Isozymes, moonlighting proteins and promiscous enzymes

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The structures of isoenzymes differ and yet these catalyse the same type of reaction. These structures evolved to suit the physiological needs and are located in different parts of cells or tissues. Moonlighting proteins represent the same structure performing very different biological functions. Biological promiscuity reveals that the same active sites can catalyse different types of reactions. These three different phenomena, all illustrate similar evolutionary strategies. Viewed together, it emerges that biologists need to take a hard look at the 'structure–function' paradigm as well as the notions of biological specificity. Meanwhile, biotechnologists continue to exploit the opportunities which 'nonspecificity' offers.

Keywords: Biological promiscuity, C–C bond formation, enzyme specificity, isoenzymes, moonlighting proteins.

'Scientists are seeing more and more evidence that we are specifically designed by mother nature to fool ourselves.'

Fooled by Randomness, Nassim Nicholas Taleb

FOR a long time, it was believed that the structure and function are so tightly correlated in biology that one protein structure corresponds to a specific biological function. This implied that the almost 'magical' quality of enzymes (the twin virtues of high catalytic efficiency and high specificity) are the result of an intelligent unique design in structure. Hence, it was perceived that only that structure could carry out that function (and no other function). The three terms in the title of this review are of different vintage but all the three represent the fact that the smartness of the biological function of proteins does not originate in the unique one-to-one relationship between structure and function. The discovery of isozymes was the first recognition of the fact that more than one structure can catalyse the same type of reaction.

Moonlighting proteins refer to a phenomenon, which in a way is the reverse of the above. One structure performs many functions. Gene fusions, splice variants or proteolytic fragments are excluded[1,2]. The phenomenon is not limited to enzymes; receptors, transmembrane channels, chaperones and ribosomal proteins are also included[1–3]. The functions performed, i.e. moonlighting are not even remotely related. The enzyme phosphoglucoisomerase, catalysing the interconversion of glucose-6-phosphate and fructose-6-phosphate during glycolysis can act as a neuroleukin! Moonlighting proteins show unambiguously that one structure can perform diverse functions. Gene sequences and bioinformatics tools are not always of much help in our search of moonlighting proteins. We do not know what we are looking for till we find it!

The third phenomenon of biological promiscuity also relates to the fact that one structure can carry out different functions. Essentially, it refers to enzymes catalysing reactions, which are quite unexpected and unrelated to the normal reactions which these are known to catalyse *in vivo* and *in vitro*. Again, so far promiscuity is unpredictable. Gene sequences or protein sequences are of limited help in predicting promiscuous reactions.

This brief review attempts to further bring out the fact that all three phenomena essentially relate to enzyme specificity and hence looking at these together may bring out an integrated view of molecular recognitions in the context of biological affinity.

Gradual changes in evolution of the concept of biological affinity

Much of what we call enzyme specificity relates to the binding energy of the (substrate) molecule to the active site of the enzyme. (In the case of allosteric enzymes, other sites on the enzyme are also capable of specific molecular recognitions.) It is now well established that thermodynamically speaking, binding energy of the substrate to the enzyme drives the catalysis[4,5]. Nevertheless, enzymes (and proteins) can bind to a wide variety of substances. Appreciation of this fact has led to an explosive growth of affinity-based bioseparations and bioanalyses[6–8]. Hence, the binding per se has been known to be highly promiscuous for a long time. Separations of protein on dye-columns and immobilized metal ion affinity chromatography (IMAC) are early known examples of promiscuous binding. Sometimes, these have been referred to as 'pseudo-affinity'[9]. Now of course, peptides and aptamers can be generated to achieve fairly specific binding with any protein[10]. So, in a way proteins have been known to be promiscuous for a long time as far as binding is concerned.

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Chemists tend to always view the large protein structures with scepticism and the literature is full of 'model enzyme systems' and biomemetics. Earlier, even biochemists have worried about it. Few of the old generation biochemists may remember the review by Koshland, which talked about 'junk amino acids' in protein structure[11]. There was an early appreciation that scaffold of the large protein structure is necessary for maintaining the integrity of the active site. Over the years, it has been slowly recognized that proteins/enzymes do not merely fulfil the 'need of the moment' for the cell (that itself is no mean feat!) by responding to the multiple stimuli in a smart manner, these structures should have 'evolvability'. A good tailor can design or 'evolve' many different kinds of dresses out of 6 yards of cloth; it may not be possible to make more than a handkerchief out of 9 inches × 6 inches of cloth. The three phenomena discussed in this review in a way also justify why proteins have to be large in size.

