Quantification of Catalysis and Measures of Enzyme Purity

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Exactness cannot be established in the arguments unless it is first introduced into the definitions

Henri Poincare

Numerical precision is the very soul of science

Sir D'Arcy Thompson

This is more so in enzymology, as it is a quantitative and exact branch of biology. It is necessary to understand how enzyme activity is measured, calculated, and presented.

14.1 Enzyme Units, Specific Activity, and Turnover Number

The molar concentration of an enzyme in a sample (at times even in the pure enzyme!) is often not known. However, with the help of a robust and reliable assay method, reaction rates can be recorded for any enzyme sample. Assay conditions like concentration of the substrate, pH, and temperature should be well defined to obtain a reproducible initial velocity (v) (Halling and Gupta 2014). The relationship "$v \propto [E]$" holds only when true initial velocities are measured. Therefore the first objective in any quantitative assay (and kinetic study) is to establish the two limits of linearity. These are the maximum [*P*] that can accumulate before the two responses, namely, $[P] \rightarrow t$ and $v \rightarrow [E]$, become nonlinear. Within these limits, the measured initial velocity (v) can be used to express [*E*], the catalyst concentration.

Enzyme Unit To facilitate comparison of enzyme activities from various samples (and from values reported in the literature), an international unit is recommended.

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The standard *enzyme unit (U)* is *the amount that catalyzes the formation of one micromole of product per minute*, under defined assay conditions. This unit has the dimensions of $\mu mol \times min^{-1}$. The more the number of units in a sample means the more enzyme catalyst present in that sample. While one U of enzyme in a standard assay produces one μmol of product per min, two U of the same enzyme gives 2.0 $\mu mol \times min^{-1}$ of the product – and so on. The enzyme concentration in a given sample is then expressed in terms of $U \times ml^{-1}$. A sample containing 2.0 $U \times ml^{-1}$ is four times more concentrated enzyme than a sample with 0.5 $U \times ml^{-1}$. We should note that, as defined, the catalysis unit by itself does not indicate anything about the purity of the enzyme sample.

The enzyme activity unit may also be expressed in terms of μmol substrate consumed per min. For any reaction with a defined substrate–product stoichiometry, this can be converted to the standard unit (U) described above. Some enzyme-catalyzed reactions may be relatively slow or fast. Accordingly, the unit may be redefined for convenience by suitably changing either the units for product formed (from μmol to nmol, mmol, etc.) or the unit of time (from min^{-1} to h^{-1}, sec^{-1}, etc.). If such changes are adopted for convenience, then they should be clearly documented. We find most enzyme literature in clearly defined units. However, the International Union of Biochemistry has recommended the use of katal – according to SI units. *A katal corresponds to the amount of enzyme that produces one mole of product per second*. From the calculations (see box below), it is obvious that katal is a very large unit and hence is not in common use.

$$\begin{aligned} 1 \text{ katal} &= 1 \text{ mol} \times \text{ sec}^{-1} \\ &= 10^6 \mu mol \times 60 \text{ min}^{-1} \\ &= 6 \times 10^7 \mu mol \times \text{min}^{-1} \\ &= 6 \times 10^7 U \end{aligned}$$

$$\text{Similarly,} \qquad 1 \text{ U} = 16.67 \text{ nkatal}$$

Specific Activity A way to express the amount and concentration of enzyme is through U and $U \times ml^{-1}$, respectively. These units reflect on the enzyme content of the given sample but do not tell us anything about the purity of the enzyme. The units of enzyme in a sample can be same regardless of the quantity and diversity of other proteins present. We could however present the quantity (U) of enzyme present in a known amount of protein. Specific activity is thus defined as the *number of units per mg of protein*. It is an index of the purity of the enzyme sample – the higher the proportion of enzyme protein in a given protein sample, the greater will be its specific activity. The purer the enzyme sample, the higher is its specific activity. If this is extended logically to the stage of highest enzyme purity, then that sample must have every protein molecule representing only that enzyme. Beyond this point (of limit of highest $U \times mg^{-1}$ protein!), it is not possible to enhance the specific activity by any method of purification. Conversely, achieving highest constant specific activity is considered a necessary criterion of enzyme purity.

