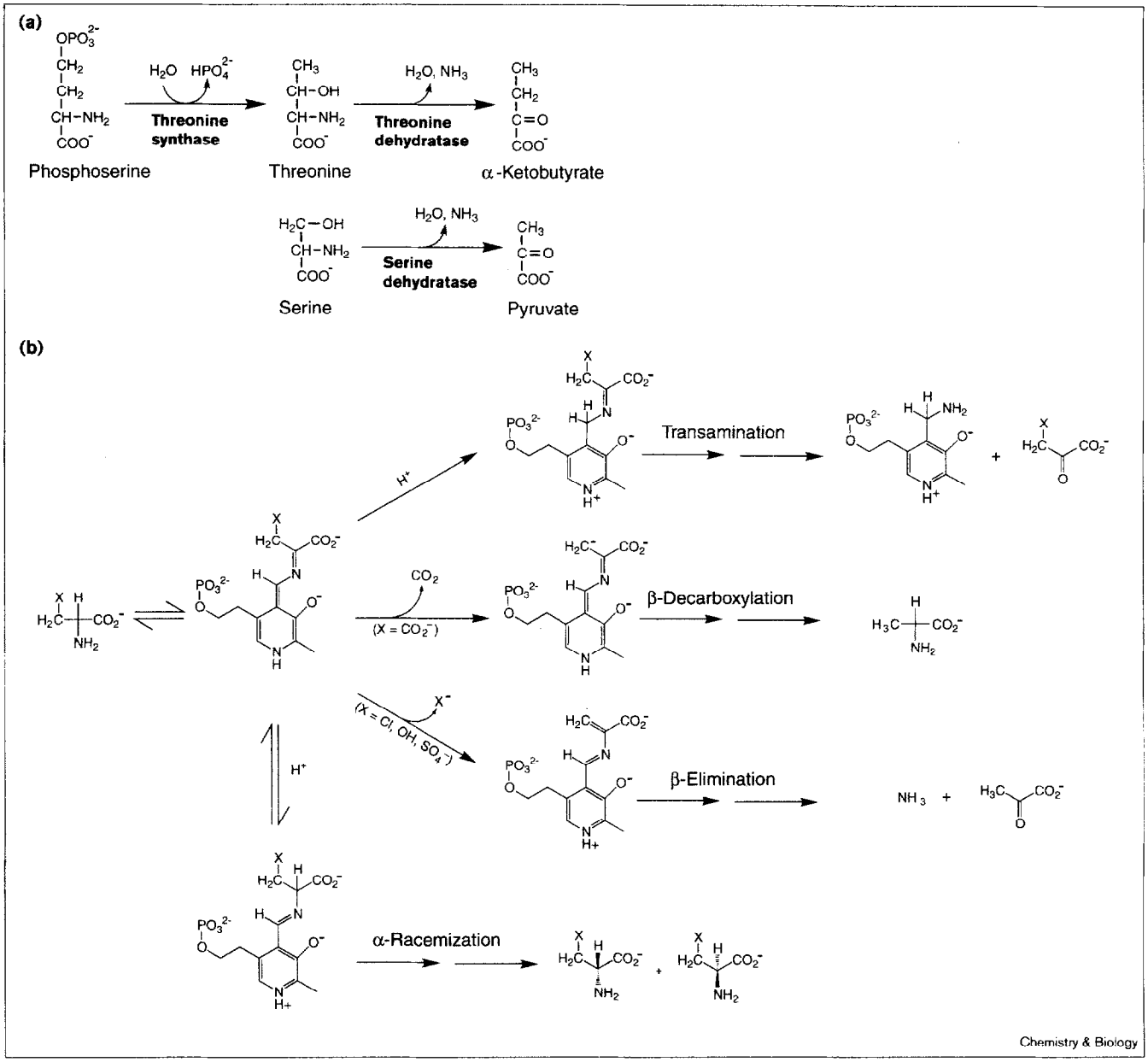
Cyclophilin: proline isomerase→proline-specific endopeptidase

Cyclophilin catalyzes proline isomerization in polypeptides. It was recently engineered to become a proline-specific endopeptidase (Table 3; [101]). A series of mutations that add functional groups to the active site, Ala91→Ser/Phe104→His/Asn106→Asp, increases k_{cat}/K_M for the endopeptidase activity by 10^6-fold relative to wild-type cyclophilin. Strikingly, the Arg91→Ser mutation alone gives an ~10^5-fold increase in endopeptidase activity, suggesting that catalytic machinery for this reaction is already present in the active site [101].

L-Ribulose-5-phosphate 4-epimerase: epimerase→aldolase

A key enzyme in the bacterial arabinose metabolic pathway is L-ribulose-5-phosphate 4-epimerase (L-Ru5P epimerase), which catalyzes the inversion of stereochemistry at C-4 of the sugars L-ribulose-5-phosphate and D-xylulose-5-phosphate, thereby connecting arabinose metabolism with the pentose phosphate pathway (Table 3). L-Ru5P epimerase has extensive sequence homology with *E. coli* L-fuculose-1-phosphate aldolase, including ligands of the active-site metal ion. These similarities suggest that this epimerase is evolutionarily related to class II aldolases [102–104]. Mutation of His94, a ligand of the epimerase active-site metal ion, to asparagine, uncovering an aldolase activity (Table 3; [104]). This mutant is able to condense dihydroxyacetone phosphate and glycoaldehyde phosphate to produce L-ribulose-5-phosphate and D-xylulose-5-phosphate, whereas the wild-type enzyme has no detectable activity for this aldol condensation. The discovery that His94→Asn L-Ru5P epimerase is an aldolase, coupled with the sequence homology to class II aldolases, suggests