Isoenzymes

An old multivolume book has described structure, function and applications of isoenzymes in various areas[12]. The areas wherein isoenzymes play a significant role include developmental biology, genetics, clinical biochemistry and physiological functions. The early work is covered extensively in the above cited reference.

The IUPAC–IUB commission on biochemical nomenclature in its 'recommendations 1976' for 'nomenclature of multiple forms of enzymes' states 'that "multiple enzyme forms" in a single species should be known as isoenzymes (or isozymes). It is known that enzymes catalysing essentially the same reaction may differ in various ways. According to the original definition, which was meant to be purely operational, all these multiple forms should be termed "isozymes". It is therefore recommended that "multiple forms of the enzyme" should be used as a broad term covering all proteins catalysing the same reaction and occurring naturally in a single species. The term "isoenzyme" or "isozyme" should apply only to those multiple forms of enzymes arising from genetically determined differences in primary structure and not to those derived by modification of the same primary sequence'[13].

The recommendations of the commission notwithstanding, the books, which do discuss isoenzymes, often tend to ignore this. Isoenzymes are widely used in clinical biochemistry. While discussing this, Palmer[14] writes 'at least some of the alkaline phosphatases isoenzymes may be products of the same gene, the difference being due to post genetic modification, particularly glycosylation'. The 'isoforms' have a special significance in the context of regulatory approval of pharmaceutical proteins. For example, the recombinant proteins produced in higher expression systems will generally contain multiple variants having different extents of post-translational modifications (such as glycosylation). Such variations may also arise during downstream processing. These different 'isoforms' may have altered specific activity, solubility and stability. The Food and Drug Administration (FDA), USA mandates that process/product validation documents the structural details for each variant and the composition of the protein preparation in terms of percentage of each variant[15].

It is interesting to note that Fersht[4] calls isoenzymes 'multiple molecular forms of the same enzyme'. Voet *et al.*[16] are more precise and define isoenzymes as 'enzymes that catalyse the same reaction but are encoded by different genes and have different kinetic or regulatory properties'.

This confusion apart, let us look at the molecular basis for isoenzymes, its biochemical significance and evolutionary aspects. The most well-known example is of lactate dehydrogenase, which converts lactate into pyruvate. The enzyme found in many species is a tetramer with different combinations of two different subunits: M-form (as it predominates in skeletal muscle) and H-form (which occurs in heart muscle). Only tetramers (5 isoforms H_4, H_3M, H_2M_2, HM_3 and M_4) are catalytically active. As discussed clearly in most of the standard textbooks (see for example, Stryer[17]), lactate dehydrogenase (LDH) isoenzymes constitute an example of gene duplication to generate protein diversity. This in turn is vital for physiological cooperation among organs. The isoenzymes may occur in the same cell but at different places. (Isocitrate dehydrogenase isoenzymes in cytoplasm and mitochondrion are another textbook example.) As we will see shortly, moonlighting proteins also have this identical feature. The difference is that isoenzymes carry out the same reaction with different kinetic properties; a moonlighting protein carries out very different biochemical functions at two different locations (in a cell/tissue). These are two different evolutionary paths. Isoforms can also change when embryonic form differentiates to become an adult. This makes sense, as that is how various tissues can have different isoforms. As Palmer[14] points out, enzyme assays as such cannot distinguish between isoforms that is why 'unit of activity' has to be defined (and used) rather than just use molar catalytic activity. Carrying out assays in the presence or absence of inhibitors/activators can of course distinguish between isoforms and form the basis of many clinical tests such as that for prostate cancer.

It has not been always possible to understand the reason for isoforms. Both muscle and nonmuscle cells contain microfilaments of actin and myosin. Many multicellular organisms contain several actin genes. Besides, actins undergo acetylation and methylation post-translationally. All actins carry out the same biochemical activity. The advantage of having isoforms is not clear[18].

Does presence of isoforms have a bearing on the application of biocatalysts? One of the major applications of biocatalysis is in the preparation of chiral compounds,

which are valuable as agrochemicals and drugs[19–21]. One important issue is whether to use whole cells as biocatalysts or isolate enzymes. The cost of isolation and purification of enzymes constitute a very high percentage of overall production costs. So, wherever possible, one prefers to use whole cells as biocatalysts. Reduction of keto compounds to obtain chirally pure secondary alcohols is an important synthetic approach[22]. Alcohol dehydrogenases are enzymes, which have shown considerable promise in this regard. Baker's yeast is a well-studied source of alcohol dehydrogenase activity. Hence, it appears to be a cheap biocatalyst and a good substitute for the isolated/purified enzyme. In fact, using whole cells as a source of alcohol dehydrogenase is also attractive for another reason. The dehydrogenases require an expensive coenzyme nicotinamide adenine dinucleotide (NADH). Using whole cells avoids the cost of coenzyme regeneration as whole cells have other activities (desirable for metabolic purposes), which regenerate NADH. However, 'reproducible performance by Baker's yeast is not guaranteed because many competing dehydrogenases are present in the cell and their relative abundance and activity depends on genetic variability between strains, growth conditions, etc.'[23]. Different enzyme 'isoforms' have different substrate specificity and stereoselectivity, which creates this hurdle. (Nevertheless, to avoid creating wrong impression, it may be added that various industrial level processes use whole cells as biocatalysts.)

