Enzyme promiscuity: mechanism and applications

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Introductory courses in biochemistry teach that enzymes are specific for their substrates and the reactions they catalyze. Enzymes diverging from this statement are sometimes called promiscuous. It has been suggested that relaxed substrate and reaction specificities can have an important role in enzyme evolution; however, enzyme promiscuity also has an applied aspect. Enzyme condition promiscuity has, for a long time, been used to run reactions under conditions of low water activity that favor ester synthesis instead of hydrolysis. Together with enzyme substrate promiscuity, it is exploited in numerous synthetic applications, from the laboratory to industrial scale. Furthermore, enzyme catalytic promiscuity, where enzymes catalyze accidental or induced new reactions, has begun to be recognized as a valuable research and synthesis tool. Exploiting enzyme catalytic promiscuity might lead to improvements in existing catalysts and provide novel synthesis pathways that are currently not available.

Introduction

A modern term in enzymology is promiscuity, but what does it mean? As with many modern words, it is used without a well-defined meaning or with different meanings, depending on the author. In this overview, we will define a promiscuous enzyme as one that does things it is not expected to do, and we will focus on the enzyme promiscuity that has been exploited to expand the use of enzymes in the synthesis of compounds, whereas promiscuity connected to enzyme evolution will only be mentioned briefly.

Promiscuity comes in many different forms, and we would like to introduce the classification of promiscuity into condition promiscuity, substrate promiscuity and catalytic promiscuity (Box 1). The classification is sometimes not straightforward, and we will see that different types of promiscuity can be combined. One example illustrating this problem is amide formation catalyzed by a lipase: amines are, undoubtedly, promiscuous substrates for a lipase, but is this also an example of catalytic promiscuity and thereby a new reaction for a lipase? Some scientists think so, whereas others regard the two transition states as being too similar to be classified as different reactions. Even if the term promiscuity is new in enzymology, there are numerous old examples in the literature that live up to the definition. An example of promiscuity was demonstrated in the reversal of hydrolytic enzyme activity shown for maltase 1898 [1] and for pancreatic lipase in 1900 [2]. Condition promiscuity was explored by Rona et al. in the early 1930s in a series of experiments using pig liver lipase and pancreatic lipase in organic solvents for resolution of chiral alcohols and esters [3]. A good example of both substrate and catalytic promiscuity is the use of pyruvate decarboxylase to form carbon–carbon bonds; this was first studied in 1921 and is, today, an industrial activity [4].

Enzyme promiscuity has become popular in terms of enzyme evolution, enzyme engineering and biocatalysis [5–7]. Some scientists regard the promiscuity of enzymes as the starting point for enzyme divergent evolution; thus, low promiscuity activity towards an unnatural substrate might, by a single point mutation, turn the enzyme into a much more proficient catalyst, affording a survival benefit to the organism. Following the first beneficial mutation, the enzyme then has time to evolve into an even better catalyst [8]. Furthermore, in the laboratory, a promiscuous function can be the starting point for the creation of a new enzyme activity [9]. The evolutionary aspect of promiscuity has recently been discussed in more detail by Tawfik et al.–they propose that a new promiscuous activity can evolve without negative trade-offs in the native activity, leading to a generalist enzyme that can later on become a specialist [10]. Understanding of substrate and transition state recognition in enzyme catalysis offers great opportunities in the development of new catalytic functions in the scaffold of old enzymes.

