Biochimica et Biophysica Acta 1864 (2016) 70–76

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Review

Measuring specificity in multi-substrate/product systems as a tool to investigate selectivity *in vivo*☆

Yin-Ming Kuo, Ryan A. Henry, Andrew J. Andrews *

Department of Cancer Biology, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

ARTICLE INFO

Article history:
Received 11 April 2015
Received in revised form 7 August 2015
Accepted 25 August 2015
Available online 29 August 2015

Keywords:
Internal competition
Kinetics
Specificity
Selectivity
Mass spectrometry

ABSTRACT

Multiple substrate enzymes present a particular challenge when it comes to understanding their activity in a complex system. Although a single target may be easy to model, it does not always present an accurate representation of what that enzyme will do in the presence of multiple substrates simultaneously. Therefore, there is a need to find better ways to both study these enzymes in complicated systems, as well as accurately describe the interactions through kinetic parameters. This review looks at different methods for studying multiple substrate enzymes, as well as explores options on how to most accurately describe an enzyme's activity within these multi-substrate systems. Identifying and defining this enzymatic activity should help clear the way to using *in vitro* systems to accurately predicting the behavior of multi-substrate enzymes *in vivo*. This article is part of a Special Issue entitled: Physiological Enzymology and Protein Functions.

1. Introduction

It is common for one enzyme to be able to catalyze multiple substrates or interact with multiple sites, as has been found from various *in vitro* enzymatic studies (for example, cytochrome P450 enzymes [1,2], lysine acetyltransferases [3], and kinases [4]). In *in vivo* systems, as a consequence, all these potential substrates/sites also have the potential to act as competitors. Enzyme preference is usually revealed by different rates or affinities for substrates. The preference of an enzyme for one specific substrate is defined as the specificity, and the preference for one substrate over another is its selectivity. As such, a single target substrates matched with a single enzyme is the most direct system for investigating enzyme specificity *in vitro* (*i.e.*, classical steady-state approach). From the kinetic parameters obtained *via* this straightforward approach, one can determine the specificity (*i.e.*, specificity constant, k_{cat}/K_m) of a substrate [5,6]. The ratio of specificity constants from two different substrates with the same enzyme may then be used to interpret the preference of that enzyme for one substrate over the other: the selectivity [7]. These enzymatic kinetic parameters create adequate applications for *in vitro* systems, but it has been shown with corresponding observations from *in vivo* assays that some predictions of these parameters fail to match up [8–10]. The complexity of *in vivo* assays may lead to the following potential factors being overlooked: protein–protein interactions [11–14], enzymatic structural/conformational changes [15–18], and internal inhibition [19–22]. Thus, recent research has utilized the method of internal competition (multiple substrates to one enzyme) to study the selectivity of an enzyme between substrates [23–26]. This experimental design can/may more closely simulate the *in vivo* environment. However, such assays also create difficulties in producing accurate detections for multiple targets, as signals from one target have to be independent of the others. Fortunately, advances in current technologies allow for the measurement of multi-substrates/products in a less labor-intensive and time-consuming manner.

In this review, we examine multiplexed, high throughput, and the potentiality of real-time methodologies applied on different enzymatic selectivity assays and we provide an overview of how to use the kinetic parameters from internal competition to interpret the potential selectivity *in vivo*. Using this approach, we also highlight the possible constraints of each method, such as choices of substrate concentrations, time frame selections (steady-state condition), and/or available cofactors/inhibitors.

2. Techniques for multiplexed, high throughput measurements of multiple substrates/products

Internal competition is a method that has been used to investigate the differences of an enzyme for individual mixed substrates by measuring either the consumption rate of individual substrates or the generation rate of individual products. This method has also been extensively

Abbreviations: KAT, lysine acetyltransferases; PAH, polycyclic aromatic hydrocarbons; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance spectrometry; Gcn5, general control nonderepressible 5; Rtt109, regulator of Ty1 transposition 109; Vps75, vacuolar protein sorting 75; CBP, CREB-binding protein.

☆ This article is part of a Special Issue entitled: Physiological Enzymology and Protein Functions.

* Corresponding author.
E-mail address: Andrew.Andrews@fccc.edu (A.J. Andrews).

http://dx.doi.org/10.1016/j.bbapap.2015.08.011
1570-9639/

used in studying kinetic isotope effects [27–29]. Since the concentrations of multi-substrates and/or multi-products need to be monitored, a multiplexed analytical technique is required to measure all of the concentrations of each of these components for data analysis. This section will discuss the recent analytical techniques applied to study the kinetics of internal enzyme competition.

This simplest method for these approaches is liquid chromatography (LC), which relies on the separation of multiple substrates by hydrophobicity or cation/anion exchange, depending on what is being separated. This is often a reverse phase column with the accompanying detection as UV absorption, fluorescence, or radio chemical. An example of this approach is the analysis of multiple substances in the bioremediation of polycyclic aromatic hydrocarbons (PAHs) [30,31]. In this case, LC alone can separate various forms of PAHs; in other cases additional verification is needed, such as mass/charge.

Mass changes (either cleavage or addition/removal of functional groups) of a substrate are often the result of catalytic reactions. Thus, mass spectrometry (MS) is a common analytical technology that is utilized for these types of studies [32–35]. The coupling of LC or gas chromatography to MS provides separation of multiple substrates, and therefore more accurate quantification, for multiple target analysis [36,37]. Furthermore, tandem MS (MS/MS) can be used to acquire more spatial or structural information of analytes. For example, LC-MS/MS has been used to quantitate the substrates and/or products from enzymatic kinetic assays [13,38–40], and a detection resolution as small as a single amino acid residue can be reached [7,41,42]. Recently, this kind of site-specific study has even been utilized for the investigation of non-enzymatic protein modifications [7,14,43].