One of the most often used lipases is the lipase from *Candida antarctica*. In fact, the organism produces two lipases: *C. antarctica* A (CAL A) and *C. antarctica* B (CAL B)[24]. Immobilized form of CAL B sold commercially by Novozyme is called Novozyme 435 and is perhaps the most versatile and often used commercially available lipase. CAL A is not known so widely. Unlike, most of the lipases which show sn-1, 3 preference during hydrolysis of triglycerides, CAL A shows sn-2 preference. CAL A also shows very high selectivity towards trans-fatty acids. The N-terminal sequences of CAL A and CAL B show no homology and both lipases probably are part of two different genes and constitute a very good example of biotechnologically useful isoenzymes. CAL A shows high chemoselectivity towards amine groups and hence is a useful catalyst for asymmetric synthesis of amino acids. CAL A also accepts stearically-hindered alcohols, which is very uncommon for hydrolases[24]. CAL A shows regioselectivity towards cyclic alcohols and has been usefully employed in the dynamic kinetic resolution of cyanohydrins[25]. The two isoenzymes of lipases occurring in *C. antarctica* together form a powerful tool for organic synthesis.

To sum up, evolution has resulted in isoforms in many cases. The essential strategy is not different from common strategies used for creating protein diversity. The structural diversification here has been tailored to keep the basic reaction type being catalysed similar although differences in substrate specificity, regioselectivity, stereoselectivity, kinetics, stability and response to the presence of other molecules often differ in a very significant way.

Moonlighting proteins

The one protein–one function has been an established paradigm in biology. It underlines the importance of specificity inherent in the binding and catalysis of substrates. Some of the early examples that challenged this notion came from (i) eye protein crystallins[26,27] which were also found to act as heat shock proteins, lactate dehydrogenase, enolase, quinone oxidoreductase, glyceraldehyde-3-phosphate dehydrogenase, etc. and (ii) role of biotin holoenzyme synthatase as repressor of the biotin operon in *Escherichia coli*[28]. Today, a conservative estimate would be that we know more than 50 such examples. As Jeffery[1] said, 'Increasingly, we are finding that gene products of higher organisms moonlight.' It should be added however that known examples of moonlighting are also from prokaryotes. Three good reviews[1,29,30] give numerous examples. Jeffery[1] provides a table consisting of good illustrative examples. It is not necessary to repeat all those examples. Instead, we will look at the salient features of this phenomenon, which perhaps needs wider awareness among both molecular biologists and enzymologists. Obviously, people working in the area of bioinformatics have to factor in this phenomenon while analysing gene or protein sequences.

- Mostly, only one function is catalytic, the other functions are either as structural proteins or receptors or regulatory proteins. It is a moot point that what are moonlighting roles and what is the main role of the protein. If we have known a particular function of an enzyme for a long time and it is a critical function in the cell metabolism, that function is likely to be recognized as the main function. Also, if the enzyme is ubiquitous and does have a role in prokaryotes, that is likely to be the main function. Moonlighting roles may have evolved in higher organisms. A good example is that of the glycolysis enzyme phosphoglucose isomerase. This intracellular enzyme is also a secretory protein in higher organisms and serves both as a cytotoxin and a nerve growth factor[31,32]. In other extreme cases, our referring to some functions as moonlighting may be quite arbitrary.
- Moonlighting roles can arise in different locations of a cell: as a result of differential expression in different cell types or due to oligomerization[1]. The last mechanism operates by a monomer or an oligomer (or multimer) having different biological activities. For example, the monomer of the tetrameric glyceraldehyde-3-phosphate dehydrogenase is uracil-DNA

glycolase[33]. It is interesting to note that while the tetramer activity is relevant in a cytoplasmic process, the monomer activity is required in the nucleus. So, again different locations of the cell are involved.