Turnover Number The specific activity of an enzyme sample is expressed as $U \times mg^{-1}$ protein (note that 60 U $\times$ mg^{-1} corresponds to 1 katal $\times$ kg^{-1}). This is nothing but velocity per unit amount of catalyst protein – i.e., μmol product formed per min per mg protein. With a pure enzyme (possessing highest limiting specific activity), the amount of enzyme protein (say in mg) can also be expressed as number of moles of that enzyme (say in μmol). However, to do this we need to know one additional bit of information – the molecular mass of the enzyme. When this is available, we can present the specific activity (see box) of the pure enzyme.

$$\begin{aligned}\text{Specific activity} &= U \times mg^{-1} \\ &= \mu\text{mol product formed} \times min^{-1} \times mg^{-1} \\ &= \mu\text{mol product formed} \times min^{-1} \times \mu mol^{-1} \text{of enzyme}\end{aligned}$$

This quantity – called the *turnover number* – has the units of dimension "$time^{-1}$" (more commonly, sec^{-1}). It indicates the number of times a single enzyme molecule converts substrate into product in 1 min. In this definition it is assumed that substrate is saturating and that the enzyme has one active site per molecule. For enzymes with multiple active sites per molecule (such as lactate dehydrogenase, a tetramer with one active site per monomer), one could define a *catalytic center activity* by accounting for the number of active sites per subunit in the calculation. The turnover numbers of different enzymes (Table 14.1) could be compared under the best and optimal assay conditions (in terms of pH, temperature, saturating [*S*], etc.). For instance, an enzyme with a turnover number of 60 min^{-1} is ten times sluggish in comparison to another of 10 s^{-1}. The turnover number of catalase is among the highest known (1.0×10^7 s^{-1}). The reciprocal of the turnover number (sometimes given as k_{cat}) actually indicates the time required for a *single catalytic cycle* – and for catalase this is 100 nanoseconds! (Which is nothing but reciprocal of 10^7 s^{-1}).

Table 14.1 Range of enzyme turnover numbers

Enzyme (substrate)	Turnover number (s^{-1})
Catalase (for H_2O_2)	1.0×10^7
Carbonic anhydrase (for CO_2)	0.6×10^6
Ketosteroid isomerase	0.7×10^5
Urease	1.0×10^4
Triosephosphate isomerase	4.3×10^3
DNA polymerase I (*E. coli*)	6.0×10^2
Adenosine deaminase	3.7×10^2
Chorismate mutase	5.0×10^1

14.2 Enzyme Purification and Characterization

Most kinetic studies do not require a pure enzyme preparation provided there are no interfering activities. However, as we have noted for turnover number calculations above, there are significant benefits of working with pure enzymes. The famous quote by Efraim Racker – "Don't waste clean thinking on dirty enzymes" – is at the core of molecular enzymology and good chemical practice. The availability of pure enzyme is very valuable in determining its molecular, mechanistic, and regulatory properties.

The objective of enzyme purification is to retain and enrich the enzyme protein of interest while eliminating most other proteins (and other biological macromolecules like DNA). This is typically achieved by a combination of protein separation techniques including fractional precipitation, ion exchange, size exclusion, and affinity chromatography. An enzyme is best isolated from a source where it is abundant, for instance, lysozyme from egg white and chymotrypsin from the pancreas. With the advent of powerful molecular biology tools, most enzymes can now be produced in a suitable heterologous overexpression system, such as in *E. coli*. We also have the option of producing the protein/enzyme with or without a tag – and tags make purification a routine chore. Every tag is designed with a purification strategy in mind. A His_6-tagged enzyme is best purified on a metal affinity (Ni-NTA) column. It is a different matter however to ensure that enzymes with tags are (a) active or not and (b) retain the original properties or are significantly altered. Clearly, for instance, His_6 tag introduces the property to bind a divalent metal ion (such as Ni^{2+}, Co^{2+}, or other similar metal ion); this complicates further analysis whenever enzyme–metal ion interactions are to be studied. Proteolytic cleavage of the tag after purification is one solution but adds an extra technical step in the process.

The theory and practice of protein (and hence enzyme) purification is a mature subject, and much literature has accumulated over the years. As the reader may access these tools through suitable books and references (e.g., Deutscher 1990; Burgess and Deutscher 2009), they are not covered here. However, it must be borne in mind that whatever steps/protocols are used to purify them, it is highly desirable to consistently obtain a stable, concentrated enzyme preparation with well-defined cofactor content, etc.

