Trends in Biochemical Sciences

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Review

The Limits of Enzyme Specificity and the Evolution of Metabolism

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The substrate specificity of enzymes is bound to be imperfect, because of unavoidable physicochemical limits. In extant metabolic enzymes, furthermore, such limits are seldom approached, suggesting that the degree of specificity of these enzymes, on average, is much lower than could be attained. During biological evolution, the activity of a single enzyme with available alternative substrates may be preserved to a significant or even substantial level for different reasons – for example when the alternative reaction contributes to fitness, or when its undesirable products are nevertheless dispatched by metabolite repair enzymes. In turn, the widespread occurrence of promiscuous reactions is a consistent source of metabolic 'messiness', from which both liabilities and opportunities ensue in the evolution of metabolic systems.

Highlights

Substrate specificity cannot be absolute and is inherently limited.

The maximum capacity to discriminate between alternative substrates can be relatively low, and in any case it is seldom approached owing to evolutionary constraints.

In some enzymes of primary metabolism, substrate promiscuity is favored, although this may interfere with high flux and efficient regulation.

In other cases, enzymes acting on alternative substrates generate toxic or useless products; however, these can be destroyed or recycled by repair enzymes.

The limited substrate specificity of enzymes often results in the production of non-standard metabolites which contribute to the complexity of the metabolome.

Substrate promiscuity helps to fuel an 'underground' network of reactions which may represent a basis for further evolution and diversification of metabolism.

Many Metabolic Enzymes Are Not Strictly Substrate-Specific

It is now well appreciated that a substantial fraction of metabolic enzymes can catalyze reactions of different types and/or with different substrates [1,2]. The former behavior is termed catalytic promiscuity [3], the latter is usually called substrate promiscuity [4] (because these terms may be equivocal, Box 1 explains the definition of 'promiscuity' used here and compares it to a stricter definition accepted by evolutionary biochemists). Although this paper is essentially concerned with substrate promiscuity, it must be noted that the two behaviors are interrelated, often co-occur (e.g., [5–7]), and have analogous impacts on metabolism, such that most points raised concerning substrate promiscuity are similarly applicable to catalytic promiscuity.

Why is it that many metabolic enzymes can transform several different substrates? Is it simply because an absolute substrate specificity cannot be attained owing to the inherent imperfection of enzymes [8,9]? Or is it mostly the result of selective pressures (or lack thereof)? Finally, what are the consequences and implications of the recurrence of substrate promiscuity for the global evolution of metabolism? Related to these issues, this review begins by showing that substrate specificity is indeed inherently limited, for reasons rooted in physical chemistry, but also that, in many cases, metabolic enzymes are less selective than they could be. The review then examines how different evolutionary factors (both positive and negative selection, as well as neutral drift) may help to shape the degree to which enzymes discriminate between potential substrates. Finally, it is suggested that the universal tendency of enzymes to show substrate promiscuity is an important source of metabolome complexity that helps to fuel an 'underground' network of reactions which may represent a basis for further evolution and diversification of metabolism.

Substrate Specificity, Discrimination, and Binding Energy

In contrast to catalytic efficiency, which can be gauged in reference to an absolute scale of 'catalytic perfection' [10,11], specificity is a relative concept because it requires comparison

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Box 1. Usage of the Term 'Promiscuity' in This Review and in the Literature

This paper examines the capacity of enzymes to transform, with variable efficiency, different physiologically available substrates. As a shorthand to indicate such a property, I employ the term 'substrate promiscuity'. This is close to the inclusive (non-rigorous) usage of the term promiscuity adopted in a large part of the metabolic literature (e.g., [1,7,33,44,52,53,56,65,66,73]). Note, however, that evolutionary biochemists assign to 'promiscuity' a much more restricted definition. According to this definition, promiscuous enzymes are solely those possessing side activities that are physiologically irrelevant (e.g., because the alternative substrates are never available in the cell, or because the secondary activity is too weak to have an impact on organismal fitness) [25,74]. Conversely, enzymes catalyzing multiple reactions that are biologically relevant are termed 'multifunctional' [35,74]. This distinction assumes the absence of evolutionary pressures on 'truly promiscuous' activities – an assumption that is useful in many analyses. However, several examples in this review suggest that (i) when dealing with enzymes acting on different metabolites, drawing clear boundaries between 'relevant' and 'irrelevant' alternative reactions may be difficult or nearly impossible, and (ii) overall, true promiscuity (as defined by evolutionary biochemists) might be less common than has frequently been assumed. Note also that the term 'multifunctionality' (or moonlighting [75]) suggests that the alternative activities of an enzyme are useful; however, side activities may sometimes be biologically relevant in the sense that they are detrimental and therefore subject to negative selective pressures – again, as suggested by some examples discussed in this review.

between given alternative substrates. In fact, specificity is formally defined as the ability of an enzyme to discriminate between two potential substrates, in the presence of both compounds [12,13]. In a biological context, specificity entails acting on a single substrate in preference to a multitude of other metabolites in the cell. Intuitively, this can be a very difficult exercise. For example, an enzyme intended to be specific for aspartate should discriminate against (among others) asparagine, glutamate, homoserine, homocysteine, phosphoserine, alanine, L-malate, oxaloacetate and succinate, all of which are common metabolites with obvious structural similarities to the proper (cognate) substrate.

Discrimination (and hence specificity) does not depend simply on the relative affinity of the substrates for the enzyme, indeed it is acknowledged that the substrates should be compared based on the ratio of K_{cat}/K_m values for their reactions [14] – a ratio also called the discrimination factor [8]. A pertinent question therefore is whether there are intrinsic limits to this ratio. One potential way to address this issue is through application of transition state theory. According to this theory, the logarithm of K_{cat}/K_m is proportional to the free-energy difference ($\Delta G^{\neq}$) between the free enzyme and substrate and the transition state complex [12] (Box 2). Thus, whenever transition state theory is applicable, differences in K_{cat}/K_m between different substrates reflect their different binding energies in the transition state.

In addition, the amount of binding energy provided by simple groups is finite. Hence, when comparing substrates with similar structures, the difference in binding energies ($\Delta\Delta G^{\neq}$) must

Box 2. Specificity and Binding Energy

In transition state theory, the transition state is treated as if it were a stable entity in thermodynamic quasi-equilibrium with the ground state [i.e., the reactant(s)]. In a simple situation in which an enzyme (E) acts on a single substrate (S), K_{cat}/K_m describes precisely such an equilibrium (Figure IA) [76]. Accordingly, the logarithm of K_{cat}/K_m will be proportional to the free-energy difference between the free enzyme and substrate and the transition state complex (ΔG; Figure IB) [12]. Thus, whenever transition state theory is applicable, differences in K_{cat}/K_m between alternative substrates reflect their differential binding interactions in the transition state. Specificity can arise from differential interactions already formed in the initial enzyme–substrate complex (in which case they will contribute mostly to K_m; Figure IC), or forming only in the transition state complex (hence contributing to K_{cat}; Figure ID), but the total amount of binding energy associated with a specific group will be the same irrespective of where, along the reaction coordinates, the group establishes interactions with the enzyme.

