From Kinetic Data to Mechanism and Back 28

We conclude our forays into enzyme kinetic mechanisms by summarizing three practical aspects. Some examples of relating mechanism with steady-state kinetic data are detailed. Secondly, a general scheme that one should follow in orderly collection of kinetic data is given. This last section (in Part III) is an attempt to bring home the point that enzyme kinetic study is not just "blue sky" research – but has real practical value.

28.1 How to Relate Mechanisms with Steady-State Kinetic Data

A product inhibits the enzyme-catalyzed reaction by virtue of its binding to one or more enzyme forms. The product inhibition patterns offer useful inputs in deciding the kinetic mechanism of a multi-substrate and/or multiproduct enzyme. In a bi-reactant mechanism, information on the slope and intercept effects due to a product is normally obtained in a systematic manner. For example, concentration of one substrate (say A) is varied at different fixed levels of product inhibitor (say P). This experiment itself should be performed at two distinct fixed concentrations of B – once with saturating (noted as $B = \infty$) and again with subsaturating (noted as $B = K_B$). As P is the inhibitory product used in this case, the reaction rates may be followed by formation of Q. For Q as the inhibitor, we need to monitor P however. Thus there are eight different product inhibition patterns possible for a bi-reactant mechanism.

We recall here that saturation with a fixed substrate (a) may result in an irreversible step in the mechanism (see Chap. 18) and (b) leads to no inhibition by the product that competes for the same enzyme form. Armed with an understanding of equilibria, irreversible steps, how slope (V_{max}/K_M; 1/slope of Lineweaver-Burk plot) and intercept (V_{max}; 1/intercept of Lineweaver-Burk plot) are affected, and the thumb rules (described in Chap. 18), we can predict various product inhibition patterns for any given mechanism. *Converse of this is what we do in practice –*

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experimentally obtain various slope/intercept effects and from these data arrive at the enzyme kinetic mechanism. The expected product inhibition patterns for three common bi-reactant mechanisms are given below.

28.1.1 Ordered Mechanism

Lactate dehydrogenase is an example of ordered bi-substrate reaction. The substrate–product pair of NAD^+/NADH is the outer pair and binds the free enzyme according to the scheme (Fig. 28.1):

The predicted slope/intercept effects and the product inhibition patterns are given in Table 28.1.

28.1.2 Random Mechanism

Hexokinase is an example of random bi-substrate reaction. The substrates and products bind various enzyme forms according to the scheme (Fig. 28.2):

The predicted slope/intercept effects and the product inhibition patterns are given in Table 28.2.

The prediction of product inhibition patterns (for both ordered and random mechanisms) shown above is made with the understanding that the *EBQ* dead-end

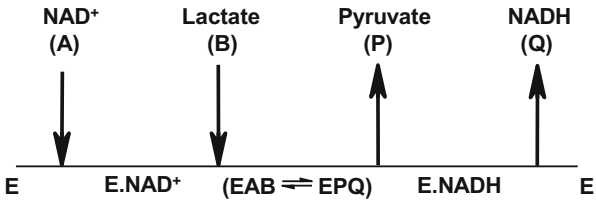

Fig. 28.1 Ordered bi-substrate reaction of lactate dehydrogenase

Table 28.1 Product inhibition patterns for an ordered bi-reactant mechanism

Product inhibitor	Substrate varied	Fixed substrate at	Enzyme parameter affected		Inhibition pattern
			Intercept ($1/V_{max}$)	Slope (K_M/V_{max})	
P	*A*	$B = \infty$	Yes	No	Uncompetitive
P	*A*	$B = K_B$	Yes	Yes	Noncompetitive
P	*B*	$A = \infty$	Yes	Yes	Noncompetitive
P	*B*	$A = K_A$	Yes	Yes	Noncompetitive
Q	*A*	$B = \infty$	No	Yes	Competitive
Q	*A*	$B = K_B$	No	Yes	Competitive
Q	*B*	$A = \infty$	No	No	No inhibition
Q	*B*	$A = K_A$	Yes	Yes	Noncompetitive