Wherever possible, it is desirable to start with a sample that is intentionally enriched. Arginase is 10–15-fold induced in *Aspergillus niger* mycelia grown on L-arginine as the sole nitrogen source. Provided the induced enzyme form is not different, it would be prudent to start the purification with induced cells. The summary of a successful strategy for the purification of *A. niger* arginase (induced on L-arginine) is shown in Table 14.2 as an example. Excellent bookkeeping through a *purification table* is a must in monitoring the course of purification process. Both the amount of protein (in mg) and activity (in U) are estimated in every step. With these experimentally measured data (given in Table 14.2, in black), all other parameters may be easily calculated. These derived parameters, after each step of purification, are shown in gray in the table.

Table 14.2 Purification of arginase from *A. niger* mycelia[a]

Step	Total volume (ml)	Activity ($U \times ml^{-1}$)	Protein ($mg \times ml^{-1}$)	Specific activity ($U \times mg^{-1}$)	Yield (%)	Fold purified
Crude protein extract	72.5	19.4	2.47	**7.85**	**100.0**	**1.0**
$(NH_4)_2SO_4$ (30–60%) fraction	50.0	28.8	3.15	**9.14**	**102.0**	**1.2**
DEAE-Sephacel	4.0	36.2	0.35	**103.43**	**10.3**	**13.2**
Hydroxylapatite	3.0	16.4	0.08	**205.00**	**3.5**	**26.1**

[a]Typically this purification is initiated with 15 g of mycelia (wet weight); U of arginase is defined as the amount that produces 1 μmol of ornithine (product) in 1 min

Obtaining meaningful information about enzyme purification from *primary data* is at the heart of all calculations. An example of how the *derived parameters* (specific activity, fold purification, and yield) are obtained from experimental data (Table 14.2) is shown in the box below.

The crude protein extract was obtained from 15 g of wet mycelial mat. Suppose 10 μl of this sample produced 0.194 μmol of ornithine (product) in 1 min, in a standard assay. It thus contained 19.40 units of arginase per ml and had a protein concentration of 2.47 mg $\times$ ml^{-1}.

$$19.40\ \text{U} \times \text{ml}^{-1} \times 72.5\ \text{ml total volume} = 1406.5\ \textbf{total U}$$

$$\text{and } 2.47\ \text{mg} \times \text{ml}^{-1} \times 72.5\ \text{ml total volume} = 179.1\ \text{mg}\ \textbf{total protein}$$

The specific activity of the crude extract will be:

$$\frac{19.40\ \text{U} \times \text{ml}^{-1}}{2.47\ \text{mg} \times \text{ml}^{-1}} = \mathbf{7.85\,U \times mg^{-1}}\ \text{protein}$$

(*Same number is obtained when we divide total U by total protein*)
After the final step, the specific activity of the purified arginase was:

$$\frac{16.40\ \text{U} \times \text{ml}^{-1}}{0.08\ \text{mg} \times \text{ml}^{-1}} = \mathbf{205.0\,U \times mg^{-1}}\ \text{protein.}$$

The number of folds this enzyme got purified from the crude sample was:

(continued)

$$\frac{205.00\ \text{U} \times \text{mg}^{-1}}{7.85\ \text{U} \times \text{mg}^{-1}} = \mathbf{26.1\ fold}$$

The *final yield* of pure enzyme was:

$$\frac{\text{Total U in pure fraction}}{\text{Total U in crude extract}} \times 100 = \frac{16.4\,\text{U} \times \text{ml}^{-1} \times 3.0\ \text{ml}}{19.4\,\text{U} \times \text{ml}^{-1} \times 72.5\ \text{ml}} \times 100 = \mathbf{3.5}\%$$

Couple of additional features can be gleaned from the purification data – provided the arginase sample after the final step is pure. If we know that all the protein is extracted from the cell mass, then the *cellular abundance* of arginase can be evaluated. A protein to be pure after 26.1-fold enrichment must form 3.83% of the total protein pool of *A. niger*. Secondly, knowing that arginase is a homohexamer (molecular mass of 219 kDa), we can calculate its turnover number. From Table 14.2, the specific activity of pure arginase is 205 U × mg^{-1} (or μmol × min^{-1} × mg^{-1}). One milligram of arginase protein corresponds to 4.57 × 10^{-3} μmol of arginase (because 219 mg ≡ 1.0 μmol). The turnover number for arginase is therefore:

$$\frac{205.0\ \mu\text{mol} \times \text{min}^{-1} \times \text{mg}^{-1}}{4.57 \times 10^{-3}\mu\text{mol}} = 44858\ \text{min}^{-1} = 748\ \ \text{sec}^{-1}$$

Since there are six active sites (per hexamer), the catalytic center activity should be one sixth, i.e., 125 s^{-1}.