Figure 7



Catalytic promiscuity of pyridoxal phosphate-dependent enzymes. **(a)** The reactions catalyzed by three evolutionarily related enzymes, threonine synthase, threonine dehydratase, and serine dehydratase, adapted from [76]. Threonine synthase has been shown to have promiscuous threonine and serine dehydratase activities [75,77]. **(b)** Aspartate aminotransferase catalyzes several reactions (adapted from [80]). Transamination, the physiological reaction, uses aspartate or glutamate as an amino donor (the half reaction is shown); β-decarboxylation of aspartate yields alanine; β-elimination from serine (dehydration), serine-O-sulfate, or β-chloroalanine yields pyruvate; and α-racemization of alanine yields D- and L-alanine [79,80,134].

divergent evolution from a common ancestor with conservation of central mechanistic features for carbon–carbon bond cleavage and formation [104].

An extensive role for catalytic promiscuity in the diversification of enzymatic function?

The results described above raise the possibility that catalytic promiscuity could have played, and could continue to play, an important role in the creation of new enzymes via divergent evolution. A low level of activity could decrease or eliminate periods of random drift, thereby greatly increasing the probability that the duplicated gene for an enzyme be fixed in the genome and optimized via Darwinian evolution to catalyze a new reaction (Figure 2; [43,105]). Several examples of enzymes with catalytic promiscuity that have more efficient evolutionary relatives

Table 3

Examples of increased promiscuous activity from protein engineering.

Enzyme	New activity	Catalytic proficiency[a]	Reference
3α-Hydroxysteroid dehydrogenase	5β-Reductase	—	[93]
Butyrylcholinesterase	Phosphotriesterase	10^{10}	[95]
Papain/asparagine synthetase B	Nitrile hydratase	10^{9}	[99,100]
Cyclophilin	Endopeptidase	10^{13}	[101]
L-Ribulose-5-phosphate 4-epimerase	Aldolase	–	[104]
Aspartate aminotransferase	Aspartate β-decarboxylase	–	[79,80]

[a]Catalytic proficiency for the new activity is defined as $[(k_{cat}/K_M)/k_2]$, in which k_2 is the second-order rate constant for nonenzymatic hydrolysis.

were described above, and point mutations have uncovered additional examples. We expect that there are many more examples, but convincing demonstration of low level alternative activities is experimentally challenging and has not been systematically investigated. Nevertheless, measurable catalytic promiscuity is not expected for all examples of enzymes with close evolutionary relationships. In some cases the alternative activity will be below the threshold for selection, but within reach of a selectable level with a limited number of mutations, and in other cases the alternative activity will have been selected against or simply eliminated over the course of evolution.

Enzyme superfamilies have been identified by common structural and mechanistic features that are shared among members, but there is also structural homology between a number of different superfamilies. For example, 18 different superfamilies share the α/β-barrel fold [106–109]. Could catalytic promiscuity have played a role in the mechanistic divergence between these structural relatives? As noted above, active sites harbor a high concentration of functional groups that can play a variety of roles in different reactions. There is no reason to expect the alternative reactions to use active-site groups in the same roles that these groups are used in the normal reactions. For example, a divalent metal ion activates a bound water for nucleophilic attack in α/β-barrel-hydrolase superfamily reactions, whereas a divalent metal ion stabilizes negative charge accumulation on the enolate intermediate in enolase superfamily reactions (Figure 1; [6,8,9,12]). As both enzymes share the overall α/β-barrel fold, it is tempting to speculate that the bound metal ion in one member of one of these superfamilies could have, along with other active-site features, fortuitously provided a low level of activity for a reaction of the other superfamily. Although we are not aware of any convincing evidence to support this particular evolutionary pathway, this example underscores that it is common to see the same functional group carrying out different roles in different enzymatic reactions. The versatility of active-site functional groups, and the congregation

of these groups within active sites, might have allowed catalytic promiscuity to participate broadly in the divergence of enzymatic catalysis.

Conclusions

Uncovering how nature has created such a wealth of enzymatic diversity remains a fascinating challenge. The diversity of alternative reactions catalyzed at enzyme active sites and the above analysis suggest that a low level of catalytic promiscuity could be a common characteristic of enzymes. A low level of activity for a different reaction can greatly increase the probability that a duplicated enzyme will evolve to catalyze that new reaction by providing, or facilitating the establishment of, a selectable activity subsequent to gene duplication. Catalytic promiscuity could have aided the evolution of new enzymes via divergent evolution, including enzymes that utilize different mechanisms and catalyze different types of reactions.

The recent explosion in the identification of evolutionarily related superfamilies and the structural insights that allow effective comparisons between different members of these superfamilies will continue to contribute greatly to our understanding of enzymes. Analysis of these superfamilies, in conjunction with mechanistic understanding of how individual enzymes function, has already provided valuable clues for understanding evolutionary relationships between distantly related enzymes [8,9,12]. We expect that increased understanding of chemical mechanisms and the role of active-site features will continue to enrich our understanding of molecular evolution.