In nature, enzymes appear to develop to a point at which they are ‘good enough’ for their task, meaning that the substrate and catalytic specificities have reached a point that satisfies the needs of the cell. There are, however, important exceptions. Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) evolved under low oxygen pressure and is unable to discriminate between carbon dioxide and oxygen. The consequence is an oxygenase reaction that creates byproducts that the cell has to cope with. In our opinion, this is a case of natural promiscuity that the cell has to live with in the absence of a better alternative. Also, as mentioned above and pointed out by O’Brien and Herschlag, promiscuity can be a part of the evolution of new enzyme activity [11]. Gerlt et al. has shown that some enzymes with similar reaction intermediates are able to catalyze each others reactions, sometimes with astonishingly high efficiency [12,13]. Furthermore, these enzymes probably originated from the same gene.
Enzymes taken out of the cell can be exposed to reaction conditions and substrates not seen in vivo that will challenge their specificity and might force them to handle substrates and catalyze reactions they were not designed for. This is why there is a low barrier to exploit enzymes as catalysts for unnatural reactions in the laboratory and industry. This could be called accidental in vitro promiscuity [14].

We will now describe the various types of promiscuity, focusing on examples of interest in applied synthesis (Table 1).

### Condition promiscuity

Both condition promiscuity and substrate promiscuity have been explored and used in enzyme-catalyzed synthesis. There are thousands of examples of these, such as reactions performed in organic solvents, in absence of solvents, at high temperature, or at extreme pH. The exploitation of lipases using modified substrates in organic solvents is particularly pronounced; this combination of substrate promiscuity and condition promiscuity has led to several industrial applications [15].

Solid–gas bioreactors represent another interesting example of condition promiscuity. There is no liquid phase; instead, the enzyme is immobilized in a dry form on a solid support, and the substrates and products are present in the gas phase only. The system enables individual fine-tuning of the thermodynamic activities of substrates and water, and is of great theoretical interest. In addition, the high productivity of the system enables the lipase-catalyzed production of esters at a commercial scale [16].

Early important examples of engineering the condition promiscuity of enzymes come from the group of Frances Arnold at the beginning of the 1990s. They explored the stability and activity of the serine protease subtilisin E in the polar organic solvent DMF – where water hydrolysis is suppressed – and improved the performance of the enzyme for peptide synthesis with random mutagenesis. The initial enzyme was further improved with three rounds of directed evolution, leading to an enzyme with ten amino acid substitutions [17]. This enzyme showed a 256-fold higher activity than the wild type in 60% DMF. The important interactions contributing to protein stability in non-aqueous solvents have been summarized as design rules by Arnold [18].

### Substrate promiscuity

As mentioned above, substrate promiscuity has been frequently exploited in the past. Here, we will mention examples of enzymes with strict substrate specificity, which can be destroyed by simple point mutations, and enzymes with an intrinsic high substrate promiscuity, which can be

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increased even more. These examples will show that even if enzymes intrinsically have high or low substrate specificity, they often can be engineered into a promiscuous substrate behavior that suits a specific application.

**Coenzyme promiscuity**

For the living cell it is important to keep the NADPH–NADP⁺ pool in a reduced state, whereas the pool of NADH–NAD⁺ must be oxidized. Consequently, dehydrogenases must be able to distinguish between the two cofactor pairs. This is an example of where the cell does not permit a few point mutations can induce it. Ashida et al. describe an interesting case in which one point mutation can destroy the NAD⁺ selectivity or even reverse it to favor NADP⁺ [19]. Alanine dehydrogenase from *She-wanella* sp. Ac10 shows a high preference for NAD⁺ compared with NADP⁺, with a ratio of specificity constants of 2500. The mutation Asp198Ala creates an enzyme with a decreased *K_M* and an increased *k_cat* for NADP⁺, leading to specificity for NADP⁺ of 14 compared with NAD⁺ – altogether, a change of 35 000-times without a drop in total activity. A similar mutation, Asp198Gly, creates an enzyme almost without cofactor specificity. Other dehydrogenases are equally sensitive to simple point mutations. For example, the NAD⁺ specificity of phosphite dehydrogenase can be abolished by one point mutation and turned into NADP⁺ specificity by two [20].