14.3 Interpreting a Purification Table: Criteria of Enzyme Purity

Reading a well-compiled purification table is very informative. The data provides a bird's-eye view on the efficiency of each step and the progress of purification. Table 14.2 reports a small but significant increase in total activity (yield goes up from 100% to 102%). This may be because (a) the $(NH_4)_2SO_4$ carried over from the fractionation process may be an activator of the enzyme, or (b) some inhibitors from the crude extract are removed by this step. The objective of each step is to take the enzyme to higher level of purity (increase in specific activity). While this is achieved, the yield drops significantly in the DEAE-Sephacel step (leading to ≈ 90% loss). A good purification step should do both – recover most of the enzyme originally present and also to enrich it. However, one may sacrifice yield for the sake of an excellent purification step. At times a good purification step is avoided because of poor yield. As a whole, a well-established purification protocol should be robust and reproducible and provide the enzyme of desired purity.

As the enzyme gets purified, its specific activity will increase to a limiting value. No matter what additional steps of purification are used, this limiting specific activity

cannot be bettered. A pure enzyme usually elutes from a chromatographic column as a symmetric peak with each fraction showing constant specific activity. This pure enzyme – of highest specific activity – is a preparation where all protein molecules present in the sample are of that enzyme alone. Native polyacrylamide gel electrophoresis is very popular among many criteria used to test the purity of an enzyme protein. Owing to its excellent resolution (based on charge and size), native PAGE is able to resolve closely similar proteins – even isozymes. Often the presence of a single protein band (on native PAGE gels) is used to describe the electrophoretic homogeneity of the purified sample. An enzyme found to be electrophoretically pure however does not necessarily guarantee that all the molecules present are active. An *active homogeneous enzyme* means only enzyme molecules are present and every protein molecule in the sample is enzymatically active. For example, in a sample containing 100 molecules of protein of which 50 are of the enzyme – then in principle – specific activity of this sample can be doubled by eliminating those molecules which are not enzyme. Similarly, even if a given enzyme sample is homogeneous, it can in principle contain both active and inactive forms of the same enzyme. There is thus scope to increase the specific activity of this sample by eliminating the inactive enzyme molecules from it. One way of achieving this is to use a *functional affinity separation* that selectively binds only the active enzyme species. Subsequently, bound molecules (of active enzyme) may be collected by suitable elution protocols. In many cases, it is possible to estimate the amount of active enzyme in the given sample by *active site titration*. Any agent that stoichiometrically reacts with the active site may be used to determine the number/concentration of active sites. The number of active molecules in an acetylcholinesterase preparation was determined using ^{32}P-labeled diisopropylfluorophosphate (DIFP). The size of the burst observed, when chymotrypsin acts on p-nitrophenylacetate, can also be used as a measure of active enzyme in a homogeneous preparation of chymotrypsin.

Homogeneous, pure enzyme sample allows us to infer about the molecular details of the catalyst. Their oligomeric state can be deduced by a combination of techniques including denaturing PAGE (such as SDS-PAGE). Enzymes come in different designs. Lysozyme, RNase A, and chymotrypsin are straightforward examples of *single subunit–single active site* enzymes. Not every subunit of an oligomeric protein may contain one active site. HIV protease (and possibly proline racemase) is dimeric but contains a single active site. The active site of the homodimeric mammalian ornithine decarboxylase spans both the subunits. The lone active site of *E. coli* RNA polymerase is generated from a holoenzyme made up of five ($\alpha_2\beta\beta'\sigma$) subunits. Mitochondrial ATP synthase consists of 22 subunits made from 8 distinct polypeptide chains. It is thus possible to define an *enzyme equivalent weight* – it is the mass of a protein expressed in grams per mole of active sites. For lysozyme it is the same as its molecular mass whereas it will be the mass of each subunit in the case of lactate dehydrogenase tetramer.