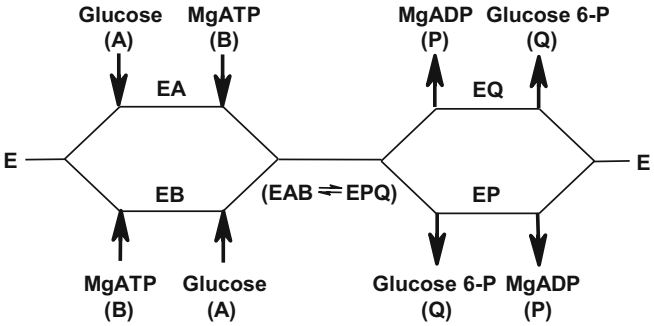

Fig. 28.2 Random bi-substrate reaction of hexokinase

Table 28.2 Product inhibition patterns for a random bi-reactant mechanism

Product inhibitor	Substrate varied	Fixed substrate	Enzyme parameter affected		Inhibition pattern
			Intercept ($1/V_{max}$)	Slope (K_M/V_{max})	
P	A	$B = \infty$	No	No	No inhibition
P	A	$B = K_B$	No	Yes	Competitive
P	B	$A = \infty$	No	No	No inhibition
P	B	$A = K_A$	No	Yes	Competitive
Q	A	$B = \infty$	No	Yes	Competitive
Q	A	$B = K_B$	No	Yes	Competitive
Q	B	$A = \infty$	No	No	No inhibition
Q	B	$A = K_A$	Yes	Yes	Noncompetitive

complex is possible and is formed. Here Q is not only acting as a product inhibitor but is also combining with EB in a dead-end fashion. We can rationalize occurrence of such complexes from the physical picture of the enzyme active site and how substrates and products occupy their respective places there. Consider a kinase reaction where the γ-Ⓟ from ATP is transferred to an acceptor R–OH. This could be hexokinase where acceptor R–OH is nothing but glucose.

$$\text{Ado} - \text{Ⓟ}\text{Ⓟ}\text{Ⓟ} + \text{HO} - \text{R} \rightleftarrows \text{Ado} - \text{Ⓟ}\text{Ⓟ} + \text{Ⓟ}\text{O} - \text{R}$$

Besides their respective single occupancy on the enzyme (EA, EB, EP, and EQ), the following four ternary complexes (Fig. 28.3) could be anticipated in principle.

Two of these (complexes 3 and 4 in Fig. 28.3) are dead-end complexes. The EAP complex (complex 3) is expected to be observed as one Ⓟ (the γ-Ⓟ) is missing. However the formation of complex 4 (the EBQ complex) depends on whether the extra piece (Ⓟ group here) can be accommodated at the active site or not. We now can generalize this to other enzyme examples – (a) EBQ should form for smaller