It may be also interesting to note that a somewhat related phenomenon wherein different subunit compositions give rise to different physiological functions has been known for a very long time. Luteinizing hormone, follicle-stimulating hormone and thyroid-stimulating hormone are composed of a common α-chain and different β-chains. However, the α-chain in the different hormones are glycosylated differently[34]. It may be recalled that many isoenzymes differ in their glycosylation patterns. Also, oligomerization has been used as a tool for regulatory purposes as well. A well-known textbook example is that of eukaryotic acetyl CoA carboxylase, an enzyme in fatty acid synthesis. The protomer is inactive; formation of active filamentous oligomer is allosterically regulated[17]. The point is that nature has a finite tool box and it is used in many contexts. Reminiscent of allosteric regulation, the presence of ligand/substrate concentration also is another way a protein can switch between main and moonlighting functions. In glyceraldehyde-3-phosphate dehydrogenase, oligomerization is influenced by concentrations of ATP, NAD^+ and protein[33].

It has been known for a long time that platelet-derived growth factor receptor is also a protein tyrosine kinase receptor. It is a single polypeptide chain of 500 amino acids consisting of five domains[35]. Neither this nor the examples of hormones cited above are normally considered moonlighting. However, the *E. coli* aspartate receptor which also doubles up as a maltose-binding protein is considered an illustration of the moonlighting mechanism in which proteins utilize distinct or overlapping binding sites[1,36]. Incidentally, considering that by now we know that the so-called 'affinity recognition' can be pretty promiscuous[6–8], promiscuity among receptors should not come as a surprise and should turn out to be more common in future. Also, cross-reacting antibodies have been known for decades. The historical work of Jenner on smallpox vaccine using cowpox serum[37] illustrates that we have known about promiscuity of proteins for a long time indeed.

Yet another mode of moonlighting described by Jeffery[1] is that some subunit/polypeptide chain with a defined function becomes part of a quaternary structure of a protein with another function altogether. *E. coli* thioredoxin recruited by T7 phage becomes part of DNA polymerase. In fact, gene duplication does show a milder manifestation of the same phenomenon. However, let us backtrack a little and look at the good old α-chymotrypsin[35]. The two domains of the enzyme have similar 3-D structures and it is believed that not only these two domains but also all the members of the chymotrypsin superfamily evolved out of a common ancestor. The two domains together form the well-known catalytic triad of chymotrypsin. Mutants produced by protein engineering, which lack the triad, still show some proteolytic activity. So gene duplication followed by mutation may have led to the present chymotrypsin with enhanced catalytic activity. So, domains get duplicated, subunits come together and polypeptides or subunits become part of different proteins, or the subunits oligomerize. These steps in protein evolution take place sometime for regulatory purposes, sometime for enhancing catalytic activity and sometime for moonlighting activities. We will see later, promiscuity may also originate in a similar way at least in some cases.

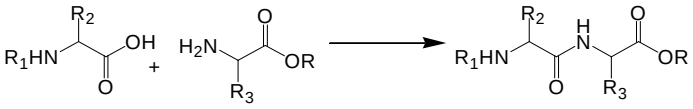
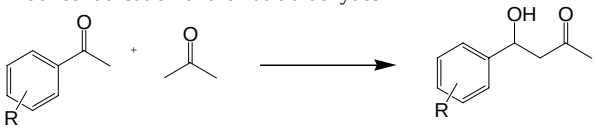
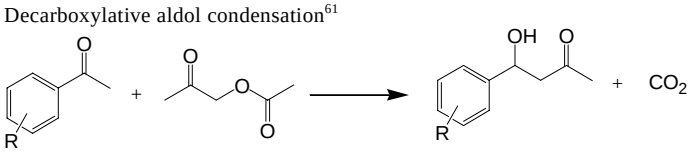
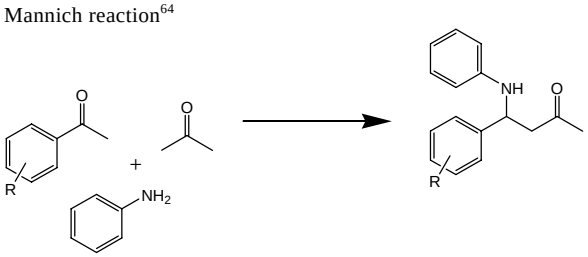
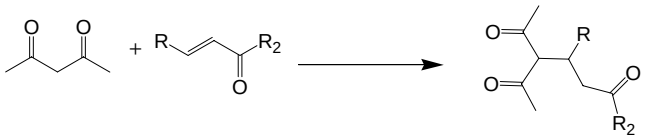
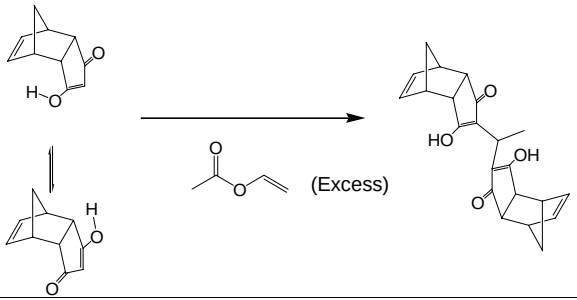
Feedback inhibition of enzyme dictates the activity of existing molecules of regulatory enzymes in metabolic cycles. The synthesis of actual number of copies of such enzymes may be under transcriptional or translational control. In a fascinating facet of the phenomenon of moonlighting, the enzyme molecules may exert transcriptional or translational control of the protein synthesis by binding to its gene or m-RNA respectively[38,39].