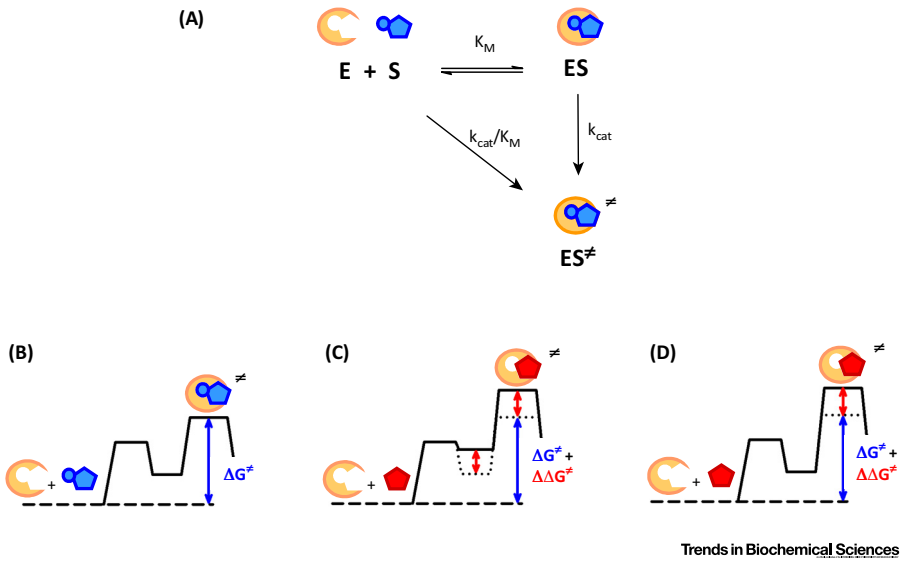


Figure I. Relationships between Substrate Discrimination and Binding Energy According to Transition-State Theory.

also be finite, relatively small, and, in some cases, calculable. To illustrate this point, I next analyze three exemplary cases in which an enzyme is asked to discriminate against alternative substrates that are either slightly smaller or slightly larger than the cognate substrate (Figure 1).

Theoretical and Empirical Limits of Substrate Specificity

That substrate specificity is inherently limited was first glimpsed by Pauling 60 years ago [15] in relation to the process of aminoacyl-tRNA synthesis. He focused in particular on the case of isoleucyl-tRNA synthetase that must distinguish between isoleucine and valine. The two amino acids differ only by one methyl group, and the difference in binding energy between them could therefore not be greater than the energy provided by the terminal CH_3 group of L-Ile. This has been estimated to be worth at most 3 kcal/mol ([12] and references therein), which sets an upper limit of about 160-fold in the discrimination between valine and isoleucine by simple binding. Such a relatively low discrimination factor seems to be biologically unacceptable, in fact extant isoleucyl-tRNA synthetases possess a distinct proofreading function that selectively deacylates any mis-aminoacylated $tRNA^{Ile}$, achieving a better accuracy in an energy-expensive manner [16].

Evidently, the limit above does not apply solely to aminoacyl-tRNA synthetases, but is general for enzymes that must distinguish between two substrates differing by only one methyl group. Pairs of this type are not infrequent among common metabolites. A survey of the Brenda database [17] shows that the observed discrimination index of extant enzymes only rarely reaches the theoretical limit (Figure 1A).

One can also try to calculate the theoretical limits of discrimination in cases when the alternative substrate lacks a hydroxyl group compared to the cognate substrate. The binding energy provided by a hydroxyl depends essentially on the ability of the group to form hydrogen bonds,

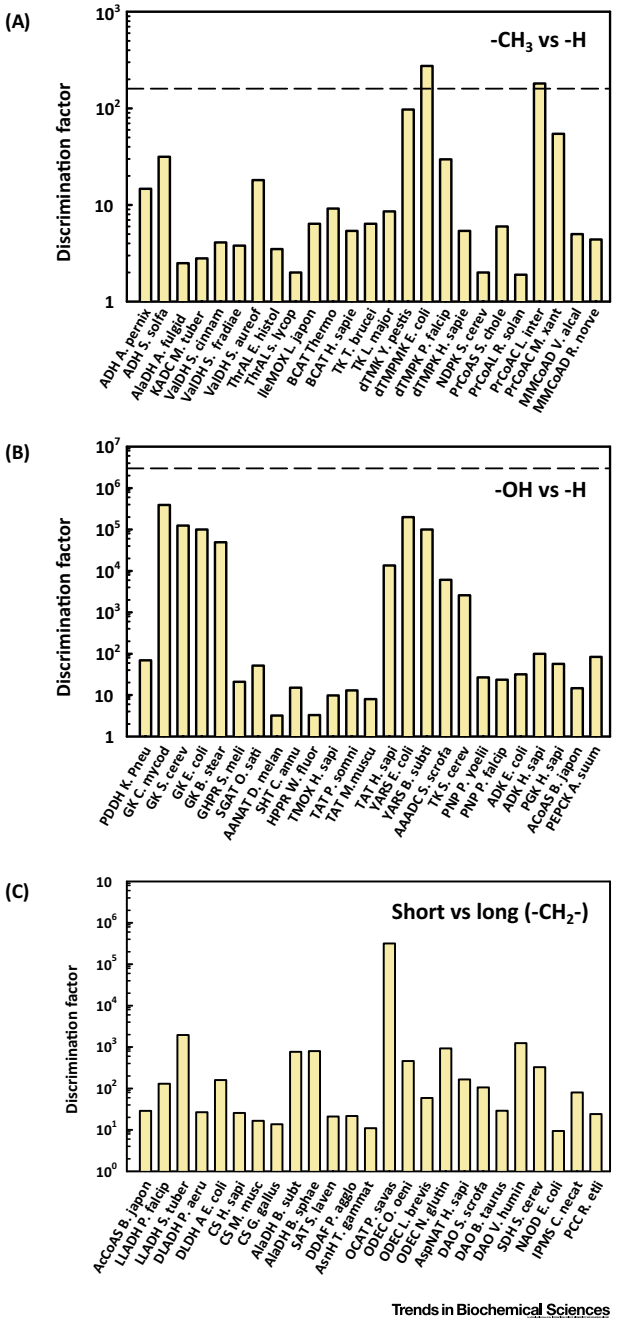


Figure 1. A Survey of Experimentally Determined Abilities of Enzymes to Discriminate between Substrates with Similar Structures. The substrates considered are metabolically available and, within each pair of substrates, the structural differences occur away from the reaction center. Information about the individual enzymes, species, and substrates, as well as references to the original studies, can be found in Tables S1–S3. (A) Cases in which the alternative substrate lacks a single CH_3 group with respect to the reference substrate. The dashed line represents a discrimination factor of 160, which is the approximate limit for selectivity at 25 °C (the limit will vary with temperature). (B) Cases in which the alternative substrate lacks one OH with respect to the reference substrate. The dashed line represents a discrimination factor of 4×10^6. (C) Cases in which the reference substrate is a linear molecule and the alternative substrate is longer by one methylene (-CH_2-) group.