14.4 Unity of the Enzyme

It should be obvious from the above discussion that enzyme specific activity is an important measure of enzyme purity. Highest attainable specific activity coupled with electrophoretic homogeneity tells us that the enzyme sample is pure. There are limits to know how much of a protein contamination is present. A contaminant which is less than a fraction of a percent may not be noticed on native PAGE gels. Enzyme activity assays are inherently more sensitive, and hence contaminating proteins may still be detected as additional interfering activities in the sample. It is equally possible that the "contaminating" activity may be an intrinsic property (side reaction!) of the enzyme itself (Table 14.3). There are documented examples where a single enzyme protein displays multiple activities – either at the same active site or on distinct sites (Kirschner and Bisswanger 1976). These also include multifunctional polypeptides (a single polypeptide harboring more than one distinct enzyme activity) and multienzyme complexes (many polypeptides form oligomeric structures with more than one distinct enzyme activity).

Table 14.3 Enzymes exhibiting multiple activities

Enzyme example	Activities
One active site with many activities	
Glutamine synthetase	Glutamine synthesis
	γ-Glutamyl transfer
RuBP carboxylase (RubisCO)	Carboxylase
	Oxygenase
Hexokinase	Phosphate transfer to sugar
	ATP hydrolysis (very weak)
Sucrose phosphorylase	Sucrose phosphorylase
	Transglucosylase
Fructose-1,6-bisphosphate (FBP) aldolase/ phosphatase (from Archaea)	FBP aldolase
	FBP phosphatase
One enzyme with many active sites	
A. *Multifunctional polypeptides*	
Aspartokinase-homoserine dehydrogenase	Aspartokinase
	Homoserine dehydrogenase
DNA polymerase I (*E. coli*)	5′-3' DNA polymerase
	3′-5′ exonuclease (proofreading)
	5′-3′ exonuclease (repair)
Fatty acid synthase (type I; mammalian)	Seven different activities
The *arom* complex	Five different activities (aromatic amino acid biosynthesis)
B. *Multienzyme complexes*	
Pyruvate dehydrogenase	Pyruvate decarboxylase
	Transacetylase
	Dihydrolipoamide dehydrogenase
Fatty acid synthase (type II; *E. coli*)	Seven different activities

Although not simple, it should be possible to demonstrate that the same polypeptide (or multienzyme complex) is responsible for the main reaction as well as the other reaction(s), if any. The criteria of unity of an enzyme may be confirmed by one or more of the following protocols:

(a) All the different activities exhibited by the same enzyme (with one or more active sites) co-purify during various chromatographic separations. The ratio of their specific activities remains constant through various steps of purification. Since a pure enzyme elutes as a symmetric peak of constant specific activity, other activities of the same protein also behave similarly. A contaminating activity can be resolved, in principle, by one or the other separation step.
(b) The two activities displayed by the same active site are not purely additive because one substrate becomes competitive inhibitor of the other.
(c) A potent inhibitor affects two activities in parallel if they are due to the same active site. Inactivation (by heat, chemical agents, etc.) studies demonstrate a simultaneous loss of both the activities. Similarly, both activities are affected in the same way by the presence/absence of cofactors, if any.
(d) Differential proteolysis sometimes provides clues to the relationship between various activities of an enzyme. If they are due to the same active site, their kinetics of inactivation will be superimposable. Activities residing in different domains of a protein (e.G., multifunctional polypeptide) may be separated by limited proteolysis. For instance, various activities of mammalian fatty acid synthase can be released as separate fragments. Also, the Klenow fragment (corresponding to 324–928 amino acid residues) from *E. coli* DNA polymerase I is obtained by the release of the first 323 residues. Accordingly, Klenow fragment is missing the 5′-3′ exonuclease (repair) function.
(e) Replacement of an essential amino acid residue from the enzyme active site, through a site-directed mutagenesis (SDM) approach, should in principle affect all the activities due to that active site. For instance, both the carboxylase and the oxygenase activities of RuBP carboxylase are knocked off simultaneously by replacement of a single active site residue. With respect to enzymes having separate active sites, however, the situation will be different. The homoserine dehydrogenase activity of aspartokinase-homoserine dehydrogenase can remain unaffected by SDM at the aspartokinase site and *vice versa*. The SDM tool thus is a powerful approach in establishing the nature of multiple activities of the same protein – On the same active site or on different active sites.