As Jeffery[29] points out, the moonlighting phenomenon makes drug discovery even more challenging. One may like to block the activity of particular enzyme with a drug but may also end up diminishing/abolishing its moonlighting function. The latter may be necessary even under pathological situation. Looking at it in a positive way, better information about moonlighting activities may lead to better understanding of the so-called side effects of the drug.

Enzyme promiscuity

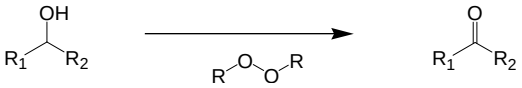
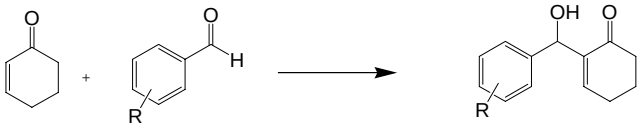
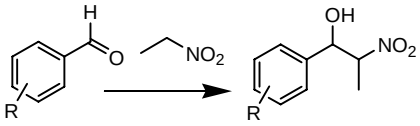
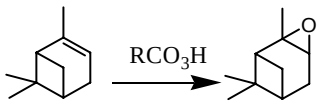
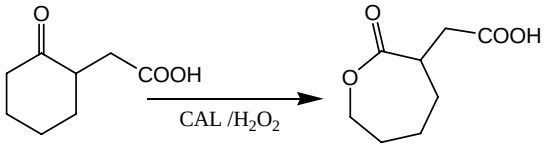
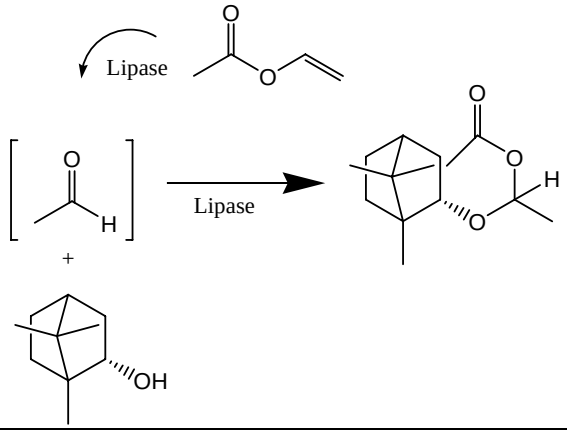
An honest admission by Hult and Berglund[40] about the meaning of the phrase 'enzyme promiscuity' captures the spirit of the current review. According to the above authors 'As with many modern words, it is used without a well defined meaning or with different meanings, depending on the author'. Hult and Berglund also bring in some clarity about the phenomenon by classifying enzyme promiscuity into: enzyme condition promiscuity, substrate promiscuity and enzyme catalytic promiscuity. Figure 1 and Table 1 show the increasing interest in the phenomenon of enzyme promiscuity. Let us examine the various classes of enzyme promiscuity and see how 'new' the phenomenon really is for the enzymologists. While most of the recent reviews[40–42] focus on the promiscuity being induced through directed evolution, the following discussion will be limited to naturally occurring promiscuity. This is not to imply that application of directed evolution in this area is not exciting; it has in fact opened up several new avenues in applied enzymology and organic synthesis. However, the above cited reviews give a very comprehensive list of results. Here, the focus is on the phenomenon as it occurs in nature, without human intervention.