Another technique used for these internal competition assays, nuclear magnetic resonance spectrometry (NMR), is used to determine the kinetic isotope effects between stable isotope labeled substrates and unlabeled substrates [27–29]. As more innovative methodologies have been developed to utilize this advanced technology, a very high degree of precision and accuracy can be obtained for the measurement of low abundance, stable isotope-labeled substrates [44–47]. Additionally, with the proper sample and safety controls, a method of radioactive remote labeling can be utilized to study internal competition. A scintillation detector with a multi-channel analyzer can record different radiation energy from different radioactive sources. With this technique, a high sensitivity and high accuracy of measurements can be achieved for the detection of various radioactively labeled substrates [48–50]. The study of hydrogen tunneling by using radioactive remote labeling is one example of the application of this technique [51,52].

Internal competition assays are not limited to proteins. Substrates can also include DNA and/or RNA. Current biochemical and labeling techniques have been developed to effectively and efficiently measure the DNA/RNA kinetics of enzymatic catalysis [53–56]. For example, Goodman (et al.) used kinetic assays to investigate DNA polymerase fidelity by comparing the competition of right and wrong nucleotide incorporations [56]. As another example, substrate competition of endoribonucleases can occur *in vivo* because endoribonucleases can cleave multiple RNA substrates [57]. Harris (et al.) examined the internal competition between different tRNA precursors for ribonuclease P by radiolabelling substrates and directly quantifying the substrate specificity [58,59]. Furthermore, it has been found that RNA sequence is very specific for ribonucleases (for example, RNase H) [60–62]. To understand the substrate sequence specificity and site specificity, deep sequencing methods (for review see [63–65]) were used to investigate the frequency and locations of ribonuclease L cleavage sites of viral RNAs [66,67]. Site-specific studies have also been carried out, using primer extension reactions to characterize ribonuclease L specific cleavage sites in hepatitis C virus RNA [68] and DNA damage sites [69]. Utilizing these analytical technologies (*i.e.*, MS), coupled with these types of assays can be useful for the detection of not only the DNA/RNA sequences [70–72] but also the modifications on the individual nucleosides [73–75]. Additionally, MS analysis has been used to investigate the substrate selectivity of artificial restriction enzymes [76], which can be applied to manipulating RNAs for biotechnology applications.

Finally, the concept of this internal competition method was also applied to developing quantitative competitive polymerase chain reaction techniques to quantitate target DNA [77]. By competing with internal DNA segment, Tompkins (et al.) demonstrated that the reverse transcription-quantitative competitive polymerase chain reaction technique can quantitate the expression of seven cytokine mRNAs in domestic mammals [78]. This approach can be extended to barcoded samples like those demonstrated by Nguyen (et al.) where they paired unique DNA sequences to specific histone modifications in nucleosomes [79]. While this would require a separation step, it would also allow simple quantitative PCR in place of expensive sequencing or other technology. Through these types of experiments, we have seen the progress in adapting the knowledge gained from internal competition assays into the utilization in practical applications. All aforementioned methods that can be utilized to measure multiple substrates/products are summarized in Table 1.

3. Analysis of enzyme kinetics for substrate selectivity

3.1. Steady-state analysis of specificity and selectivity for multiple substrates/sites in simple systems

The enzyme kinetics of one substrate under multiple turnover steady-state conditions can be described by the Michaelis–Menten equation (Eq. (1)). The Michaelis–Menten equation describes a hyperbolic relationship when plotting the initial rate (v) *versus* substrate concentrations, [S]. Where [E] is the concentration of enzyme, and k_{cat} and K_m are steady-state kinetic parameters, representing the catalytic constant and the Michaelis constant, respectively. Conceptually, k_{cat} represents the number of turnover events occurring per unit time, and K_m is a relative measure of substrate binding affinity.

$$\frac{v}{[E]} = k_{cat}\frac{[S]}{([S]+K_m)}. \tag{1}$$

Differences in substrate specificity by a single enzyme have been studied since the 1920s [80], but it wasn't until the 1960s that a usable definition was articulated [81]. Specificity is "… defined as a higher rate of reaction with respect to some reference substrate or reaction … to measure the special contribution of the enzyme to the catalysis, we should compare the velocity of the enzymatic reaction to the velocity of a nonenzymatic reaction" [81]. From this point Brot and Bender use the term specificity constant to refer to k_{cat}/K_m [82], but it wasn't until 1974 that Fersht linked specificity and selectivity together, by using $(v/[E])_1/(v/[E])_2$, to show that induced fit and non-productive complexes are not represented in the specificity of an enzyme (in a simple system) [83]. From this point we can see specificity is linked to the ability to choose one substrate over another, or selectivity, and that k_{cat}/K_m, is the best description of specificity for a substrate because it will predict selectivity in a mixture of substrates in a simple system (Eq. (2)).

$$\left(\frac{v}{[E]}\right)_A \Big/ \left(\frac{v}{[E]}\right)_B = [A]\left(\frac{k_{cat}}{K_m}\right)_A \Big/ [B]\left(\frac{k_{cat}}{K_m}\right)_B. \tag{2}$$

We can apply this foundation to modern methods to understand selectivity between larger numbers of substrates. To do this we need to keep the standard steady-state assumptions, namely; total substrate concentration should remain close to the free substrate concentration, enzyme should be much less than the substrate concentration and less than 10% of total substrate should be consumed [6]. This gets more complicated when you have either one substrate that can produce multiple products as is the case with histone acetyltransferases [7,14,41], or multiple different substrates. In this simple system one substrate will not impact the specificity or selectivity of another substrate or product