Acknowledgements

We have attempted to cite representative references, but recognize and apologize for necessary omission of direct citation of many interesting and pertinent references. The authors thank members of the Herschlag lab for helpful comments on the manuscript. This work was supported by a David and Lucile Packard Foundation Fellowship in Science and Engineering to D.H., and P.J.O. was supported in part by a NIH Biotechnology Training Grant.

References

1. Lewis, E.B. (1951). Pseudoallelism and gene evolution. *Cold Spring Harbor Symp. Quant. Biol.* **16**, 159-163.
2. Ohno, S. (1970). *Evolution by Gene Duplication*. George Allen and Unwin, London.
3. Jensen, R.A. (1976). Enzyme recruitment in evolution of new function. *Annu. Rev. Microbiol.* **30**, 409-425.
4. Rossman, M.G., Liljas, A., Branden, C.-I. & Banaszak, L.J. (1975). Evolutionary and structural relationships among dehydrogenases. In *The Enzymes*. (Boyer, P.D., ed.), pp. 61-102, Academic Press, New York.
5. Wilmanns, M., Hyde, C.C., Davies, D.R., Kirschner, K. & Jansonius, J.N. (1991). Structural conservation in parallel β/α-barrel enzymes that catalyze three sequential reactions in the pathway of tryptophan biosynthesis. *Biochemistry* **30**, 9161-9169.
6. Babbitt, P.C., *et al.*, & Gerlt, J.A. (1995). A functionally diverse enzyme superfamily that abstracts the α protons of carboxylic acids. *Science* **267**, 1159-1161.
7. Babbitt, P., *et al.*, & Gerlt, J.A. (1996). The enolase superfamily: a general strategy for enzyme-catalyzed abstration of the α-protons of carboxylic acids. *Biochemistry* **35**, 16489-16501.
8. Babbitt, P.C. & Gerlt, J.A. (1997). Understanding enzyme superfamilies. *J. Biol. Chem.* **272**, 30591-30594.
9. Holm, L. & Sander, C. (1997). An evolutionary treasure: unification of a broad set of amidohyrolases related to urease. *Proteins* **28**, 72-82.
10. Ollis, D.L., *et al.*, & Goldman, A. (1992). The α/β hydrolase fold. *Protein Eng.* **5**, 197-211.
11. Bernat, B.A., Laughlin, L.T. & Armstrong, R.N. (1997). Fosfomycin resistance protein (FosA) is a manganese metalloglutathione transferase related to glyoxalase I and the extradiol dioxygenases. *Biochemistry* **36**, 3050-3055.
12. Gerlt, J.A. & Babbitt, P.C. (1998). Mechanistically diverse enzyme superfamilies: the importance of chemistry in the evolution of catalysis. *Cur. Opin. Chem. Biol.* **2**, 607-612.
13. Armstrong, R.N. (1998). Mechanistic imperatives for the evolution of glutathione transferases. *Cur. Opin. Chem. Biol.* **2**, 618-623.
14. Alexander, F.W., Sandmeier, E., Mehta, P.K. & Christen, P. (1994). Evolutionary relationships among pyridoxal-5′-phosphate-dependent enzymes: Regio-specific α, β and γ families. *Eur. J. Biochem.* **219**, 953-960.
15. Aravind, L. & Koonin, E.V. (1998). A novel family of predicted phosphoesterases include *Drosophila* prune protein and bacterial RecJ exonuclease. *Trends Biol. Sci.* **23**, 17-19.
16. Baker, P.J., *et al.*, & Rice, D.W. (1997). Determinants of substrate specificity in the superfamily of amino acid dehydrogenases. *Biochemistry* **36**, 16109-16115.
17. Baker, A.S., *et al.*, & Dunaway-Mariano, D. (1998). Insights into the mechanism of catalysis by the P-C bond-cleaving enzyme phosphonoacetaldehyde hydrolase derived from gene sequence analysis and mutagenesis. *Biochemistry* **37**, 9305-9315.
18. Bork, P. & Koonin, E.V. (1994). A new family of carbon-nitrogen hydrolases. *Protein Sci.* **3**, 1344-1346.
19. Bork, P., Holm, L., Koonin, E.V. & Sander, C. (1995). The cytidyltransferase superfamily: Identification of the nucleotide-binding site and fold prediction. *Proteins* **22**, 259-266.
20. Bork, P., Brown, N.P., Hegyi, H. & Schultz, J. (1996). The protein phosphatase 2C (PP2C) superfamily: Detection of bacterial homologues. *Protein Sci.* **5**, 1421-1425.
21. Galperin, M.Y., Bairoch, A. & Koonin, E.V. (1998). A superfamily of metalloenzymes unifies phosphopentomutase and cofactor-independent phosphoglycerate mutase with alkaline phosphatases and sulfatases. *Protein Sci.* **7**, 1829-1835.
22. Holm, L. & Sander, C. (1995). Evolutionary link between glycogen phosphorylase and a DNA modifying enzyme. *EMBO J.* **14**, 1287-1293.
23. Holm, L. & Sander, C. (1997). Enzyme HIT. *Trends Biochem. Sci.* **22**, 116-117.
24. Jez, J.M., Bennett, M.J., Schlegel, B.P., Lewis, M. & Penning, T.M. (1997). Comparative anatomy of the aldo-keto reductase superfamily. *Biochem. J.* **326**, 625-636.
25. Koonin, E.V. & Tatusov, R.L. (1994). Computer analysis of bacterial haloacid dehalogenases defines a large superfamily of hydrolases with diverse specificity. *J. Mol. Biol.* **244**, 125-132.
26. Koonin, E.V. (1996). A duplicated catalytic motif in a new superfamily of phosphohydrolases and phospholipid synthases that include poxvirus envelope proteins. *Trends Biochem. Sci.* **21**, 237-277.
27. Petsko, G.A., Kenyon, G.L., Gerlt, J.A., Ringe, D. & Kozarich, J.W. (1993). On the origin of enzymatic species. *Trends Biochem. Sci.* **18**, 372-376.
28. Murzin, A.G. (1993). Can homologous proteins evolve different enzymatic activities? *Trends Biol. Sci.* **18**, 403-405.
29. Murzin, A.G. (1998). How far divergent evolution goes in proteins. *Curr. Opin. Struct. Biol.* **8**, 380-387.
30. Aravind, L., Galperin, M.Y. & Koonin, E.V. (1998). The catalytic domain of the P-type ATPase has the haloacid dehalogenase fold. *Trends Biochem. Sci.* **23**, 127-129.
31. Niedhart, D.J., Kenyon, G.L., Gerlt, J.A. & Petsko, G.A. (1990). Mandelate racemase and muconate lactonizing enzyme are mechanistically distinct and structurally homologous. *Nature* **347**, 692-694.
32. Wagner, U.G., Hasslacher, M., Griengl, H., Schwab, H. & Kratky, C. (1996). Mechanism of cyanogenesis: the crystal structure of hydroxynitrile lyase from *Hevea brasiliensis*. *Structure* **4**, 811-822.
33. Díaz, E. & Timmis, K.N. (1995). Identification of functional residues in a 2-hydroxymuconic semialdehyde hydrolase: a new member of the α/β hydrolase-fold family of enzymes which cleaves carbon-carbon bonds. *J. Biol. Chem.* **270**, 6403-6411.
34. Lebioda, L. & Stec, B. (1988). Crystal structure of enolase indicates that enolase and pyruvate kinase evolved from a common ancestor. *Nature* **333**, 683-686.