Table 1. Promiscuity exhibited by naturally occurring enzymes

Biocatalyst	Promiscuity exhibited naturally
Serine proteases Subtilisin and α-chymotrypsin	Synthesis of a peptide[75,76] 
Lipases and proteases	Esterification[77–80] $R_1COOH + R_2OH \rightarrow R_1COOR_2 + H_2O$ Transesterification[77–82] $R_1COOR + R_2OH \rightarrow R_1COOR_2 + ROH$
Lipases	Interesterification[83] $R_1COOR_3 + R_2COOR \rightarrow R_1COOR + R_2COOR_3$ Aldol condensation of aromatic aldehydes[58]  Decarboxylative aldol condensation[61]  Mannich reaction[64]  Michael addition of 1,3 diketones[59]  Aldol condensation of a tricyclic 1,3-diketone[63] 

(*Contd*)

Table 1. (*Contd*)

Biocatalyst	Promiscuity exhibited naturally
	Oxidation of alcohols[84] 
	Morita–Bayles–Hillman reaction[65] 
	Henry reaction[85] 
	Epoxidation[86,87] 
	Bayer–Villiger oxidation[88] 
	Hemiacetal formation followed by acetylation[89] 

Enzyme condition promiscuity

The enzyme catalysis in low water media[43–47] has not yet made an appearance in standard textbooks of biochemistry. However, it is now a well-established area of research. In 1913, Bourquelot and Bridel used a crude preparation of a hydrolase to synthesize alkyl glucosides in dry alcohol[47]; in 1966–67, Dastoli and coworkers[47] used chymotrypsin and xanthine oxidase in dry organic solvents. The 1980–90 decade showed convincingly that: (i) Enzymes can carry out unexpected and unusual reactions in low water containing organic solvents or in

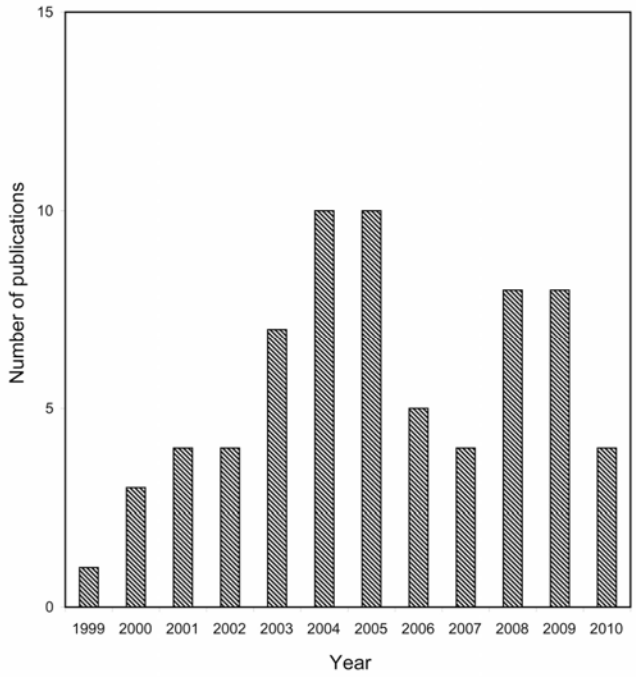


Figure 1. Number of papers published on promiscuity from 1999 to 2010 (till March). These are the papers published on promiscuity from the enzymes obtained from both wild and mutant organism sources. (The data were collected by combining the search results by search engines: Google, Science Direct and PubMed.)

reverse micelles. For example, proteases can carry out peptide synthesis, lipases can catalyse esterification, transesterification and interesterification. (ii) Amount of water or strictly speaking water activity a_w dictates the catalytic efficiency and selectivity of the enzyme. (iii) The choice of the medium (the nature of the organic solvent) can decide not only the initial rates but regioselectivity and enantioselectivity as well. The list of reaction conditions can be expanded to high temperature (enzymes from thermophiles), extreme pH (enzymes from acidophiles and alkalophiles), high salt concentrations (enzymes from halophiles), gaseous phase, solvent free conditions, ionic liquids and solid phase[48–53]. Different isoenzymes are known to show different conditions of promiscuity. This also underlines that how blurred the lines of demarcation between the different phenomena can be! As we will see, lipases have been most extensively studied with respect to enzymes promiscuity. However, it is debatable whether lipases constitute the most promiscuous class of enzymes or just happen to be a 'visible tip of an iceberg'! However, it should be added that directed evolution has been able to create a much larger variety of promiscuous activities[40–42].

Enzyme substrate promiscuity

Lipases have been used for resolution of racemic acids and alcohols[54]. The enantioselectivity towards acids is much lower than that for alcohols. In addition to this, resolutions of even amines and peroxides have been carried out with lipase by using these compounds as acyl donors[54]. At times, it may be difficult to distinguish between substrate promiscuity and catalytic promiscuity.