**Lipase substrate promiscuity**

Lipases are promiscuous with regard to substrates: they accept a wide range of esters and are extensively used in the resolution of racemic acids and alcohols, although they normally show low enantioselectivity towards chiral acids. The research group around Reetz showed that it was possible to evolve lipases with preference for either the R- or S-enantiomer. They started with the lipase from *Pseudomonas aeruginosa*, which has no enantioselectivity towards 2-methyldecanolic acid p-nitrophenol ester, and subjected its gene to random mutagenesis to obtain, first, a lipase with an enantiomeric ratio of 51 favoring the S-enantiomer and, later on, a new mutant showing an R-preference with an enantiomeric ratio of 38 [21]. These experiments were performed without knowledge of the enzyme structure but were later followed up by a paper describing the structure and the effects of the mutations [22].

It has been determined that all lipases have the same preference for secondary alcohols, where the preferred enantiomers have the same geometrical configuration – called the Kazlauskas rule [23]. Lipase B from *Candida antarctica* has a stereospecificity pocket in which the chiral alcohol binds during catalysis. Through a single point mutation, Magnusson et al. were able to change the enantioselectivity 8 × 10⁷-fold and reverse it from R- to S-selectivity, mainly by increasing the activity towards the S-enantiomer [24]. Interestingly, the change was only because of an increase in the entropic term favoring the S-enantiomer. Although only a small change in the protein is needed to obtain a lipase with inverted enantioselectivity, no enzyme is known from natural sources.

**Catalytic promiscuity**

Enzyme catalytic promiscuity refers to the ability of an enzyme active site to catalyze more than one different chemical transformation. Kazlauskas proposes that transformations are different if the types of bonds cleaved and/or made are different, and if the transition states of the two reactions are different [25]. A detailed mechanistic insight can be used to predict promiscuous reactions in an existing enzyme active site. One challenge in current enzyme redesign is to turn a low promiscuous activity of an enzyme into the main activity or to use part of the existing mechanism and divert the reaction path to new products. We will discuss a few such examples in this section.

**Catalytic promiscuity and enzyme evolution**

The enzymes mandelate racemase, mucunate lactonizing enzyme and enolase share a common partial reaction, namely the Mg²⁺-assisted α-proton abstraction and enolization of carboxylic acids (Figure 1). The enol intermediate consequently reacts differently in each enzyme to produce their specific products [14]. The enzymes show a similar bi-domain structure in which the active site has a conserved location. With these common features it is easy to accept the hypothesis that these enzymes have evolved by divergent evolution from a common progenitor. In some cases, the evolution has not only changed the substrate specificity but also the reaction specificity. Gerlt et al. found one member of the enolase super-family that had a promiscuous activity as a N-acylamino acid racemase, with a *k_cat/K_M* equal to 590 M⁻¹s⁻¹ for the best substrate, N-acyctylnmethionine, compared with 2 × 10⁵ M⁻¹s⁻¹ for the natural reaction as o-succinylbenzoate synthase. The discovery of enzymes with dual activity indicates that at least some keep their original activity at the same time as new activities start to develop, resulting in an enzyme showing natural catalytic promiscuity [26].

**Markovnikov additions catalyzed by a hydrolase – accidental catalytic promiscuity**

The Markovnikov addition is an important reaction in synthetic chemistry. Wu et al. have demonstrated the promiscuous addition of nitrogen-containing heterocycles to vinyl esters, catalyzed by the enzymes d-aminoacylase [27], penicillin G acylase [28] and acylase from *Aspergillus oryzae* [29] in an organic solvent. Several different N-heterocyclic compounds were used, including purines and pyrimidines, and several reactions reached close to a quantitative yield. No product was observed when using vinyl ethers. This suggests that the carbonyl in the substrate is needed, and the authors propose that its oxygen is bound in an oxyanion hole or, alternatively, coordinated.

![Figure 1](https://www.sciencedirect.com)
to the zinc ion in the enzyme active site, which, according to them, would polarize the vinyl double bond. The heterocyclic nucleophile is deprotonated by a general base and simultaneously attacks the partial positive charge of the C=O double bond. It is somewhat surprising to note that none or low enantioselectivity was observed in the Markovnikov additions. This is unexpected and hard to explain.

