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MULTIFUNCTIONAL ENZYMES AND PATHWAY MODELLING

STEFAN SCHUSTER[1,*] AND IONELA ZEVEDEI-OANCEA[2]

[1]Friedrich Schiller University Jena, Faculty of Biology and Pharmaceutics, Section of Bioinformatics, Ernst-Abbe-Platz 2, D-07743 Jena, Germany

[2]Humboldt University Berlin, Section of Theoretical Biophysics, Invalidenstr. 42, D-10115 Berlin, Germany

E-Mail: *schuster@minet.uni-jena.de

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ABSTRACT

The analysis of network properties of metabolic systems has recently attracted increasing interest. While enzymes are usually considered to be specific catalysts, many enzymes in living cells are characterized by broad substrate specificity. Here we discuss some aspects of the treatment of such multifunctional enzymes in metabolic pathway analysis, for example, their suitable representation. The fact that the choice of independent functions of multifunctional enzymes is non-unique is explained. We comment on the annotation of such enzymes in metabolic databases and give some suggestions to improve this. We then explain the proper definition of metabolic pathways (elementary flux modes) for systems involving multifunctional enzymes and discuss some ontological problems.

INTRODUCTION

Metabolic pathway analysis has become a widely used tool in biochemical modelling [1-5]. It is instrumental in metabolic engineering [6,7] and functional genomics [8,9]. The analysis is based on the decomposition of metabolic networks into their smallest functional entities - the metabolic pathways. One of its major advantages is that it does not require any knowledge of kinetic parameters. It only uses the stoichiometric coefficients and information about the directionality of enzymatic reactions.

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A central concept in metabolic pathway analysis is that of elementary flux modes [10,11]. An elementary mode is a minimal set of enzymes that can operate at steady state, with all irreversible reactions used in the appropriate direction and the enzymes weighted by the relative flux they carry. Any flux distribution in the living cell is a superposition of elementary modes.

While enzymes are usually considered as specific biocatalysts, it should be acknowledged that many biochemical reactions in living cells are catalysed by enzymes with broad substrate specificity. That is, the same enzyme can alternatively convert various substrates. For example, 5'-nucleotidase (EC 3.1.3.5) can dephosphorylate AMP, IMP and other ribonucleotide monophosphates. Further examples are provided by transketolase (EC 2.2.1.1), hexokinase (EC 2.7.1.1) and branched-chain amino acid transaminase (EC 2.6.1.42). It has been argued that enzymes with high specificity have developed from low-specificity ancestors during biological evolution [12]. In pathway analysis and in biochemical modelling in general, only a few studies on enzymes with broad substrate specificity have been presented so far [12-14]. Such enzymes are often called multifunctional enzymes. However, the latter also include enzymes with more than one active site (e.g. multi-enzyme complexes). Here, we consider enzymes with only one active site, at which different substrates can be converted.

In bioinformatics and in the modelling of biochemical systems, an ever increasing role is played by online metabolic databases. Prominent examples are KEGG (http://www.genome.ad.jp/kegg/kegg2.html), BioCyc (http://biocyc.org/), ExPASY ENZYME (http://us.expasy.org/enzyme/), and BRENDA (www.brenda.uni-koeln.de). The former two include information on metabolic pathways, as well as information on individual enzymes. The latter has the advantage of including values of kinetic parameters of enzymes. KEGG has the helpful feature that in maps of metabolic networks, the enzymes present in particular organisms can be highlighted. Between these databases, numerous cross-links exist. By metabolic databases, the search for enzyme information is facilitated and accelerated in comparison to literature search. However, the data are sometimes less reliable.

In the present contribution, we discuss some aspects of the treatment of multifunctional enzymes in metabolic pathway analysis, for example, their suitable representation. The fact that the choice of independent functions of such enzymes is non-unique will be explained. We comment on the annotation of such enzymes in metabolic databases and give some suggestions to improve this. We then explain the proper definition of elementary modes for systems involving multifunctional enzymes and discuss some ontological problems.

REPRESENTATION OF REACTIONS CATALYSED BY ENZYMES WITH BROAD SUBSTRATE SPECIFICITY

Linearly independent functions

Let us first consider the example of transketolase. This enzyme can bind, and transfer two-carbon units between, glyceraldehyde-3-phosphate (G3P), erythrose-4-phosphate (E4P), ribose-5-phosphate (R5P), xylulose-5-phosphate (X5P), fructose-6-phosphate (F6P), sedoheptulose-7-phosphate (S7P), and several other substances which we will not consider here. Usually, two different functions are given in biochemistry textbooks:

Tkt1: R5P + X5P = G3P + S7P

Tkt2: E4P + X5P = G3P + F6P

with the equality sign denoting a reversible reaction (see, e.g. [15] and references given in [14]. However, the linear combination

Tkt3 = **Tkt1** - **Tkt2**: R5P + F6P = E4P + S7P

is equally simple. From among the three functions Tkt1, Tkt2 and Tkt3, only two are linearly independent. Any two linearly independent functions can be chosen - one might use, alternatively, Tkt1 and Tkt3, or Tkt2 and Tkt3.