and the thermodynamic stability of hydrogen bonds within macromolecules has been addressed in several mutagenesis studies. The maximum energy associated to hydrogen bonding in an uncharged donor–acceptor pair is ~2 kcal/mol, whereas a hydrogen bond to a charged partner can be worth up to 5 kcal/mol [18]. Because interactions with more than one charged partner would generate electrostatic interference, and because hydroxyl groups can form up to three hydrogen bonds, one rough estimate is that an extra hydroxyl group could be worth up to 9 kcal/mol of binding energy, or a factor of $\sim 4 \times 10^6$-fold in selectivity [19]. Figure 1B shows a set of data from the literature pertaining to this case. Although the sample may be somewhat biased (because catalytic parameters for substrates showing very low reactivity are less likely to be determined), it nevertheless suggests that discrimination is often much lower than the theoretical maximum (Figure 1B and Table S2 in the supplemental information online).

Repulsive interactions towards undesirable substrates are arguably a very efficient means to implement specificity [8]. In particular, it could be assumed that discrimination against a substrate that is larger than the cognate substrate may be achieved easily by restricting the active site and exploiting steric repulsion [12]. However, enzymes are relatively flexible (and there is experimental evidence that flexibility may correlate with substrate promiscuity; e.g., [20–22]) while active sites must have an opening towards the solvent; it has been suggested that such features in specific cases may allow conversion of bulkier alternative substrates [9].

In this instance the actual limits to specificity are nearly impossible to estimate *a priori*. Figure 1C examines a practical case, considering how enzymes that act on a linear substrate discriminate against a competitor metabolite containing one additional methylene group. In this case too, many enzymes show relatively modest selectivity. Note that alternative activities may occur even with substrates that are much larger than the canonical substrate. For example, it was shown that several mammalian transaminases, whose standard substrates are simple amino acids, can transaminate the tripeptide glutathione with low efficiency [23].

Reactivity with Alternative Substrates May Be Advantageous for Fitness

It is assumed that, in the earliest phases of metabolism, enzymes were generalists that acted on many substrates. In the course of evolution, most tended to become specialists – more and more selective (and arguably efficient) for a given substrate [1,24,25]. Nevertheless, as shown in Figure 1, it seems that the maximum possible discrimination between available substrates is seldom reached by extant metabolic enzymes. This observation is consistent with the idea that the actual degree of substrate specificity in these catalysts depends largely on evolutionary factors and dynamics.

In the context of metabolism, the activity of an enzyme with alternative substrates could conceivably produce three types of evolutionary responses. Sometimes, activity towards different substrates is simply advantageous for fitness, and is hence retained or selected for during evolution. In addition to digestive enzymes or detoxifying catalysts such as cytochromes P450 [26], there are textbook instances of substrate-promiscuous enzymes operating in the middle of primary metabolism, such as ketol-acid reductoisomerase [27] and branched-chain amino acid transaminase [28], each of which is involved in the biosynthesis of both valine and isoleucine, catalyzing distinct (but analogous) reactions in the two pathways. Similarly, transketolase [29] and adenylosuccinate lyase [30] catalyze reactions with different substrates in the same pathway. Other enzymes catalyzing distinct reactions in primary metabolism have been described, often in specific organisms (e.g., [27,31–35]). There have even been reports of bifunctional pathways, such as one in which the biosynthesis of both L-lysine and L-ornithine is accomplished using the same set of enzymes [36].

To provide an advantage, a substrate-promiscuous enzyme does not need to act with comparable efficiency against two alternative substrates because even a slow side reaction may sometimes be beneficial – for example when its product is required at low levels by the cell. For instance, in microorganisms, some enzymes from primary metabolism can also participate (acting on different substrates) in the biosynthesis of antibiotics [37,38]. In mammals, a similar case may perhaps be the synthesis of D-aspartate. This D-amino acid occurs at small but significant levels in the brain and acts as an endogenous co-agonist for synaptic *N*-methyl D-aspartate receptors [39]. Contrary to earlier claims on the occurrence of a specific aspartate racemase, it is now believed that D-aspartate may be mainly produced by serine racemase, even though this enzyme catalytically converts L-aspartate ~50-fold less efficiently than L-serine [40].

The immediate advantage of using of a single catalyst for different metabolic reactions is parsimony [41]; however, there are also obvious drawbacks. One potential problem is related to flux efficiency – both because it may be difficult to combine substrate versatility with high rate of the catalyzed reactions in the same enzyme ([8,42] and references therein) and because the alternative substrates may interfere with each other (e.g., [30,43]). Another drawback is that two activities carried out by a single enzyme cannot be regulated separately by modulating the expression level or subcellular localization of the enzyme (intuitively, this is much less of a problem if the two activities are required in the same pathway). A network analysis study on *Escherichia coli* [1] has provided support to the notion that promiscuous enzymes are less abundant in pathways where a high metabolic flux is needed, or where the flux must be regulated more tightly.

Detrimental Activities with Alternative Substrates May Be Redressed by Repair Enzymes

An opposite scenario arises when the activity of a metabolic enzyme with an alternative substrate is seriously detrimental to fitness. In fact, every such reaction may generate a product that is useless for the cell (a metabolic dead-end, implying a waste of resources) or that is even toxic [44], and this is expected to elicit strong evolutionary pressure to improve the specificity of the enzyme. However, as discussed, there are limits to such an improvement. Preventing access of the enzyme to the alternative substrate by compartmentalization may also be often impossible or insufficient.

Instead, evolution can lead to the development of *ad hoc* enzymes that destroy or recycle the unwanted products of these side reactions. In fact, there is a growing list of 'metabolite repair' enzymes whose sole purpose appears to be the correction of 'errors' committed by enzymes of intermediary metabolism [45,46], most often attributable to imperfect substrate specificity. These resemble the proofreading activities that improve the accuracy of aminoacyl-tRNA synthesis [45]; the repair task is sometimes even allocated to a separate domain of the promiscuous metabolic enzyme, not unlike what happens in many aminoacyl-tRNA synthetases [47].