**Aldol and Michael additions as accidental and induced catalytic promiscuity in a lipase**

In the reaction mechanism of serine hydrolases, the oxyanion hole is an important feature: it polarizes the carbonyl double bond in the ester substrate and renders the carbonyl carbon susceptible for nucleophilic attack by the active-site serine (Figure 2a). If the serine is removed by mutation, the oxyanion hole is still able to activate carbonyl functions. This is the basis for several of the promiscuous reactions that are shown to take place in the active site of serine hydrolases.

Carbon–carbon bond formation is important in synthesis, and many biotransformation examples exist [30]. Branneby et al. have shown that lipase B from *Candida antarctica* catalyzes carbon–carbon bond formation through aldol addition, with the substrates hexanal and propanal [31]. The proposed reaction mechanism involves binding of the carbonyl in the oxyanion hole of the lipase while the active-site histidine acts as a base to abstract a α-proton so that a carbon–carbon bond can be formed with the second substrate (Figure 2b). The specific activity increased by a factor of four when the active-site serine was replaced with an alanine to avoid the nucleophilic attack present in the natural reaction. Wild-type lipase covalently inhibited with methyl *p*-nitrophenyl n-hexyolphosphonate was completely inactive, confirming that the active site is needed for catalysis of this reaction. The aldolase reaction was carried out in organic solvent, with unnatural substrates and catalyzed by a lipase; it is, therefore, an example that combines all three types of promiscuity: condition, substrate and catalytic.

Conjugate addition on α,β-unsaturated carbonyl compounds is long known to be catalyzed by serine hydrolases [32]. Recently, conjugate additions by N-nucleophiles in organic solvents have been studied when catalyzed by a protease [33,34] or by a lipase [35]. Thiols were mainly used as nucleophiles by Carlqvist et al., who proposed that the mechanism involves the active-site histidine as a base that activates the nucleophile for attack at the β-carbon of the substrate, with the carbonyl oxygen hydrogen of the substrate bonded in the oxyanion hole of the enzyme (Figure 2c) [36]. This was supported by quantum chemical calculations on a minimal model system for *C. antarctica* lipase B, based on docking studies and molecular dynamics simulations. Furthermore, in that study, the Ser105Ala mutant of *C. antarctica* lipase B was found to catalyze conjugate additions of thiols and amines on α,β-unsaturated aldehydes, ketones and methyl esters in cyclohexane, toluene or diethyl ether. The reactions followed saturation kinetics with *k*ₘₐₓ values in the range of 0.001 to 4.000 min⁻¹. The enzyme proficiency was 10⁷ for the Ser105Ala mutant, a figure approaching that of natural enzymes. The conjugate-addition activity was, in many cases, improved by the Ser105Ala mutation for the tested substrates.

Svedendahl et al. has recently shown that Michael additions forming a carbon–carbon bond between a 1,3-dicarbonyl compound and an α,β-unsaturated aldehyde or ketone are efficient promiscuous reactions for *Candida antarctica* lipase B [37]. The reactions were run with various substrates in cyclohexane or in absence of solvent, with specific rates recorded in the range of 0.01–1000 s⁻¹. The wild type showed, in all cases, ~100-fold lower specific rate than the mutant Ser105Ala.

It is interesting to note that the wild type enzyme was able to catalyze all the reactions mentioned above, which could be a good example of accidental promiscuity found in vitro. It is, although, highly improbable that those reactions would occur in nature because it is unlikely that the lipase would encounter such substrates. The increased efficiency of the active-site serine-deficient mutant is an example of induced catalytic promiscuity.