The question arises whether reaction Tkt3 really proceeds without the detour via Tkt1 and Tkt2. BRENDA provides the information that in *Oryctolagus cuniculus* (European rabbit), the reaction

F6P + R5P = ?

occurs. The question mark means that the reaction product has not yet been verified experimentally. However, it is quite obvious that this reaction is just Tkt3, so that the products must be E4P and S7P. BRENDA is here "over-correct" in our opinion, because the products can often be inferred from other reactions, by linear combination of other functions of the same enzyme.

Nevertheless, the question remains whether for all multifunctional enzymes, all conceivable functions really occur. For non-catalysed reactions, often the assumption is made that such a transitive conclusion can be made: if reactions A and B proceed, then also reaction A-B proceeds [16].

However, this is subject to debate. Bauer [17] wrote: "Others insist, on purely statistical considerations, that all possible reactions should be included for the simple reason that since these could take place it is most likely they do, given the enormous number of molecular events involved. However, there are rational arguments in favour of a minimalist approach, to seek out the lowest number of dominant reactions required to account for the available data." For enzyme-catalysed reactions, steric hindrance may imply that reaction A-B virtually does not occur. The principle of microreversibility [18] says that the rate constants of the reactions around a cycle of reactions (such as Tkt1, Tkt2, Tkt3) fulfil the equation

$$\frac{k_{+A} \times k_{+B} \times k_{+C}}{k_{-A} \times k_{-B} \times k_{-C}} = 1.$$

It does not, however, say anything about the magnitude of the particular rate constants. It might be, for example, that k_C and k_{-C} are extremely small, so that reaction C does not proceed at a measurable rate.

Determining the rates of particular reactions in experiment meets with the difficulty of how to distinguish between the rate of reaction C itself and the "detour" reaction A-B. The convention to use Tkt1 and Tkt2 as "basic reactions" is originally due to the measurements of the entire pentose phosphate pathway by Horecker et al. [19]. They concluded that reactions Tkt1 and Tkt2 (together with transaldolase, EC 2.2.1.2, and the other monofunctional enzyme reactions of the pathway) provide the most plausible explanation for the measured exchange of radioactive label. Later, an alternative, the so-called L-type pentose phosphate pathway, has been proposed [20], which has not, however, become generally accepted. The correct sequence of events is very difficult to measure [15].

Some indications about the relative importance of the individual rates can be derived from the reaction mechanism of the enzyme and the chemical structure of the substrates. According to BRENDA, Tkt obeys a ping-pong bi-bi mechanism. This must be an ordered mechanism because Tkt catalyses a transfer reaction, which implies that the donor must bind first:

Donor1 + Tkt = Acceptor1 + $TktC_2$

Acceptor2 + $TktC_2$ = Donor2 + Tkt

As any potential donor can be followed in the mechanism by any potential acceptor, steric hindrance should not play a role.

Moreover, the various substrates are very similar to each other (monophosphates of monosaccharides with 3-7 carbons). Therefore, also Tkt3 is likely to proceed at a measurable rate.

The branched-chain amino acid transaminase catalyses the reversible reaction

L-leucine + 2-oxoglutarate = 4-methyl-2-oxopentanoate + L-glutamate

According to the database ExPASY ENZYME, instead of L-leucine, also L-isoleucine and L-valine can be used as substrates. BRENDA mentions, in addition, methionine, aspartate and several non-proteinogenic amino acids such as norvaline, aminopimelate, and aminobutanoate. Here, we restrict the analysis to leucine, isoleucine, valine, and glutamate. (The latter is a possible substrate as well since the reaction is reversible.) Thus, there are 4*3/2=6 different reactions in total. The number of linearly independent reactions equals three. Usually, the three reactions involving glutamate are taken as a "basis".

As far as chemical structure is concerned, the three branched amino acids are very similar, while glutamate differs in that it is not branched. BRENDA provides the information that in *Rattus norvegicus* and *Escherichia coli*, also the reaction

L-isoleucine + 3-methyl-2-oxobutanoate = 3-methyl-2-oxopentanoate + L-valine

occurs. Thus, the detour via glutamate does not appear to be necessary. This makes it likely that also the direct, reversible reactions from isoleucine to leucine and from valine to leucine can occur.