One exemplary case is the activity of malate dehydrogenase with α-ketoglutarate instead of with the standard substrate oxaloacetate. The relative efficiency of the promiscuous reaction is very low, and the discrimination index of malate dehydrogenase is estimated to be $\sim 10^6$ [48], in other words higher than for any of the enzymes in Figure 1C. Despite this, the alternative reaction is not physiologically insignificant because both α-ketoglutarate and malate dehydrogenase are abundant in cells, whereas L-2-hydroxyglutarate is a metabolic dead-end, which favors its accumulation over time. Normally, such accumulation is prevented by a dedicated

repair enzyme, a FAD-dependent dehydrogenase that irreversibly reoxidizes L-2-hydroxyglutarate to α-ketoglutarate. The fact that, in humans, a deficiency of the repair enzyme causes L-2-hydroxyglutaric aciduria (a severe neurological disorder) [49] underscores the potential dangers of even slow side-activities of substrate-promiscuous enzymes.

Similarly, the slow transamination of glutathione carried out by many transaminases (mentioned earlier) generates an apparently useless product, deaminated glutathione. This compound, however, can be hydrolyzed and recycled by a 'repair' amidase that has been identified in mammals, yeast, and several glutathione-producing bacteria [23]. It is notable that the compound processed by this amidase appears to originate from the side activities of an entire class of enzymes. This attests to the efficiency of metabolite repair as an evolutionary solution to the inherent imperfection of metabolic catalysts. An analogous and even more striking example is that of a 'repair' phosphatase that degrades inhibitory compounds generated by the substrate-promiscuous activities of two glycolytic enzymes – glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase (Figure 2) [46].

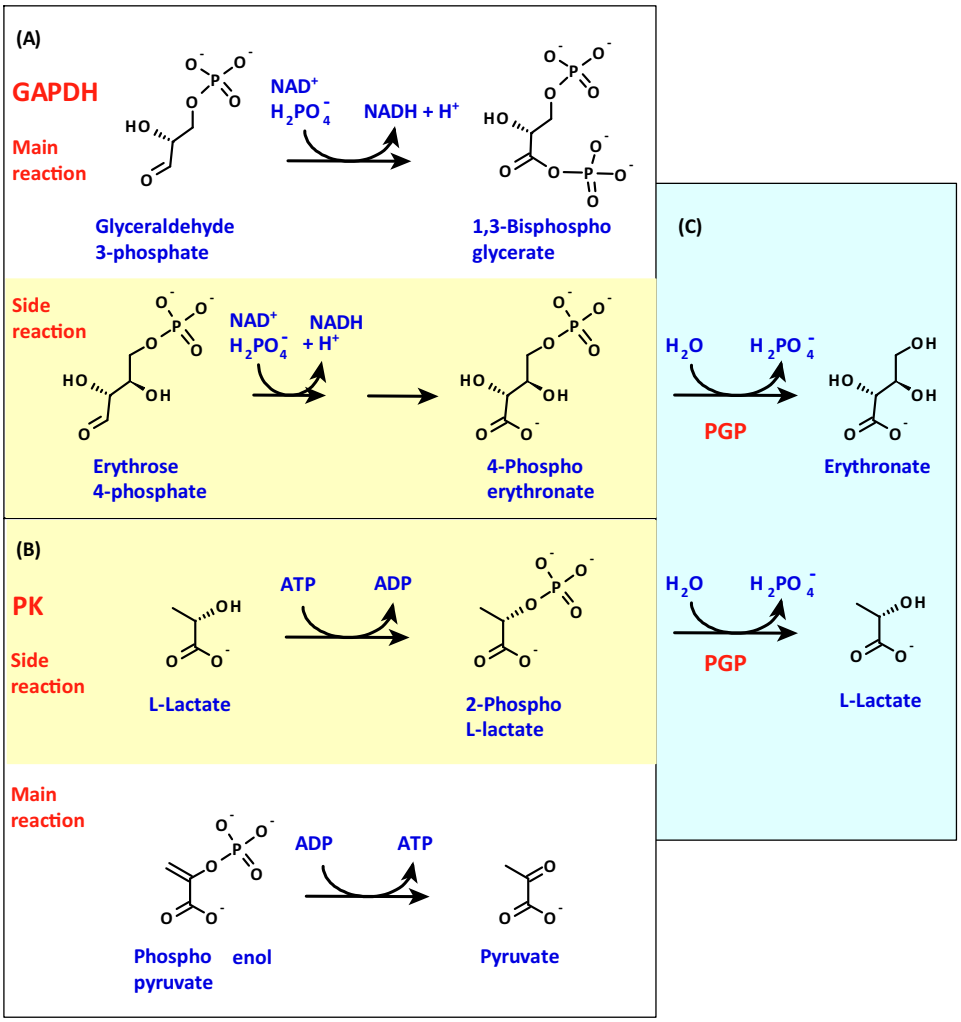
It must be noted that metabolite repair enzymes, by themselves, do not prevent the formation of useless or toxic side-reaction products, but simply curb their accumulation. Furthermore, the very presence of a repair system that remedies the effects of an undesired side reaction may be expected, somewhat paradoxically, to lower the evolutionary pressure for metabolic enzymes to maximize their specificity.

When Does Activity with Alternative Substrates Depend on Neutral Drift?

In a final scenario, the alternative reaction catalyzed by an enzyme might have no significant (positive or negative) effects on the system fitness, and thus it would be invisible to natural selection and essentially subject to neutral drift. This is often assumed as the 'default' case [11], but positive proof is scarce. We have seen above that some reactions which, based on the discrimination factor, would appear very negligible, do become liabilities because the promiscuous enzyme and the alternative substrate are abundant in the cell. As a minimum, an estimate of the biological significance of the alternative reaction (and hence of the selective pressure to which it is subject) should take into account the effective rate of its occurrence *in vivo*.

In addition, one may think that secondary activities that generate mainstream metabolites should be essentially irrelevant for fitness, and therefore depend on neutral drift. Perhaps the best case in point comes from a study by Khanal *et al.* [50], who analyzed the ability of nine microbial γ-glutamylphosphate reductases (ProA) to use the alternative substrate *N*-acetyl-glutamyl phosphate (which is usually processed by a distinct reductase, ArgC). The discrimination indices for the nine enzymes were always very high (>40 000) while the absolute efficiency of the secondary activity varied up to 50-fold between species. Furthermore, the enzyme from *E. coli* could not compensate for the loss of ArgC. All these features were consistent with the side activity of ProA being biologically irrelevant [50].