**Induced catalytic promiscuity in artificial metalloenzymes**

There are several interesting examples of artificial metalloenzymes that can catalyze enantioselective oxidation. Okrasa and Kazlauskas made a thorough study of manganese-substituted carbonic-anhydrase-catalyzed oxidation of styrene analogues. They created the manganese enzyme by dialysis, where the natural Zn²⁺ was changed to Mn²⁺. The reaction was run in a buffer containing bicarbonate using hydrogen peroxide as oxidant. The reaction rate was lower for the manganese-substituted carbonic anhydrase than for free manganese or bicarbonate; however, the produced epoxide had enantiomeric

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**Figure 2.** In serine hydrolases, the carbonyl of the substrate ester is coordinated in the oxyanion hole and attacked by the active site serine (a). Mutation of the active site serine opens up the possibility for other reaction types that depend on polarization of the carbonyl function, such as aldol addition (b) or Michael type additions (c). The oxyanion hole of *Candida antarctica* lipase B can form three hydrogen bonds to the oxygen of the substrate in the transition state, whereas, in ground state, only two would be developed.

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excesses of up to 67%, and no aldehyde product could be seen. Unfortunately, the conversion was low owing to degradation of the anhydrase by reactive intermediates generated during catalysis [38].

Catechol oxidase activity was introduced in an aminopeptidase by da Silva and Ming. They prepared a di-Cu$^{2+}$-substituted aminopeptidase that oxidized catechol with hydrogen peroxide, recording a $k_{cat}/K_M$ in the range of $3300 \text{ M}^{-1}\text{s}^{-1}$, which is only ten times lower than that of a natural catechol oxidase [39]. Interestingly, the natural di-zinc aminopeptidase produced a $4 \times 10^3$-fold acceleration of phosphodiester hydrolysis in addition to its natural peptide hydrolytic activity [40]. Thus, this protein scaffold has been shown to perform reactions that pass through totally different transition states: hydrolysis of peptides and phosphodiester with the natural di-zinc enzyme, and oxidation with the artificial di-copper enzyme. All reactions are catalyzed with efficiencies of the same magnitude as their natural counterparts.

**Induced catalytic promiscuity in pyridoxal-phosphate-dependent enzymes**

Pyridoxal-phosphate (PLP) is a versatile cofactor used by more than 140 different enzymes from five out of the six main enzyme EC classes [41]. These enzymes mainly accept $\alpha$-amino acids and catalyze reactions such as $\alpha$-racemization, transamination, $\alpha$- or $\beta$-decarboxylation, and retroaldol reactions. Their active site is similar, and the cofactor is covalently bound through an imine linkage to a lysine residue. The common mechanistic feature is the formation of an external imine formed by the amino group of the substrate and the carbonyl of the cofactor [42,43]. Many of the PLP-dependent enzymes subsequently form a quinonoid intermediate, from which the mechanism diverges depending on the type of PLP-dependent enzyme (Figure 3). Mechanistic details suggested by Toney indicate the consequence of a fully developed quinonoid intermediate for the discrimination between racemization and transamination, and it is clear that small changes are needed in one enzyme to alter the activity into that of another PLP enzyme [44]. Examples of such induced catalytic promiscuity have, for instance, been published by Esaki et al. [45].

Seebeck and Hilvert describe another example of induced catalytic promiscuity of a PLP-dependent enzyme. They converted a racemase into an aldolase by a single point mutation. The mutation, Tyr265Ala, in a racemase from *Geobacillus stearothermophilus* resulted in a $3 \times 10^3$-fold reduction in racemase activity and a $2 \times 10^5$-fold increase in retro-aldol activity with the substrate $\text{D-phenylserine}$, compared with the wild-type enzyme. Replacement of Tyr265 with Ala was expected to promote the nearby His166 to initiate a retro-aldol reaction of a $\beta$-hydroxy-amino acid, similar to the retro-aldol reaction of the non-related enzyme $\text{l-threonine aldolase}$ (Figure 4). Tyr265 is acting as a base in the active site and is necessary for converting $\text{l-}$ to $\text{d}$-alanine. Removal of Tyr265 was, therefore, also expected to drastically lower the racemase.
activity. Interestingly, the retro-aldol reaction catalyzed by the alanine racemase mutant was found to be highly enantioselective because the conversion of racemic threo-β-phenylserine stopped at 50% conversion [46].