A likely reason for the usual choice of independent functions for branched-chain amino acid transaminase is the physiological role of glutamate as a molecule used for amino group transfer. Wagner and Fell [21] have shown by metabolic network analysis that glutamate is the most central metabolite except from cofactors such as ATP, ADP and NADH in that the mean pathway distance to all other metabolites is shortest. In fact, glutamate can be considered as a cofactor as well, since it transfers the amino group just as ATP transfers the phosphate group.

A further interesting example is aldolase (EC 4.1.2.13). It catalyses the reaction

F6P = G3P + DHAP,

where DHAP stands for dihydroxyacetone phosphate.

In photo-autotrophic plants and some bacteria, aldolase also catalyses the reaction (see, e.g., BRENDA)

S7P = E4P + DHAP.

Subtracting these two reactions gives

S7P + G3P = F6P + E4P.

This reaction equation is not, however, as equally simple as the other two. Therefore, we will not consider such linear combinations here, although, depending on the reaction mechanism, such a reaction might be relevant as well.

Annotation in databases

It is interesting to look into metabolic databases and see which functions of enzymes with low substrate specificity are indicated. In BRENDA, for transketolase, the reaction

S7P + G3P = R5P + X5P

is given as the main reaction. Moreover, several other reactions such as

X5P + E4P = F6P + G3P

F6P + R5P = ?

Hydroxypyruvate + G3P = CO2 + Ru5P

Hydroxypyruvate + R5P = S7P + ?

with the names of biological species in which they have been detected are listed.

In the KEGG database (and identically in the database ExPASy ENZYME), it is indicated that the main reaction is S7P + G3P = R5P + X5P and the following comment is given:

"A thiamine-diphosphate protein. Wide specificity for both reactants, e.g. converts hydroxypyruvate and R-CHO into CO2 and R-CHOH-CO-CH2OH. Transketolase from *Alkaligenes faecalis* shows high activity with D-erythrose as acceptor." (http://www.genome.ad.jp/dbget-bin/www_bget?enzyme+2.2.1.1).

KEGG includes a subdatabase called REACTION, which can be downloaded by anonymous ftp. REACTION comprises, for transketolase, the functions Tkt1 and Tkt2. Surprisingly, the information given in the clickable front-end of KEGG does not coincide with the data in the REACTION database.

BRENDA is much more comprehensive for this enzyme and most other enzymes in that it lists more reactions and because it includes information about the species in which they have been found.

In BioCyc, it is indicated that transketolase usually occurs in the form of two isoenzymes, TktA and TktB. According to BioCyc, TktA only performs function Tkt1, while TktB performs Tkt1 and Tkt2. According to our knowledge, however, TktA can also catalyse both reactions.

Let us now consider uridine kinase (EC 2.7.1.48). It catalyses reactions such as

ATP + uridine = ADP + UMP

GTP + uridine = GDP + UMP

uridine + dUTP = UMP + dUDP

uridine + dTTP = UMP + dTDP .

The REACTION database of KEGG indicates these and 14 other reactions. The total number of 18 reactions arises from the fact that nine phosphate donors (ATP, GTP, UTP, ITP, dUTP, dTTP, dGTP, dCTP, and dATP) and two phosphate acceptors (uridine and cytidine) are indicated. However, only 10 of these reactions are linearly independent. (This number can be calculated as 22 metabolites minus 12 conserved moieties, see [14]). In contrast to transketolase, for uridine kinase, the REACTION database also gives linearly dependent reactions.

IS ANNOTATION IN TERMS OF HALF-REACTIONS AN OPTION?

Enzyme reactions can be described at two different levels. First, one may consider the half-reactions of formation, isomerization and decay of the enzyme-substrate complex. This has been done, for example, by Nuño et al. [13] and Alberty [22]. The much more common way, however, is to consider the overall reactions of conversion of substrate into product. In the former case, one needs to know the reaction mechanism. This information is not always available.

It is of interest to compare the number of half-reactions with the number of overall reactions of a multifunctional enzyme. Let us take uridine kinase as an example. Differing views on its reaction mechanism can be found in the literature.

If it is an ordered, sequential mechanism and the phosphate donor binds first [23], then there are 9 half-reactions of binding the various donors, 2*9=18 half-reactions of binding the phosphate acceptors to the 9 different enzyme-phosphate-donor complexes, and 9+18=27 different half-reactions of release (Fig. 1A). In total, there are 54 different half-reactions.