In contrast to the ProA case, however, enzymes whose secondary activities are metabolically redundant, and which can under some circumstances surrogate the function of more specialized catalysts, are often observed (e.g., [51,52]), and it cannot be excluded that the non-strict substrate specificity of these enzymes may be retained because it is beneficial. Speaking more generally, there are suggestions that side activities generating mainstream metabolites may provide advantages or disadvantages under particular conditions and therefore be under active selection [44].



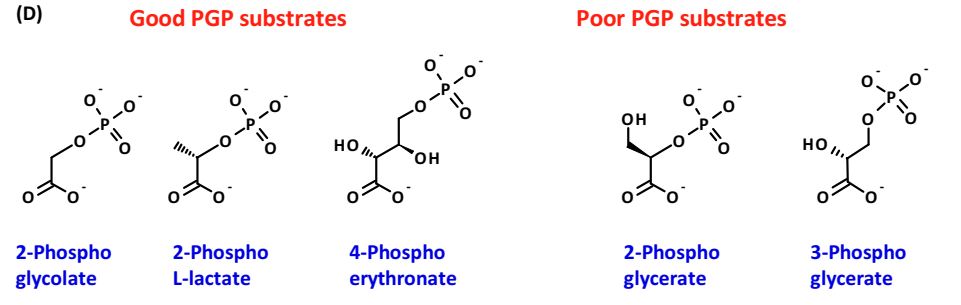


Figure 2. Detrimental Activities of Two Glycolytic Enzymes on Alternative Substrates Are Corrected by a Single Repair Enzyme. Panels (A) and (B) show the side activities of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) on erythrose 4-phosphate and of pyruvate kinase (PK) on lactate, leading ultimately to the formation of 4-phosphoerythronate and 2-phospho-L-lactate, respectively. As detailed by Collard *et al.* [46], these nonstandard metabolites are inhibitory for other enzymes and severely interfere with carbohydrate metabolism. (C) A repair enzyme, known as phosphoglycolate phosphatase (PGP) in mammals, is able to dephosphorylate both compounds, reconverting them to harmless products. (D) The mammalian PGP is also very active with phosphoglycolate, but it shows little or no detectable activity towards standard glycolytic intermediates such as 2-phosphoglycerate.

For example, consider an aminotransferase whose primary substrate is not alanine. It might be assumed that a side activity on alanine may not be strongly favored or unfavored by evolution because the product, pyruvate, is a standard metabolic intermediate. However, the alternative reaction can contribute to metabolic redundancy, which may increase fitness under particular circumstances while ensuring the resilience of the metabolic system. In *E. coli*, for example, there are three major alanine aminotransferases plus up to seven other enzymes with substantial activity towards alanine. Such a remarkable redundancy has been proposed to be important to ensure a supply of D-alanine for peptidoglycan synthesis [53]. Conversely, there may be significant counter-selection against side activities that, despite generating standard metabolites, represent an objective waste of energy or resources. An example could be the transamination of glutamine, which tends to be reserved to processes that need to be metabolically irreversible [54].

Another case worth considering is that of kinases and ligases. Many of these enzymes show a strong specificity for ATP, while others can use different nucleoside triphosphates (NTPs) with comparable efficiencies (e.g., [55,56]). These different behaviors could be the random results of distinct evolutionary histories, but again positive selection for different levels of specificity cannot be ruled out *a priori*. ATP is usually the most abundant NTP, and the activity of enzymes strictly dependent on ATP will therefore respond more promptly to the energy status of the cell, and this may be important in the perspective of regulating metabolic fluxes. On the other hand, kinases or ligases that are less selective may allow an organism to scavenge alternative NTPs depending upon their availability, thus responding to the need to preserve the efficiency of an important reaction under diverse metabolic conditions [55,56].

In sum, although it is reasonable to assume that the activities of an enzyme on alternative (metabolically available) substrates may often depend on neutral drift, the point is difficult to prove because various types of selective pressures can be at play, and these will be different in different organismal lineages [57,58]. For many enzymes, the boundaries between an evolutionarily selected degree of substrate promiscuity and an evolutionarily irrelevant degree of sloppiness may be difficult to establish (See Outstanding Questions).

Substrate Promiscuity Contributes to Underground Metabolism

As seen in the sections above, enzymes have an unavoidable tendency to act on alternative, available substrates. Even when (perhaps not very often) this tendency is restrained to near the minimum level allowed by chemistry, and even in the presence of 'repair' systems, these enzymes will generate alternative products, many of which will be nonstandard metabolites. Substrate promiscuity must be hence considered as a major contribution to the complexity of the metabolome – together with catalytic promiscuity and other factors such as the non-enzymatic transformation of metabolites and the introduction of xenobiotics [44,45]. The contribution of substrate promiscuity is in part predictable [59], and understanding it may be necessary for thorough mapping the metabolome, one of the current challenges of biomolecular sciences [60].

The compounds generated by side activities of metabolic enzymes sometimes contribute to a subterranean ensemble of reactions, proceeding in general at extremely low fluxes, that are collectively termed 'underground metabolism' [61,62]. It is believed that this messy network of reactions, while somehow inevitable, may represent a basis for the further evolution and diversification of metabolism [54,63].

That messiness may be central to the evolution of new beneficial functions is well exemplified by many pathways in secondary (specialized) metabolism. In contrast to primary metabolism,

specialized metabolism contributes to fitness by producing molecules that interfere with the biology of other organisms (e.g., environmental competitors). Many pathways in specialized metabolism tend to include multiple enzymes with non-strict substrate specificity and to produce an entire suite of chemically similar, but distinct, compounds [64–66]. The most direct advantage, in this case, is that the ability to generate a multiplicity of secondary metabolites offers more flexibility in responding to different, and evolving, organisms in the environment.

In primary metabolism, the 'messy' underground reactions could also, in some cases, facilitate evolutionary adaptation to new environments or provide bypass to genetic lesions [61,62]. For example, Kim *et al.* found that deletion of gene *PdxB*, that is required in *E. coli* for biosynthesis of pyridoxal phosphate, could be bypassed by at least three 'underground' routes (one of which was elucidated in detail, and was found to involve the promiscuous activity of homoserine kinase on an alternative substrate) [67]. A fourth route was reported by another group, using promiscuity prediction software [68]. Earlier, Patrick and coworkers had