Enzyme catalytic promiscuity

Most of the time, this is the type of promiscuity which is meant when people just talk of enzyme promiscuity. In this, the same active site (although different side chain residues in the active site may participitate to a different extent or not at all) catalyses different biotransformations. Obviously, the transition state and mechanism may differ[40,55]. Proteases acting as esterases (known for a long time indeed!) is an example of catalytic promiscuity wherein the catalytic mechanism is very similar. Some other examples of catalytic promiscuity are: (i) asparaginase cleaving the nitrile β-cyanoalanine[56] (ii) pyruvate decarboxylase catalysing acyloin condensation of acetaldehyde and benzaldehye to form a precursor for ephedrine manufacture[57] (iii) lipase catalysed aldol condensation[58], Michael addition of primary and secondary amines to acrylonitrile[59], Mannich reaction[60], decarboxylative aldol reaction and decarboxylative Knoevenagel reaction[61], synthesis of thioether containing ester functions by C–S bond formation (both Markowinkoff addition and anti-Markownikoff addition was possible through medium engineering)[62] and condensation of acetaldehyde (formed *in situ* by enzymatic hydrolysis of vinyl acetate) with a tricyclic diketone (1, Figure 2)[63] (iv) acylase catalysed aza-Michael additions of aromatic N-heterocycles[64].

Interesting examples of catalytic promiscuity[55] include the oxidation reaction catalysed by myoglobin, Kemp elimination, β-elimination of 3-ketobutyl umbelliteryl ethers and enantioselective oxidation of amines with sodium periodate catalysed by bovine serum albumin (BSA). Earlier, Reetz *et al.*[65] reported the Morita–Baylis–Hillman reaction catalysed by BSA (and lipases). Haemoglobin displaying low peroxidase activity has been known for a long time[66]. These examples appear to resemble moonlighting considering that carrier proteins are switching over to catalytic functions. Mostly, moonlighting concept has been invoked when the same protein does another job *in vivo*. This distinction is valuable as increasingly these phenomena are being viewed purely from the viewpoint of protein plasticity or their usefulness in organic synthesis[30].

Listing some important features of enzyme promiscuity may bring further clarity to the discussion:

- 'Promiscuous activities are generally considerably less efficient than the primary functions of an enzyme, but second order rate constants (k_{cat}/K_M) as high as 10^5 M^{-1} S^{-1} and rate accelerations [$(k_{cat}/K_M)/K_2$] up to

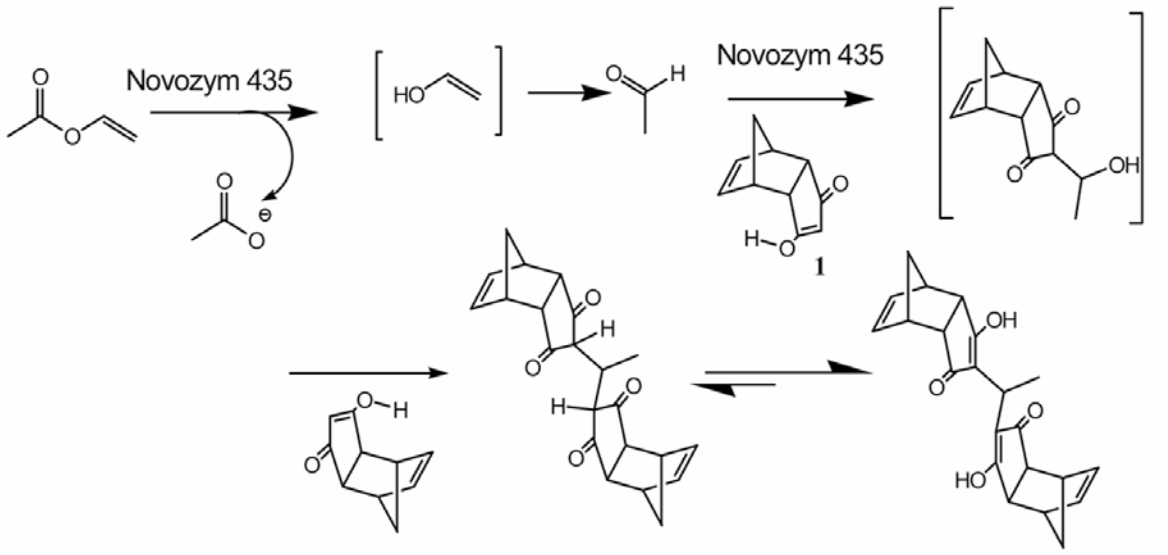


Figure 2. An unusual reaction sequence that involves: Enzymatic deacylation of vinyl esters to produce acetaldehyde even under anhydrous conditions, condensation between 1 and acetaldehyde gives an initial product *in situ* followed by formation of the bis-adduct (diketo form), which tautomerizes in its enolic form (adapted from ref. 63).