If the enzymatic mechanism of uridine kinase is ordered ping-pong [24], then there are 2*(9+2)=22 half-reactions (Fig. 1B). In both cases, however, one can reduce the number of reactions to be considered by lumping sequential steps without branching in between. For example, the steps ATP + Urk = Urk-ATP and Urk-ATP = ADP + Urk-P can be combined into ATP + Urk = ADP + Urk-P (where Urk stands for uridine kinase).

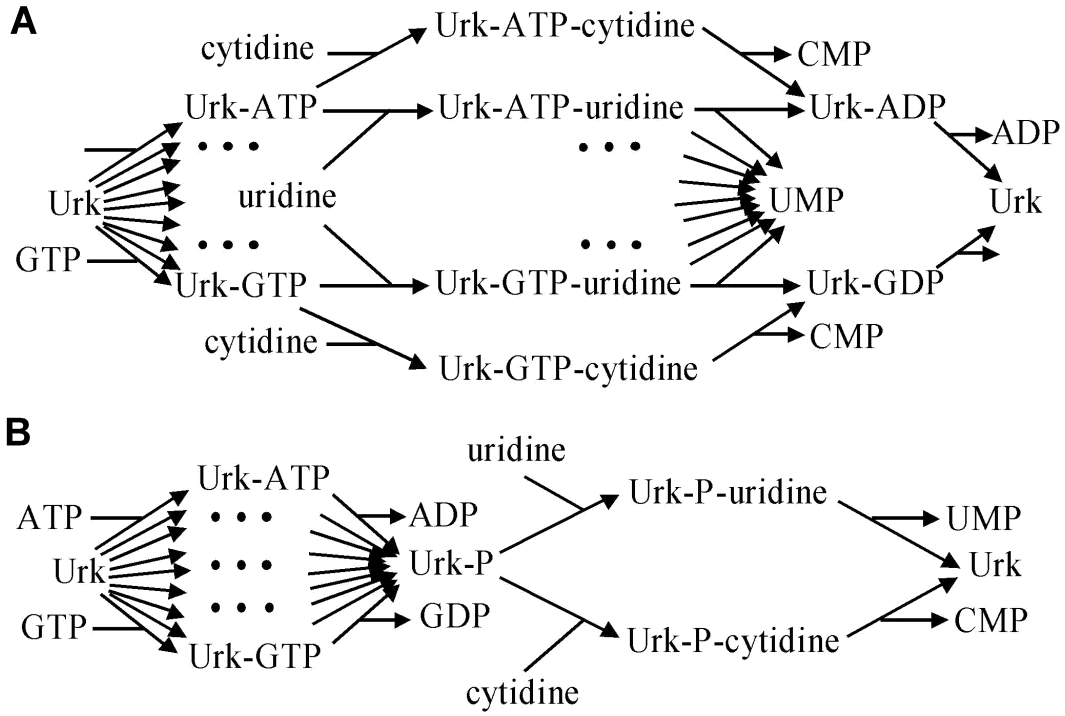

Figure 1. Possible reaction mechanisms for uridine kinase. Out of the 9 phosphate donors mentioned in the text, only two are shown explicitly for the sake of clarity. The others are referred to by "loose" arrows. Urk, uridine kinase. **A)** Ordered sequential mechanism. **B)** Ordered ping-pong mechanism.

This reduces the total number to 11. So we see that for some enzymes, the list of half-reactions can be shorter than the complete list of overall reactions if sequential steps are lumped.

Nevertheless, annotation of multifunctional enzymes in terms of half-reactions in databases appears to be inappropriate because of the frequent uncertainty about the reaction mechanism and the generally high number of half-reactions.

Importantly, the number of linearly independent functions is always smaller than the number of half-reactions because it is equal to the number of independent fluxes, which cannot be greater than the number of reaction steps. Thus, an annotation in terms of linearly independent functions is certainly better suited in spite of the arbitrariness of choosing these functions.

ONTOLOGICAL PROBLEMS AND SPECIES-SPECIFIC INFORMATION

In enzyme databases as well as in the literature, substances are given at different levels of specificity. For example, in some databases, the term "branched-chain amino acids" is used while in others, the specific amino acids are mentioned separately. Another example is provided by alcohol dehydrogenase (EC 1.1.1.1). In REACTION (and similarly in several other databases), the following reactions (amongst others) are given:

$$\text{Ethanol} + \text{NAD}^+ = \text{Acetaldehyde} + \text{NADH} + \text{H}^+ .$$

$$\text{\_1-Alcohol} + \text{NAD}^+ = \text{Aldehyde} + \text{NADH} .$$

If a multifunctional enzyme has been detected in an organism, it is not yet clear whether all functions of it are performed in that organism. Some substrates may be missing or the enzyme may be species-specific such that some functions are not performed. While information about the occurrence of particular functions of multifunctional enzymes in different organisms is missing in most databases, BRENDA provides much information about this. It gives lists of particular functions detected in particular organisms, e.g. for the particular functions of transketolase and branched chain amino acid transaminase in *Saccharomyces cerevisiae*, *E. coli*, *Triticum aestivum*, *Sus scrofa* and many other organisms.