35. Hasson, M.S., *et al.*, & Ringe, D. (1998). Evolution of an enzyme active site: the structure of a new crystal form of muconate lactonizing enzyme compared with mandelate racemase and enolase. *Proc. Natl Acad. Sci. USA* **95**, 10369-10401.
36. Palmer, D.R.J., Hubbard, B.K. & Gerlt, J.A. (1998). Evolution of enzymatic activities in the enolase superfamily: Partitioning of reactive intermediates by (D)-glucarate dehydratase from *Pseudomonas putida*. *Biochemistry* **37**, 14350-14357.
37. Gulick, A.M., Palmer, D.R.J., Babbitt, P.C., Gerlt, J.A. & Rayment, I. (1998). Evolution of enzymatic activities in the enolase superfamily: crystal structure of (D)-glucarate dehydratase from *Pseudomonas putida*. *Biochemistry* **37**, 14358-14368.
38. Hubbard, B.K., Koch, M., Palmer, D.R.J., Babbitt, P.C. & Gerlt, J.A. (1998). Evolution of enzymatic activities in the enolase superfamily: characterization of the (D)-glucarate/galactarate catabolic pathway in *E. coli*. *Biochemistry* **37**, 14369-14375.
39. Galperin, M.Y., Walker, D.R. & Koonin, E.V. (1998). Analogous enzymes: independent inventions in enzyme evolution. *Genome Res.* **8**, 779-790.
40. Hedstrom, L. (1994). Engineering for redesign. *Curr. Opin. Struct. Biol.* **4**, 608-611.
41. Wedemayer, G.J., Patten, P.A., Wang, L.H., Schultz, P.G. & Stevens, R.C. (1997). Structural insights into the evolution of an antibody combining site. *Science* **276**, 1665-1669.
42. Yano, T., Oue, S. & Kagamiyama, H. (1998). Directed evolution of an aspartate aminotransferase with new substrate specificities. *Proc. Natl Acad. Sci. USA* **95**, 5511-5515.
43. Hughes, A.L. (1994). The evolution of functionally novel proteins after gene duplication. *Proc. Roy. Soc. London B* **256**, 119-124.
44. Parsot, C. (1987). A common origin for enzymes involved in the terminal step of the threonine and tryptophan biosynthetic pathways. *Proc. Natl Acad. Sci. USA* **84**, 5207-5210.
45. Jensen, R.A. & Gu, W. (1996). Evolutionary recruitment of biochemically specialized subdivisions of family I within the protein superfamily of aminotransferases. *J. Bacteriol.* **178**, 2161-2171.
46. Walsh, C. (1979). *Enzymatic Reaction Mechanisms*. W.H. Freeman & Co., New York.
47. Kovach, I.M., Larson, M. & Schoweu, R.L. (1986). Catalytic recruitment in the inactivation of serine proteases by phosphonate esters. Recruitment of acid-base catalysis. *J. Am. Chem. Soc.* **108**, 5490-5495.
48. Zhao, Q., Kovach, I.M., Bencsura, A. & Papathanassiu, A. (1994). Enantioselective and reversible inhibition of trypsin and α-chymotrypsin by phosphonate esters. *Biochemistry* **33**, 8128-8138.
49. Pocker, Y. & Sarkanen, S. (1978). Oxonase and esterase activities of erythrocyte carbonic anhydrase. *Biochemistry* **17**, 1110-1118.
50. Earnhardt, J.N. & Silverman, D.N. (1998). Carbonic anhydrase. In *Comprehensive Biological Catalysis.* (Sinnott, M., ed.), pp. 483-495, Academic Press, London.
51. Koester, M.K., Pullan, L.M. & Noltmann, E.A. (1981). The *p*-nitrophenyl phosphatase activity of muscle carbonic anhydrase. *Arch. Biochem. Biophys.* **211**, 632-642.
52. Pullan, L.M. & Noltmann, E.A. (1985). Specific arginine modification at the phosphatase site of muscle carbonic anhydrase. *Biochemistry* **24**, 635-640.
53. Cabiscol, E. & Levine, R.L. (1995). Carbonic anhydrase III: oxidative modification *in vivo* and loss of phosphatase activity during aging. *J. Biol. Chem.* **270**, 14742-14747.
54. Cabiscol, E. & Levine, R.L. (1996). The phosphatase activity of carbonic anhydrase III is reversibly regulated by glutathiolation. *Proc. Natl Acad. Sci. USA* **93**, 4170-4174.
55. Means, G.E. & Bender, M.L. (1975). Acetylation of human serum albumin by *p*-nitrophenyl acetate. *Biochemistry* **14**, 4989-4994.
56. Hollfelder, F., Kirby, A.J. & Tawfik, D.S. (1996). Off-the-shelf proteins that rival tailor-made antibodies as catalysts. *Nature* **383**, 60-63.
57. Kikuchi, K., Thorn, S.N. & Hilvert, D. (1996). Albumin-catalyzed proton transfer. *J. Am. Chem. Soc.* **118**, 8184-8185.
58. Awad-Elkarim, A. & Means, G.E. (1988). The reactivity of *p*-nitrophenyl acetate with serum albumins. *Comp. Biochem. Physiol. B.* **91**, 267-272.
59. Shaw, E. (1970). Chemical modification by active-site-directed reagents. In *The Enzymes.* (Boyer, P.D., ed.), pp. 91-146, Academic Press, New York.
60. Bond, C.S., *et al.*, & Guss, J.M. (1997). Structure of a human lysosomal sulfatase. *Structure* **5**, 277-289.
61. Lukatela, G., *et al.*, & Saenger, W. (1998). Crystal structure of human arylsulfatase A: the aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis. *Biochemistry* **37**, 3654-3664.