**Induced catalytic promiscuity in glycoside synthesis**

In 1898, Hill prepared maltose from glucose by equilibrium-controlled synthesis using maltase as a catalyst [1]. Owing to the need for water to dissolve the substrate, this methodology produces low yields. Kinetically controlled synthesis of glycosides requires activated substrates, and might reach yields of up to 40%. In 1998, Withers and colleagues reported how the yield could be increased considerably by introducing catalytic promiscuity to suppress hydrolysis [47]. This first paper has led to an abundance of extremely useful glycosynthases [48].

Inverting glycosidases have two carboxylates in the active site: one acts as a nucleophile to form a

![Diagram of glycosidase and glycosynthase catalytic mechanisms](https://example.com/diagram.png)

**Figure 5.** Induced catalytic promiscuity. Glycosidases were transformed into glycosynthases, thioglycoligases or thioglycosynthases by rerouting reaction intermediates achieved by deletion of catalytic amino acid side chains. The different mutations are shown in bold.
glycosyl–enzyme intermediate, whereas the other one acts as an acid/base catalyst. Removing the nucleophilic carboxylate produces an enzyme that is unable to hydrolyze glycosidic bonds. However, the otherwise intact active site can bind a suitable activated α-glycosyl fluoride and an acceptor sugar in such a way that the remaining acid/base carboxylate can catalyze the formation of a glycosidic bond. Because the mutant enzyme is devoid of hydrolytic activity, the product can accumulate without being hydrolyzed. A mutant in which the nucleophilic carboxylate was changed not to an alanine but to serine is especially good as a glycosynthase (Figure 5) [49].

An alternative modification of the reaction mechanism is to cripple the ability of the enzyme to hydrolyze the glycosyl–enzyme intermediate by removing the acid/base carboxylic acid. The glycosyl enzyme can still be formed from substrates with good leaving groups, such as di-nitrophenol or fluoride; however, water and hydroxyl-containing sugars are too weak nucleophiles to react at an appreciable reaction rate without the acid/base catalytic group. Instead, thiol-containing sugars, which are much more reactive, have to be used. The thioglycosides produced are not hydrolyzed by the enzyme and can, therefore, be prepared in good yields. Withers et al. named this activity thiyoglycoligase (Figure 5) [50].

In the two examples above, either of the two carboxylates in the active site was removed, but what would happen if both were removed? The double mutant would not be able to form a glycosyl–enzyme intermediate and, in addition, there would not be an acid/base to facilitate glycoside formation. Again, Withers’ group conducted the experiments and found an enzyme that could form glycosidic bonds between activated sugars and thioglycosides. Presumably, the protein scaffold provided a proximity effect by binding and orienting the substrates in a favorable position for reaction (Figure 5) [51].

Conclusions
Enzyme promiscuity, a property shown by many enzymes, is nowadays exploited in applied enzymology. In this review we have discussed some examples of various types of enzyme promiscuity and some applications based on it. We propose that the term, enzyme promiscuity, can be classified into enzyme condition promiscuity, substrate promiscuity and catalytic promiscuity. The applied aspects of condition promiscuity and substrate promiscuity have been explored for a long time, resulting in many industrial applications. Enzyme catalytic promiscuity, however, has only recently been exploited for synthetic applications. Catalytic promiscuity can be further divided into accidental catalytic promiscuity and induced catalytic promiscuity. It is evident that enzyme catalytic promiscuity, and particularly induced enzyme catalytic promiscuity, offers great opportunities in the design and development of new catalytic functions in the scaffold of stable enzymes. This is particularly promising because metagenomics has increased the availability of enzymes from extremophiles, providing new, stable templates for this technology. Exploiting enzyme catalytic promiscuity might lead to new, efficient and stable catalysts with, as yet, unprecedented activity for reactions where no practically useful enzyme alternatives exist today.

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