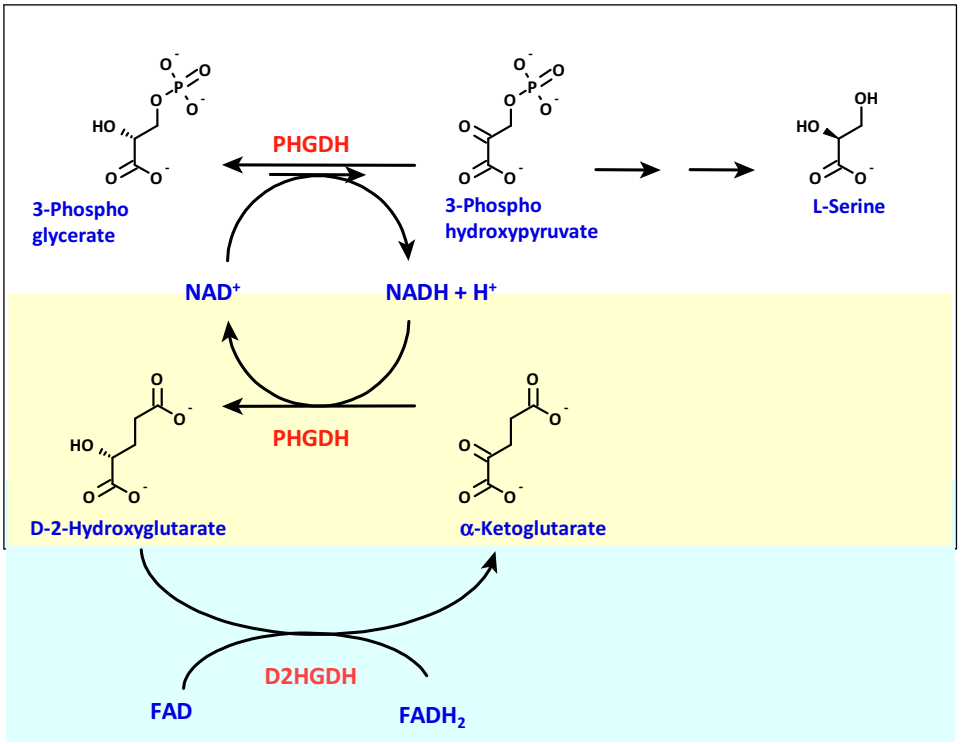


Figure 3. Reactions Catalyzed by 3-Phosphoglycerate Dehydrogenase (PGDH). PGDH interconverts the glycolytic intermediate D-3-phosphoglycerate into phosphohydroxypyruvate. This is the first step of the phosphorylated pathway for L-serine synthesis (upper part of the panel). The human enzyme also catalyzes, as a side reaction, the reduction of α-ketoglutarate to D-2-hydroxyglutarate (middle part of the panel) [70]; this compound is considered to be an oncometabolite, and its accumulation in the cell is countered by a dedicated repair enzyme, D-2-hydroxyglutarate dehydrogenase (D2HGDH; bottom part of the panel) [45]. Although in humans the canonical and side reactions of PGDH are apparently not connected, they are tightly coupled in the *E. coli* enzyme. This enzyme does not release NADH at the end of the canonical reaction, and requires that the coenzyme is reoxidized *in situ* by α-ketoglutarate [72]. The cycle that ensues (including the activity of D2HGDH to recycle D-2-hydroxyglutarate) helps to thermodynamically drive the production of phosphohydroxypyruvate and the overall pathway [71].

found that 41 of 104 *E. coli* knockout strains, that were unable to grow on minimal medium, could be rescued by overexpression of at least one gene different from the deleted gene. In several cases, the multicopy suppressor was not homologous to the deleted gene, and its effect was attributed to a promiscuous activity of the encoded enzyme or to a metabolic pathway bypass [69].

Underground reactions may also become – at some point and in some organisms – incorporated into mainstream primary metabolism. A possible example is the promiscuous activity of phosphoglycerate dehydrogenase with α-ketoglutarate, yielding D-2-hydroxyglutarate. In humans, D-2-hydroxyglutarate is an oncometabolite [70] and requires a repair enzyme to be removed [45]. In bacteria such as *E. coli*, however, the promiscuous activity of phosphoglycerate dehydrogenase is enhanced and is tightly coupled to the repair activity to thermodynamically drive L-serine biosynthesis [71] (Figure 3).

Concluding Remarks

The results and arguments reviewed here show that, although perfect substrate specificity is essentially unattainable, metabolic enzymes are often much less selective than they could be. Furthermore, enzyme activities with alternative substrates are subject to distinct selective pressures. They can be fostered by natural selection until they reach levels that are most useful for fitness, or repressed to levels at which they are no longer harmful. When deleterious side-reactions cannot be controlled efficiently enough, metabolite repair enzymes may evolve which limit the buildup of unwanted products. In any case, enzymes that act on alternative substrates create complexity in the systems they contribute to. Although this complicates our understanding of cellular behavior, it is likely to be important for future metabolic adaptations of the host organisms.

Supplemental Information

Supplemental information associated with this article can be found online at https://doi.org/10.1016/j.tibs.2018.09.015.

Outstanding Questions

The approximate limits of selectivity have been calculated when comparing substrates that differ by one methyl or by one hydroxyl group. Is it possible to establish the limits of selectivity for any given pairs of alternative substrates?

What is the nature and role of evolutionary pressures and genome dynamics in the evolution of substrate specificity?

'Multifunctional' enzymes catalyzing distinct reactions in primary metabolism have been occasionally identified in specific organisms. Are there systematic (e.g., computational, metabolomic, . . .) methods to identify more of these?

Is it possible to predict systematically the occurrence of non-standard metabolites derived from the activity of substrate-promiscuous enzymes? Which of these predicted compounds can be actually identified in metabolomics experiments?

Recognition of the role of metabolite repair enzymes is relatively recent, but the list of such enzymes is steadily growing. Many more repair enzymes are likely to exist. Are there general strategies to identify them?