62. O'Brien, P.J. & 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**, 12369-12370.
63. Claire, T., Lee, H.Y., Liotta, L.A. & Stracke, M.L. (1997). Autotaxin is an exoenzyme possessing 5′-nucleotide phosphodiesterase/ATP pyrophosphatase and ATPase activities. *J. Biol. Chem.* **272**, 996-1001.
64. Oda, Y., Kuo, M.-D., Huang, S.S. & Huang, J.S. (1993). The major acidic fibroblast growth factor (aFGF)-stimulated phosphoprotein from bovine liver plasma membranes has aFGF-stimulated kinase, autoadenylation, and alkaline phosphodiesterase activities. *J. Biol. Chem.* **268**, 27318-27326.
65. Kakuta, Y., Pedersen, L.G., Carter, C.W., Negishi, M. & Pedersen, L.C. (1997). Crystal structure of estrogen sulphotransferase. *Nat. Struct. Biol.* **4**, 904-908.
66. Peliska, J.A. & O'Leary, M.H. (1989). Sulfuryl transfer catalyzed by pyruvate kinase. *Biochemistry* **28**, 1604-1611.
67. Hemrika, W., Renirie, R., Dekker, H.L., Barnett, P. & Wever, R. (1997). From phosphatases to vanadium peroxidases: A similar architecture of the active site. *Proc. Natl Acad. Sci. USA* **94**, 2145-2149.
68. Neuwald, A.F. (1997). An unexpected structural relationship between integral membrane phosphatases and soluble haloperoxidases. *Protein Sci.* **6**, 1764-1767.
69. Stukey, J. & Carman, G.M. (1997). Identification of a novel phosphatase sequence motif. *Protein Sci.* **6**, 469-472.
70. van Etten, R.L., Waymack, P.P. & Rehkop, D.M. (1974). Transition metal ion inhibition of enzyme-catalyzed phosphate ester displacement reaction. *J. Am. Chem. Soc.* **96**, 6782-6785.
71. Stankiewicz, P.J. & Gresser, M.J. (1988). Inhibition of phosphatase and sulfatase by transition-state analogues. *Biochemistry* **27**, 206-212.
72. Messerschmidt, A. & Wever, R. (1996). X-ray structure of a vanadium-containing enzyme: chloroperoxidase from the fungus *Curvalaria inaequalis*. *Proc. Natl Acad. Sci. USA* **93**, 392-396.
73. Messerschmidt, A., Prade, L. & Wever, R. (1997). Implications for the catalytic mechanism of the vanadium-containing enzyme chloroperoxidase from the fungus *Curvularia inaequalis* by X-ray structures of the native and peroxide form. *Biol. Chem.* **378**, 309-315.
74. Butler, A. (1998). Vanadium haloperoxidases. *Curr. Opin. Chem. Biol.* **2**, 279-285.
75. Skarstedt, M.T. & Greer, S.B. (1973). Threonine synthetase of *Bacillus subtilis*: the nature of an associated dehydratase activity. *J. Biol. Chem.* **248**, 1032-1044.
76. Parsot, C. (1986). Evolution of biosynthetic pathways: a common ancestor for threonine synthase, threonine dehydratase, and D-serine dehydratase. *EMBO J.* **5**, 3013-3019.
77. Laber, B., Gerbling, K.-P., Harde, C., Neff, K.-H., Nordhoff, E. & Pohlenz, H.-D. (1994). Mechanisms of interaction of *E. coli* threonine synthase with substrates and inhibitors. *Biochemistry* **33**, 3413-3423.
78. John, R.A., (1998). Pyridoxal phosphate-dependent enzymes. In Comprehensive Biological Catalysis. pp173-200 (Sinnot, M., ed.) Academic Press, London.
79. Graber, R., *et al.*, & Christen, P. (1995). Changing the reaction specificity of a pyridoxal-5′-phosphate-dependent enzyme. *Eur. J. Biochem.* **232**, 686-690.
80. Vacca, R.A., Giannattasio, S., Graber, R., Sandmeier, E., Marra, E. & Christen, P. (1997). Active-site Arg → Lys substitutions alter reaction and substrate specificity of aspartate aminotransferase. *J. Biol. Chem.* **272**, 21932-21937.
81. Cronin, C.N., Malcolm, B.A. & Kirsch, J.F. (1987). Reversal of substrate charge specificity by site-directed mutagenesis of aspartate aminotransferase. *J. Am. Chem. Soc.* **109**, 2222-2223.
82. Malashkevich, V.N., Onuffer, J.J., Kirsch, J.F. & Jansonius, J.N. (1995). Alternating arginine-modulated substrate specificity in an engineered tyrosine aminotransferase. *Nat. Struct. Biol.* **2**, 548-553.
83. Chen, R., Greer, A. & Dean, A.M. (1995). A highly active decarboxylating dehydrogenase with rationally inverted coenzyme specificity. *Proc. Natl Acad. Sci. USA* **92**, 11666-11670.
84. Sakowicz, R., Gold, M. & Jones, J.B. (1995). Partial reversal of the substrate stereospecificity of an L-lactate dehydrogenase by site-directed mutagenesis. *J. Am. Chem. Soc.* **117**, 2387-2394.
85. Zhang, J.H., Dawes, G. & Stemmer, W.P.C. (1997). Directed evolution of a fucosidase from a galactosidase by DNA shuffling and screening. *Proc. Natl. Acad. Sci. USA* **94**, 4504-4509.
86. Dean, A.M. & Golding, G.B. (1997). Protein engineering reveals ancient adaptive replacements in isocitrate dehydrogenase. *Proc. Natl Acad. Sci. USA* **94**, 3104-3109.