10^{18} have been reported[67].' These rate accelerations are comparable to those shown by many other enzymes.

- Conformational diversity and flexibility are known to be important in enzyme catalysis. This is especially so for promiscuous enzymes. 'In particular, the mobility of active site loops appears to play a key role in mediating promiscuity[68].'
- The binding between a substrate and an enzyme consists of H-bonds, electrostatic interactions and hydrophobic bonds. The first two depend upon complementarity of structures of the substrate and the active site residues. Hydrophobic bonds depend upon desolvation and are entropy-driven. These are much less dependent upon the specific structures. Hence, it is not surprising that many promiscuous reactions depend upon substrate hydrophobicity. However, often this alone is not enough[67].

Sufficient attention has been paid by different authors on the evolutionary aspects of enzyme promiscuity. The best discussion on this is probably in the two articles by Dan Tawfik and co-workers[68,69].

It makes sense to say that primitive enzymes had broader specificities and lower catalytic efficiencies. Duplication of genes as part of divergent evolution led to 'specialization' by enzymes. 'The vestiges of these divergence processes are the scaffold and active site architecture shared by all family members of the enzyme families and super families[68].' So, promiscuity is a trait which survived during this specialization. Darwinian processes imply that these changes during divergent evolution were gradual. However, in some cases, natural evolution of new catalytic activity can occur very rapidly. An example is atrazine chlorohydrolase (which can degrade herbicide atrazine) which seems to have evolved between the 1950s and 1990s from melamine hydrolase. The new enzyme hydrolyses C–Cl bond whereas older enzyme hydrolyses C–N bond. The rate of evolution notwithstanding, it is relevant to note that melamine hydrolase does have a low atrazine chlorohydrolase activity. The newly evolved enzyme seems to be a 'specialist', i.e. it has no promiscuous activity and does not have melamine hydrolase activity[55,70].

According to James and Tawfik[69], it is necessary to realize that the energy landscape for protein conformation consists of an ensemble of conformers of similar free energy. Induced fit by a substrate would shift the equilibria in favour of the conformation corresponding to the main function. The gene duplication would leave the gene copy to evolve and may stabilize a conformation corresponding to promiscuous function. Many of the results of directed evolution confirm the above ideas about promiscuity[42,55].

Promiscuity as a black swan

In the prologue of his best selling book, Taleb[71] writes 'Before the discovery of Australia, people in the Old World were convinced that all swans were white, an unassailable belief as it seemed to be completely confirmed by empirical evidence. The sighting of the first black swan. . . . It illustrates a severe limitation to our learning from observations or experience and the fragility of our knowledge. . . an event with the following three attributes. First, it is an outlier, as it lies outside the realm of regular expectations, second it carries an extreme impact. Third, in spite of its outlier status, human nature makes

us concoct explanations for its occurrence after the fact, making it explainable and predictable'.

Looking retrospectively, we knew for a long time that in 'RNA World'[72] RNA showed high functional promiscuity: it acted as genetic material as well as catalysed reactions. In an old review on perhaps still the most well-studied enzyme RNase A[73], it was mentioned that RNase A shows poor DNase activity. We have known for a long time that proteases show esterase activity[74]. So, sometimes we are uncomfortable with an outlier observation, we rationalize it to make that fit in with our existing belief. So, sometime we called it 'broad specificity'. Often it takes sometime before it sinks in that black swans may after all exist! The textbooks of biochemistry still talk about the specificity as a virtue of these biocatalysts. We need to unlearn some of that. From the application point of view, it is seldom an advantageous trait. Lipases have dominated the area of biotransformations (including and especially those which take place in low water media). It is because these enzymes are highly non-specific when it comes to catalysing substrates with a wide range of structures and size[54].

Organic chemists have often wondered why one needs large molecules such as proteins to carry out biocatalysis. Good textbooks list some answers. We can add the need for biological promiscuity as an additional reason. It will be difficult for a small biomimetic molecule to either do moonlighting or evolve. The isoforms of biomimetic compounds, isomers, may not be able to neatly tailor their activity and specificity to the regulatory needs of a living cell in real time.

Finally, biological promiscuity of enzymes also questions our belief that the tight correlation between structure and function is a hallmark of biological systems. One structure can have many functions. What then happens to the 'perfect enzymes' concept?[4] We have tended to look at enzymes as the final finished product with an optimized design. We have perhaps overlooked that evolution is an ongoing process. Many years hence, the future generations may look at biological activity and enzyme specificity very differently. It may be as they say; future is already here!

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