References

1. Nam, H. *et al.* (2012) Network context and selection in the evolution to enzyme specificity. *Science* 337, 1101–1104
2. Martínez-Núñez, M.A. *et al.* (2017) Tracing the repertoire of promiscuous enzymes along the metabolic pathways in archaeal organisms. *Life (Basel)* 7, 30
3. O'Brien, P.J. and Herschlag, D. (1999) Catalytic promiscuity and the evolution of new enzymatic activities. *Chem. Biol.* 6, R91–R105
4. Hult, K. and Berglund, P. (2007) Enzyme promiscuity: mechanism and applications. *Trends Biotechnol.* 25, 231–238
5. Taylor Ringia, E.A. *et al.* (2004) Evolution of enzymatic activity in the enolase superfamily: functional studies of the promiscuous o-succinylbenzoate synthase from *Amycolatopsis*. *Biochemistry* 43, 224–229
6. Magnani, G. *et al.* (2013) Completing the folate biosynthesis pathway in *Plasmodium falciparum*: *p*-aminobenzoate is produced by a highly divergent promiscuous aminodeoxychorismate lyase. *Biochem. J.* 455, 149–155
7. Tarcz, S. *et al.* (2014) Substrate and catalytic promiscuity of secondary metabolite enzymes: O-prenylation of hydroxyxanthones with different prenyl donors by a bisindolyl benzoquinone C- and N-prenyltransferase. *RSC Adv.* 4, 17986
8. Tawfik, D.S. (2014) Accuracy-rate tradeoffs: how do enzymes meet demands of selectivity and catalytic efficiency? *Curr. Opin. Chem. Biol.* 21C, 73–80
9. Copley, S.D. (2015) An evolutionary biochemist's perspective on promiscuity. *Trends Biochem. Sci.* 40, 72–78
10. Bar-Even, A. *et al.* (2011) The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry* 50, 4402–4410
11. Newton, M. *et al.* (2018) Enzyme evolution: innovation is easy, optimization is complicated. *Curr. Opin. Struct. Biol.* 48, 110–116
12. Fersht, A.R. (1999) *Structure and Mechanism in Protein Science – A Guide to Enzyme Catalysis and Protein Folding,* Freeman & Co
13. Herschlag, D. (1988) The role of induced fit and conformational changes of enzymes in specificity and catalysis. *Bioorg. Chem.* 16, 62–96
14. Cornish-Bowden, A. and Cardenas, M.L. (2010) Specificity of non-Michaelis-Menten enzymes: necessary information for analyzing metabolic pathways. *J. Phys. Chem. B* 114, 16209–16213
15. Pauling, L. (1958) The probability of errors in the process of synthesis of protein molecules. In *Festschrift fur Prof Dr Arthur Stoll*, pp. 597–602, Birkhauser Verlag
16. Perona, J.J. and Gruic-Sovulj, I. (2014) Synthetic and editing mechanisms of aminoacyl-tRNA synthetases. *Top. Curr. Chem.* 344, 1–41
17. Schomburg, I. *et al.* (2017) The BRENDA enzyme information system – from a database to an expert system. *J. Biotechnol.* 261, 194–206
18. Fersht, A.R. and Serrano, L. (1993) Principles of protein stability derived from protein engineering experiments. *Curr. Opin. Struct. Biol.* 3, 75–83
19. Wolfenden, R. and Kati, W.M. (1991) Testing the limits of protein-ligand binding discrimination with transition-state analogue inhibitors. *Acc. Chem. Res.* 24, 209–215
20. Kristiansson, H. and Timson, D.J. (2011) Increased promiscuity of human galactokinase following alteration of a single amino acid residue distant from the active site. *Chembiochem* 12, 2081–2087