87. Cronin, C.N. (1998). Redesign of choline acetyltransferase specificity by protein engineering. *J. Biol. Chem.* **273**, 24465-24469.
88. Hopfner, K.-P., Kopetzki, E., Krebe, G.-B., Bode, W., Huber, R. & Engh, R.A. (1998). New enzyme lineages by subdomain shuffling. *Proc. Natl Acad. Sci. USA* **95**, 9813-9818.
89. Nixon, A.E., Warren, M.S. & Benkovic, S.J. (1997). Assembly of an active enzyme by the linkage of 2 protein modules. *Proc. Natl Acad. Sci. USA* **94**, 1069-1073.
90. Crameri, A., Raillard, S.-A., Bermudez, E. & Stemmer, W.P.C. (1998). DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature* **391**, 288-291.
91. Shuman, S. (1998). Polynucleotide ligase activity of eukaryotic topoisomerase I. *Molec. Cell* **1**, 741-748.
92. Brown, P., Shanklin, J., Whittle, E. & Somerville, C. (1998). Catalytic plasticity of fatty acid modification enzymes underlying chemical diversity of plant lipids. *Science* **282**, 1315-1317.
93. Jez, J. & Penning, T. (1998). Engineering 5β-reductase activity into rat liver 3α-hydroxysteroid dehydrogenase. *Biochemistry* **57**, 9695-9705.
94. Millard, C.B., Lockridge, O. & Broomfield, C.A. (1995). Design and expression of organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase. *Biochemistry* **34**, 15925-15933.
95. Lockridge, O., Blong, R.M., Masson, P., Foment, M.-T., Millard, C.B. & Broomfield, C.A. (1997). A single amino acid substitution, Gly117His, confers phosphotriesterase (organophosphorus acid anhydride hydrolase) activity on human butyrylcholinesterase. *Biochemistry* **36**, 786-795.
96. Millard, C.B., Lockridge, O. & Broomfield, C.A. (1998). Organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase: synergy results in a somanase. *Biochemistry* **37**, 237-247.
97. Campbell, P.M., Trott, J.F., Claudianos, C., Smyth, K.A., Russell, R.J. & Oakeshott, J.G. (1997). Biochemistry of esterases associated with organophosphate resistance in *Lucilia cuprina* with comparisons to putative orthologues in other *Diptera*. *Biochem. Genet.* **35**, 17-40.
98. Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russell, R.J. & Oakeshott, J.G. (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**, 7464-7468.
99. Dufour, E., Storer, A.C. & Menard, R. (1995). Engineering nitrile hydratase activity into a cysteine protease by a single mutation. *Biochemistry* **34**, 16382-16388.
100. Boehlein, S.K., Rosa-Rodriguez, J.G., Schuster, S.M. & Richards, N.G.J. (1997). Catalytic activity of the N-terminal domain of *Escherichia coli* asparagine synthetase B can be reengineered by a single-point mutation. *J. Am. Chem. Soc.* **119**, 5785-5791.
101. Quemeneur, E., Moutiez, M., Charbonnier, J.-B. & Menez, A. (1998). Engineering cyclophilin into a proline-specific endopeptidase. *Nature* **391**, 301-304.
102. Dreyer, M.K. & Shulz, G.E. (1996). Refined high-resolution structure of the metal-ion dependent L-fuculose-1-phosphate aldolase (class II) from *Escherichia coli*. *Acta Crystallogr. D* **52**, 1082-1091.
103. Dreyer, M.K. & Schulz, G.E. (1996). Catalytic mechanism of the metal-dependent fuculose aldolase from *Escherichia coli* as derived from the structure. *J. Mol. Biol.* **259**, 458-466.
104. Johnson, A.E. & Tanner, M.E. (1997). Epimerization via carbon-carbon bond cleavage. L-ribulose-5-phosphate 4-epimerase as a masked class II aldolase. *Biochemistry* **37**, 5764-5754.
105. Piatigorsky, J. & Wistow, G. (1991). The recruitment of crystallins: new functions precede gene duplication. *Science* **252**, 1078-1079.
106. Farber, G.K. & Petsko, G.A. (1990). The evolution of α/β barrel enzymes. *Trends Biochem. Sci.* **15**, 228-234.
107. Farber, G.K. (1993). An α/β-barrel full of evolutionary trouble. *Curr Opin. Struct. Biol.* **3**, 409-412.
108. Reardon, D. & Farber, G.K. (1995). The structure and evolution of α/β barrel proteins. *FASEB J.* **9**, 497-503.
109. Murzin, A.G., Brenner, S.E., Hubbard, T. & Chothia, C. (1995). SCOP: A structural classification of proteins database for the investigation of sequences and structures. *J. Mol. Biol.* **247**, 536-540.
110. Radzicka, A. & Wolfenden, R. (1995). A proficient enzyme. *Science* **267**, 90-93.
111. Jackson, R.C. & Handshumacher, R.E. (1970). *Escherichia coli* L-asparaginase. catalytic activity and subunit nature. *Biochemistry* **9**, 3585-3590.
112. Gan, K.N., Smolen, A., Eckerson, H.W. & Du, B.N.L. (1991). Purification of human serum paraoxonase/arylesterase: evidence for one esterase catalyzing both activities. *Drug Metab. Dispos.* **19**, 100-106.