Moonlighting and Promiscuous Enzymes A single enzyme protein could have multiple activities – either at the same active site or on sites distinct from the active site. Such activities are closely related to each other – either as part of the same

Table 14.4 Moonlighting activities of common metabolic enzymes

Enzyme example	Moonlighting activity
Structural components	
Lactate dehydrogenase, argininosuccinate lyase, enolase, GSH S-transferase	Lens crystallins
Transcriptional/translational regulation	
Aconitase	Iron-responsive element binding protein (IRE-BP)
E. coli thioredoxin	Subunit of T7 DNA polymerase
Proline dehydrogenase (PutA)	Transcriptional repressor
DNA repair and maintenance	
Glyceraldehyde 3-phosphate dehydrogenase, aconitase, fumarase	Cytosolic/nuclear component of the DNA damage response
Differentiation and maturation	
Phosphoglucose isomerase	Neuroleukin, autocrine motility factor, differentiation and maturation mediator
Ribonuclease 5 (angiogenin)	Angiogenesis (new blood vessel formation)

function or as related reaction steps in the metabolism (see Table 14.3). Many enzymes are known to "moonlight" and found to serve additional functions that are not related to their catalytic aptitude. The protein structural features outside of the enzyme active site often participate in their moonlighting action. The moonlighting functions of enzymes/proteins were usually discovered by chance. Otherwise normal members of the intermediary metabolism, the "moonlighting" activities of enzymes may involve structural and/or regulatory role(s) within or outside the cell. Enzymes known to display moonlighting activities with well-defined functions include transcriptional/translational regulation, differentiation and maturation, DNA repair and maintenance, as well as growth factors and structural components. A few well-known examples are listed in Table 14.4, and more may be found at the website http://moonlightingproteins.org/ and the reviews on this subject (Sriram et al. 2005; Gancedo and Flores 2008).

Besides their moonlighting activities, it is being recognized that many enzymes are also catalytically promiscuous. Due to their catalytic promiscuity, such enzymes are capable of catalyzing secondary (unrelated) reactions at an active site that is specialized to catalyze a primary reaction. The potential for catalytic promiscuity (see Chap. 5) can be an advantage in generating novel catalysts for industry. Both moonlighting by and catalytic promiscuity of enzymes are valuable playing fields for evolution to work (Copley 2003, Khersonsky et al. 2006). They also help in our understanding of enzyme structure/function relationships and in the directed evolution of new functions from existing protein scaffolds (see Chap. 39).

14.5 Summing Up

Quantitative tools to measure the amount of catalyst (enzyme) present in a given sample are a prerequisite to all of enzymology. Well-defined units allow us to express the amount of enzyme in a sample and its relative purity. With good bookkeeping practices, it is possible to follow the course of enzyme purification. A qualitative and quantitative analysis of a pure enzyme gives us the first description of its molecular features. While it is desirable, a homogeneous preparation of an enzyme is not an absolute necessity for kinetic analysis. Ensuring that the contaminants are not interfering is sufficient. In fact, cell extracts are by their nature "dirty enzymes"; they contain other enzymes that act before and after the enzyme of our interest acts. Study of enzymes in intact cells and organisms is ultimately necessary to take the in vitro enzyme data into in vivo situations (see Chap. 38).

References

Burgess R, Deutscher M (eds) (2009) Guide to protein purification, Meth Enzymol, vol 463, 2nd edn. Academic, San Diego

Copley SD (2003) Enzymes with extra talents: moonlighting functions and catalytic promiscuity. Curr Opin Chem Biol 7:265–272

Deutscher MP (ed) (1990) Guide to protein purification, Meth Enzymol, vol 182. Academic, San Diego

Gancedo C, Flores C-L (2008) Moonlighting proteins in yeasts. Microbiol Mol Biol Rev 72:197–210

Halling PJ, Gupta MN (2014) Measurement and reporting of data in applied biocatalysis. Perspect Sci 1:98–109

Khersonsky O, Roodveldt C, Tawfik D (2006) Enzyme promiscuity: evolutionary and mechanistic aspects. Curr Opin Chem Biol 10:498–508

Kirschner K, Bisswanger H (1976) Multifunctional proteins. Annu Rev Biochem 45:143–166

Sriram G, Martinez JA, McCabe ERB, Liao JC, Dipple KM (2005) Single-gene disorders: what role could moonlighting enzymes play? Am J Hum Genet 76:911–924