21. Noda-García, L. *et al.* (2013) Evolution of substrate specificity in a recipient's enzyme following horizontal gene transfer. *Mol. Biol. Evol.* 30, 2024–2034
22. Zou, T. *et al.* (2015) Evolution of conformational dynamics determines the conversion of a promiscuous generalist into a specialist enzyme. *Mol. Biol. Evol.* 32, 132–143
23. Peracchi, A. *et al.* (2017) Nit1 is a metabolite repair enzyme that hydrolyzes deaminated glutathione. *Proc. Natl. Acad. Sci. U. S. A.* 114, E3233–E3242
24. Mannervik, B. and Runarsdottir, A. (2010) The quest for molecular quasi-species in ligand-activity space and its application to directed enzyme evolution. *FEBS Lett.* 584, 2565–2571
25. Khersonsky, O. and Tawfik, D.S. (2010) Enzyme promiscuity: a mechanistic and evolutionary perspective. *Annu. Rev. Biochem.* 79, 471–505
26. Behrendorff, J. *et al.* (2015) Directed evolution of cytochrome P450 enzymes for biocatalysis: exploiting the catalytic versatility of enzymes with relaxed substrate specificity. *Biochem. J.* 467, 1–15
27. Verdel-Aranda, K. *et al.* (2015) Molecular annotation of ketol-acid reductoisomerases from *Streptomyces* reveals a novel amino acid biosynthesis interlock mediated by enzyme promiscuity. *Microb. Biotechnol.* 8, 239–252
28. Yu, X. *et al.* (2014) The specificity and kinetic mechanism of branched-chain amino acid aminotransferase from *Escherichia coli* studied with a new improved coupled assay procedure and the enzyme's potential for biocatalysis. *FEBS J.* 281, 391–400
29. Kochetov, G.A. and Solovjeva, O.N. (2014) Structure and functioning mechanism of transketolase. *Biochim. Biophys. Acta* 1844, 1608–1618
30. Ray, S.P. *et al.* (2013) Inherent properties of adenylosuccinate lyase could explain S-Ado/SAICAr ratio due to homozygous R426H and R303C mutations. *Biochim. Biophys. Acta* 1834, 1545–1553
31. Poon, W.W. *et al.* (1999) Yeast and rat Coq3 and *Escherichia coli* UbiG polypeptides catalyze both O-methyltransferase steps in coenzyme Q biosynthesis. *J. Biol. Chem.* 274, 21665–21672
32. Barona-Gómez, F. and Hodgson, D.A. (2003) Occurrence of a putative ancient-like isomerase involved in histidine and tryptophan biosynthesis. *EMBO Rep.* 4, 296–300
33. Adams, N.E. *et al.* (2014) Promiscuous and adaptable enzymes fill 'holes' in the tetrahydrofolate pathway in *Chlamydia* species. *MBio* 5, e01378-01314
34. Shimizu, T. *et al.* (2017) Structure and function of an ancestral-type β-decarboxylating dehydrogenase from *Thermococcus kodakarensis*. *Biochem. J.* 474, 105–122
35. Ferla, M.P. *et al.* (2017) Primordial-like enzymes from bacteria with reduced genomes. *Mol. Microbiol.* 105, 508–524
36. Yoshida, A. *et al.* (2016) Lysine biosynthesis of *Thermococcus kodakarensis* with the capacity to function as an ornithine biosynthetic system. *J. Biol. Chem.* 291, 21630–21643
37. Mahlert, C. *et al.* (2007) Stereospecific enzymatic transformation of α-ketoglutarate to (2S,3R)-3-methyl glutamate during acidic lipopeptide biosynthesis. *J. Am. Chem. Soc.* 129, 12011–12018
38. Ishikawa, F. *et al.* (2016) *In vitro* investigation of crosstalk between fatty acid and polyketide synthases in the andrimid biosynthetic assembly line. *Chembiochem* 17, 2137–2142
39. Errico, F. *et al.* (2015) D-Aspartate: an endogenous NMDA receptor agonist enriched in the developing brain with potential involvement in schizophrenia. *J. Pharm. Biomed. Anal.* 116, 7–17
40. Uda, K. *et al.* (2016) Distribution and evolution of the serine/aspartate racemase family in invertebrates. *Amino Acids* 48, 387–402
41. Price, D.R. and Wilson, A.C. (2014) A substrate ambiguous enzyme facilitates genome reduction in an intracellular symbiont. *BMC Biol.* 12, 110
42. Gatti-Lafranconi, P. and Hollfelder, F. (2013) Flexibility and reactivity in promiscuous enzymes. *Chembiochem* 14, 285–292
43. Sun, R. and Wang, L. (2014) Thymidine kinase 2 enzyme kinetics elucidate the mechanism of thymidine-induced mitochondrial DNA depletion. *Biochemistry* 53, 6142–6150
44. Piedrafita, G. *et al.* (2015) The impact of non-enzymatic reactions and enzyme promiscuity on cellular metabolism during (oxidative) stress conditions. *Biomolecules* 5, 2101–2122
45. Linster, C.L. *et al.* (2013) Metabolite damage and its repair or preemption. *Nat. Chem. Biol.* 9, 72–80
46. Collard, F. *et al.* (2016) A conserved phosphatase destroys toxic glycolytic side products in mammals and yeast. *Nat. Chem. Biol.* 12, 601–607
47. Manandhar, M. and Cronan, J.E. (2013) Proofreading of non-cognate acyl adenylates by an acyl-coenzyme a ligase. *Chem. Biol.* 20, 1441–1446
48. Van Schaftingen, E. *et al.* (2013) Metabolite proofreading, a neglected aspect of intermediary metabolism. *J. Inherit. Metab. Dis.* 36, 427–434
49. Rzem, R. *et al.* (2004) A gene encoding a putative FAD-dependent L-2-hydroxyglutarate dehydrogenase is mutated in L-2-hydroxyglutaric aciduria. *Proc. Natl. Acad. Sci. U. S. A.* 101, 16849–16854
50. Khanal, A. *et al.* (2015) Differential effects of a mutation on the normal and promiscuous activities of orthologs: implications for natural and directed evolution. *Mol. Biol. Evol.* 32, 100–108
51. Vorobieva, A.A. *et al.* (2014) *Escherichia coli* D-malate dehydrogenase, a generalist enzyme active in the leucine biosynthesis pathway. *J. Biol. Chem.* 289, 29086–29096
52. Jerlstrom Hultqvist, J. *et al.* (2018) A bacteriophage enzyme induces bacterial metabolic perturbation that confers a novel promiscuous function. *Nat. Ecol. Evol.* 2, 1321–1330
53. Lal, P.B. *et al.* (2014) The redundant aminotransferases in lysine and arginine synthesis and the extent of aminotransferase redundancy in *Escherichia coli*. *Mol. Microbiol.* 94, 843–856
54. Danchin, A. and Sekowska, A. (2013) The logic of metabolism and its fuzzy consequences. *Environ. Microbiol.* 16, 19–28
55. Gallois-Montbrun, S. *et al.* (2004) Broad specificity of human phosphoglycerate kinase for antiviral nucleoside analogs. *Biochem. Pharmacol.* 68, 1749–1756
56. Salaemae, W. *et al.* (2015) Nucleotide triphosphate promiscuity in *Mycobacterium tuberculosis* dethiobiotin synthetase. *Tuberculosis* 95, 259–266
57. Verduzco-Castro, E.A. *et al.* (2016) Co-occurrence of analogous enzymes determines evolution of a novel (βα)8-isomerase subfamily after non-conserved mutations in flexible loop. *Biochem. J.* 473, 1141–1152
58. Juárez-Vázquez, A.L. *et al.* (2017) Evolution of substrate specificity in a retained enzyme driven by gene loss. *Elife* 6, e22679
59. Jeffryes, J. *et al.* (2015) MINEs: open access databases of computationally predicted enzyme promiscuity products for untargeted metabolomics. *J. Cheminform.* 28, 44
60. da Silva, R.R. *et al.* (2015) Illuminating the dark matter in metabolomics. *Proc. Natl. Acad. Sci. U. S. A.* 112, 12549–12550
61. D'Ari, R. and Casadesús, J. (1998) Underground metabolism. *Bioessays* 20, 181–186
62. Notebaart, R.A. *et al.* (2018) Underground metabolism: network-level perspective and biotechnological potential. *Curr. Opin. Biotechnol.* 49, 108–114
63. Noda-Garcia, L. *et al.* (2018) Metabolite–enzyme coevolution: from single enzymes to metabolic pathways and networks. *Annu. Rev. Biochem.* 87, 187–216
64. Fischbach, M.A. and Clardy, J. (2007) One pathway, many products. *Nat. Chem. Biol.* 3, 353–355
65. Weng, J.K. and Noel, J.P. (2012) The remarkable pliability and promiscuity of specialized metabolism. *Cold Spring Harb. Symp. Quant. Biol.* 77, 309–320
66. Guo, J. *et al.* (2014) Specificity and promiscuity at the branch point in gentamicin biosynthesis. *Chem. Biol.* 22, 608–618
67. Kim, J. *et al.* (2010) Three serendipitous pathways in *E. coli* can bypass a block in pyridoxal-5′-phosphate synthesis. *Mol. Syst. Biol.* 6, 436
68. Oberhardt, M.A. *et al.* (2016) Systems-wide prediction of enzyme promiscuity reveals a new underground alternative route for

pyridoxal 5′-phosphate production in *E. coli*. *PLoS Comput. Biol.* 12, e1004705

69. Patrick, W.M. *et al.* (2007) Multicopy suppression underpins metabolic evolvability. *Mol. Biol. Evol.* 24, 2716–2722
70. Fan, J. *et al.* (2015) Human phosphoglycerate dehydrogenase produces the oncometabolite D-2-hydroxyglutarate. *ACS Chem. Biol.* 10, 510–516
71. Zhang, W. *et al.* (2017) Coupling between D-3-phosphoglycerate dehydrogenase and D-2-hydroxyglutarate dehydrogenase drives bacterial L-serine synthesis. *Proc. Natl. Acad. Sci. U. S. A.* 114, E7574–E7582
72. Grant, G.A. (2018) Elucidation of a self-sustaining cycle in *Escherichia coli* L-serine biosynthesis that results in the conservation of the coenzyme, NAD^+. *Biochemistry* 57, 1798–1806
73. Holman, K.M. *et al.* (2017) Insights into substrate promiscuity of human seryl-tRNA synthetase. *RNA* 23, 1685–1699
74. Copley, S.D. (2017) Shining a light on enzyme promiscuity. *Curr. Opin. Struct. Biol.* 47, 167–175
75. Flores, C.L. and Gancedo, C. (2011) Unraveling moonlighting functions with yeasts. *IUBMB Life* 63, 457–462
76. Lienhard, G.E. (1973) Enzymatic catalysis and transition-state theory. *Science* 180, 149–154