113. Ba-Saif, S.A., Davis, A.M. & Williams, A. (1989). Effective charge distribution for attack of phenoxide ion on aryl methyl phosphate monoanion: Studies related to the action of ribonuclease. *J. Org. Chem.* **54**, 5483-5486.
114. Hay, R.W. & Govan, N. (1996). The reactivity of metal-hydroxo nucleophiles and a range of bases in the hydrolysis of the phosphate triester 2,4-dinitrophenyl diethyl phosphate. *Polyhedron* **15**, 2381-2386.
115. Kirby, A.J. & Jencks, W.P. (1965). The reactivity of nucleophilic reagents roward the *p*-nitrophenyl phosphate dianion. *J. Am. Chem. Soc.* **87**, 3209-3216.
116. Shaw, E. & Ruscica, J. (1971). The reactivity of His-57 in chymotrypsin to alkylation. *Arch. Biochem. Biophys.* **145**, 484-489.
117. Nakagawa, Y. & Bender, M.L. (1969). Modification of α-chymotrypsin by methyl *p*-nitrobenzenesulfonate. *J. Am. Chem. Soc.* **91**, 1566-1567.
118. Yebra, M.J. & Bhagwat, A.S. (1995). A cytosine methyltransferase converts 5-methylcytosine in DNA to thymine. *Biochemistry* **34**, 14752-14757.
119. Ozaki, S., Matsui, T. & Watanabe, Y. (1996). Conversion of myoglobin into a highly stereospecific peroxygenase by the L29H/H64L mutation. *J. Am. Chem. Soc.* **118**, 9784-9785.
120. van de Velde, F., Konemann, L., Rantwijk, R.V. & Sheldon, R.A. (1998). Enantioselective sulfoxidation mediated by vanadium-incorporated phytase: a hydrolase acting as a peroxidase. *Chem. Commun.* **17**, 1891-1892.
121. Matsui, T., Nagano, S., Ishimori, K., Watanabe, Y. & Morishima, I. (1996). Preparation and reactions of myoglobin mutants bearing both proximal cysteine ligand and hydrophobic distal cavity: protein models for the active site of P-450. *J. Am. Chem. Soc.* **35**, 13118-13124.
122. Ozaki, S.-I., Matsui, T. & Watanabe, Y. (1997). Conversion of a myoglobin into a peroxygenase: A catalytic intermediate of sulfoxidation and epoxidation by the F34H/H64L mutant. *J. Am. Chem. Soc.* **119**, 6666-6667.
123. Wan, L., Twitchett, M.B., Eltis, L.D., Mauk, A.G. & Smith, M. (1998). *In vitro* evolution of horse heart myoglobin to increase peroxidase activity. *Proc. Natl Acad. Sci. USA* **95**, 12825-12831.
124. Reid, T.W. & Fahrney, D. (1967). The pepsin-catalyzed hydrolysis of sulfite esters. *J. Am. Chem. Soc.* **89**, 3941-3943.
125. Shim, H., Hong, S. & Raushel, F.M. (1998). Hydrolysis of phosphodiesters through transformation of the bacterial phosphotriesterase. *J. Biol. Chem.* **273**, 17445-17450.
126. Chin, J., Banaszczyk, M., Jubian, V. & Zou, X. (1989). Co(III) complex promoted hydrolysis of phosphate diesters: comparison in reactivity of rigid *cis*-diaquotetraazacobalt(III) complexes. *J. Am. Chem. Soc.* **111**, 186-190.
127. Jencks, W.P. & Carriuolo, J. (1959). Reactivity of nucleophilic reagents toward esters. *J. Am. Chem. Soc.* **82**, 1778-1786.
128. Andrews, R.K., Dexter, A., Blakeley, R.L. & Zerner, B. (1986). Jack bean urease (EC 3.5.1.5). On the inhibition of urease by amides and esters of phosphoric acid. *J. Am. Chem. Soc.* **108**, 7124-7125.
129. Peliska, J.A. & O'Leary, M.H. (1991). Sulfuryl transfer catalyzed by phosphokinases. *Biochemistry* **30(4)**, 1049-1057.
130. Rezende, A.A., Pizauro, J.M., Ciancaglini, P. & Leone, F.A. (1994). Phosphodiesterase activity is a novel property of alkaline phosphatase from osseous plate. *Biochem. J.* **301**, 517-522.
131. Smile, D.H., Donohue, M., Yeh, M., Kenkel, T. & Trela, J.M. (1976). Repressible alkaline phosphatase from *Thermus aquaticus*. *J. Biol. Chem.* **252**, 3399-3401.
132. Uchida, T., Egami, F. & Roy, A.B. (1981). 3',5'-cyclic nucleotide phosphodiesterase activity of the sulfatase A of ox liver. *Biochimica Biophys. Acta* **657**, 356-363.
133. Chin, J. & Zou, X. (1987). Catalytic hydrolysis of cAMP. *Can. J. Chem.* **65**, 1882-1884.
134. John, R.A. & Tudball, N. (1972). Evidence for induced fit of a pseudo-substrate of aspartate aminotransferase. *Eur. J. Biochem.* **31**, 135-138.
135. John, R.A. & Fasella, P. (1969). The reaction of *L*-serine *O*-sulfate with aspartate aminotransferase. *Biochemistry* **8**, 4477-4482.
136. Fujimoto, M., Kuninaka, A. & Yoshino, H. (1974). Identity of phosphodiesterase and phosphomonesterase activities with nuclease P1 (a nuclease from *Penicillium citrinum*). *Agr. Biol. Chem.* **38**, 785-790.
137. Polya, G.M., Brownlee, A.G. & Hynes, M.J. (1975). Enzymology and genetic regulation of a cyclic nucleotide-binding phosphodiesterase-phosphomonoesterase from *Aspergillus nidulans*. *J. Bacteriol.* **124**, 693-703.