This information is of importance for the reconstruction of metabolic maps. For example, the ability of aldolase to cleave S7P in some organisms may enable an alternative pentose phosphate pathway to work (for a theoretical analysis of potential monosaccharide pathways, though only up to six-carbon sugars, see [25]).

Of course, the substance class "primary alcohol" includes, for example, ethanol. This annotation makes automatic data-mining and usage for metabolic modelling difficult because common computer programs for metabolic modelling do not know that "ethanol" is a sub-concept of "alcohol". Thus, in the future, metabolic databases and/or simulation software should be extended to include a substance class ontology.

ARE THE ELEMENTARY MODES INDEPENDENT OF THE LEVEL OF DESCRIPTION?

The different functions of multifunctional enzymes should be treated as distinct reactions in the computation of elementary modes (pathways). Importantly, only a set of independent functions must be taken because linearly dependent reactions cause the occurrence of irrelevant cyclic elementary modes with no net transformation. For example, Tkt1 and Tkt2 have been used for the determination of the elementary modes of monosaccharide metabolism [11]. When using Tkt3 instead of one of the other two, the number of resulting elementary modes is the same.

As mentioned above, enzymes can be described at two different levels. The question arises as to whether the elementary modes are independent of the level of description. If all reactions are irreversible, this is really the case [14]. If some reactions are reversible, this is not necessarily so because two elementary modes may use the same reversible half-reaction in opposite directions, so that this reaction cancels when the modes are summed up. To resolve this discrepancy, it is helpful to examine the definition of elementary modes.

In the Introduction section, we have defined this concept as the "minimal set of enzymes" that fulfils certain properties. At the level half-reactions, one might be tempted to interpret this definition in terms of half-reactions rather than enzymes, and this is formally done by computer programs for computing elementary modes, e.g. METATOOL [26]. An example is the reaction system of monosaccharide metabolism studied by Nuño et al. [13]. In terms of overall reactions, this system gives rise to 296 elementary flux modes, while in terms of half-reactions, 866 modes would arise if the reaction steps were considered as basic units.

However, in the contradictory cases, several modes should be lumped because they are equivalent in terms of enzymes. If this is done, the elementary modes are indeed independent of the level of description [14]. The question of whether enzymes or reactions are the basic units in metabolism also occurs in Metabolic Control Analysis [27].

DISCUSSION

Using several example enzymes for illustration, we have outlined some problems in the modelling of enzymes with broad substrate specificity, as well as in the storage of data about such enzymes in online databases. We argue that in enzyme databases and for metabolic modelling, only independent functions of multifunctional enzymes need to be indicated, noting that linear combinations are possible.

Moreover, in many cases where not all reaction substrates or products are known, they can be inferred from other reactions by linear combination. It is worth investigating whether only such linear combinations need be considered that are equally simple (in terms of the number of reactants and products) as the "original" reactions

It is of interest to find criteria by which an appropriate choice of linearly independent functions can be made. One possibility is to choose the functions with the highest reaction rates, which requires, however, specific measurements. Another option is to choose the functions that occur in the convex basis [26] of the metabolic network in which the enzyme in question is embedded [14]. This is in line with the historic determination of the basic functions of transketolase within the pentose phosphate pathway [19]. In some cases, a third option is to seek for those reactions in which central metabolites, such as glutamate, energy currency metabolites and redox equivalents, are involved as much as possible.

In future modelling work, it will be worth analysing the evolutionary reasons why some biochemical reactions are catalysed by highly specific enzymes and others by less specific enzymes.

A compromise appears to have been found between efficient regulation and economy of the genome. For example, the branched-chain amino acid transaminase acts on very similar amino acids. If for each of these, a separate transaminase (with glutamate as amino group donor) existed, the synthesis of the amino acids could be regulated more specifically. However, more genes would be necessary in the genome. Another point may be that enzymes need to have a concentration greater than a certain lower bound in order to allow sufficiently frequent collision events. Since the synthesis of enzymes requires metabolic effort, two specialized enzymes need more effort than one less specific enzyme. A further interesting question is why enzymes exist (e.g. glucokinase, EC 2.7.1.2) the specificity of which covers part of the range of some other enzymes (e.g. hexokinase).

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