138. Richardson, C.C. & Kornberg, A. (1964). A deoxyribonucleic acid phosphatase-exonuclease from *Escherichia coli*. I. Purification of the enzyme and characterization of the phosphatase activity. *J. Biol. Chem.* **239**, 242-250.
139. Yamane, K. & Maruo, B. (1978). Alkaline phosphatase possessing alkaline phosphodiesterase activity and other phosphodiesterases in *Bacillus subtilis*. *J. Bacteriol.* **134**, 108-114.
140. Palmer, D.R.J. & Gerlt, J.A. (1996). Evolution of enzymatic activities: Multiple pathways for generating and partitioning a common enolic intermediate by glucarate dehydratase from *Pseudomonas putida*. *J. Am. Chem. Soc.* **118**, 10323-10324.
141. Tokuyama, S. & Hatano, K. (1995). Cloning, DNA sequencing, and heterologous expression of the gene for thermostable *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60 in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **42**, 884-889.
142. Chang, Y.-Y. & Cronan, J.E. (1988). Common ancestory of *Escherichi coli* pyruvate oxidase and the acetohydroxy acid synthases of the branched-chain amino acid biosynthetic pathway. *J. Bacteriol.* **170**, 3937-3945.
143. Kim, E.E. & Wyckoff, H.W. (1991). Reaction mechanism of alkaline phosphatase based on crystal structures. *J. Mol. Biol.* **218**, 449-464.
144. Berry, M.B. & Phillips, G.N. (1998). Crystal-structures of *Bacillus-Stearothermophilus* adenylate kinase with bound AP(5)A, Mg^{2+} + AP(5)A, and Mn^{2+} + AP(5)A reveal an intermediate lid position and 6 coordinate octahedral geometry for bound Mg^{2+} and Mn^{2+}. *Proteins* **32**, 276-288.