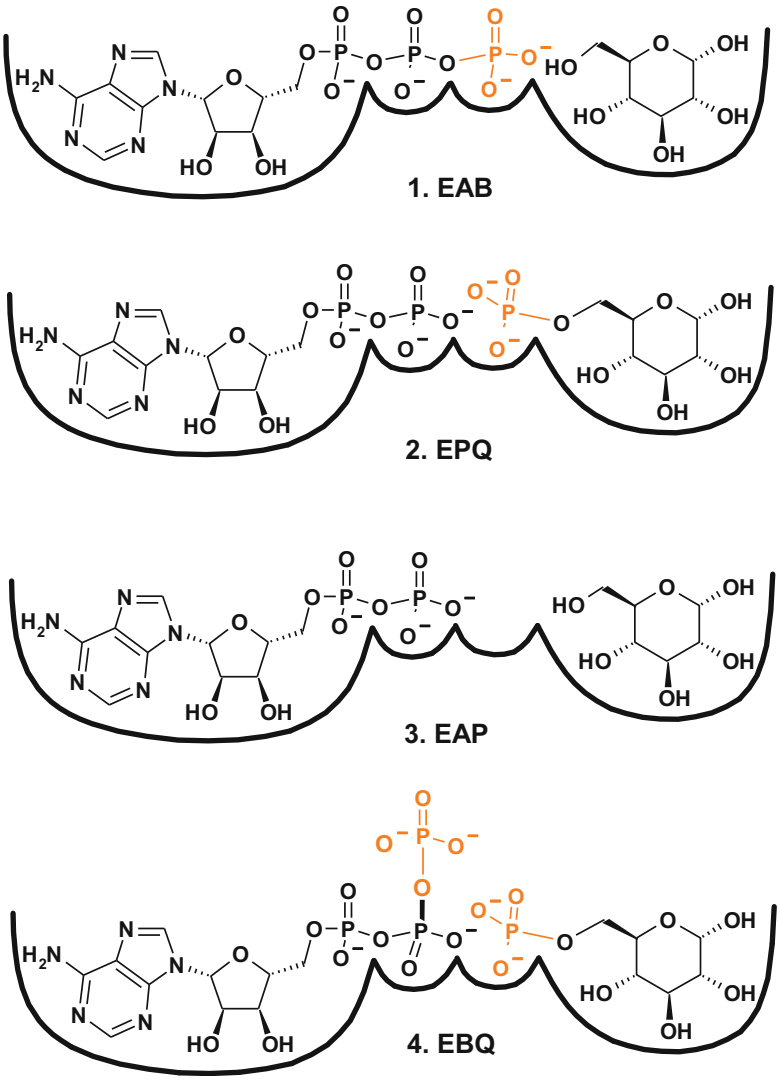

Fig. 28.3 Different ternary complexes possible with the enzyme in a kinase mechanism. The scheme is for hexokinase with glucose and is simplified by not showing Mg^{2+} in Mg-ATP. The four complexes are (1) *EAB* (E.Mg-ATP.glucose), (2) *EPQ* (E.Mg-ADP.glucose 6-phosphate), (3) *EAP* (E.Mg-ADP.glucose), and (4) *EBQ* (E.Mg-ATP.glucose 6-phosphate)

groups (like hydride from NADH), (b) *EBQ* may form with reduced affinity for groups like acetyl or phosphoryl, and (c) not at all for larger ones like glycosyl or adenosyl group. The above predictions (Tables 28.1 and 28.2) will obviously change if dead-end complexes other than *EAP* (like *EBQ*) also are formed.

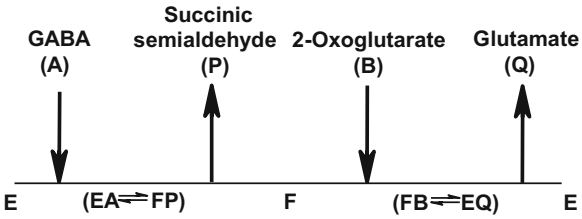

Fig. 28.4 Ping-pong bi-bi mechanism of GABA transaminase. The two enzyme forms *E* (pyridoxal phosphate-enzyme) and *F* (pyridoxamine phosphate-enzyme) are shown

Table 28.3 Product inhibition patterns for a ping-pong bi-reactant mechanism

Product inhibitor	Substrate varied	Fixed substrate	Enzyme parameter affected		Inhibition pattern
			Intercept ($1/V_{max}$)	Slope (K_M/V_{max})	
P	*A*	$B = \infty$	No	No	No inhibition
P	*A*	$B = K_B$	Yes	Yes	Noncompetitive
P	*B*	$A = \infty$	No	Yes	Competitive
P	*B*	$A = K_A$	No	Yes	Competitive
Q	*A*	$B = \infty$	No	Yes	Competitive
Q	*A*	$B = K_B$	No	Yes	Competitive
Q	*B*	$A = \infty$	No	No	No inhibition
Q	*B*	$A = K_A$	Yes	Yes	Noncompetitive

28.1.3 Ping-Pong Mechanism

4-Aminobutyrate transaminase (GABA transaminase) is an example of ping-pong bi-substrate reaction. GABA and succinic semialdehyde form one substrate–product pair for partial reaction that defines the *ping* part, and 2-oxoglutarate and L-glutamate form the other pair (defining the *pong* part). Schematically this may be shown as (Fig. 28.4).

The predicted slope/intercept effects and the product inhibition patterns are given in Table 28.3.

These product inhibition patterns are predicted for a ping-pong mechanism involving a single active site – both the half reactions occurring in the same site. However with multisite ping-pong mechanism, the product inhibition patterns are distinct. Transcarboxylase is an interesting example of two-site ping-pong mechanism involving a biotin shuttle.

$$\text{Oxaloacetate} + \text{Propionyl CoA} \rightleftarrows \text{Pyruvate} + \text{Methylamlonyl CoA}$$

Reactions on the transcarboxylase enzyme surface with two sites are shown in Fig. 28.5. Since the thioesters (propionyl CoA and methylmalonyl CoA) combine at one site, they compete with each other while oxaloacetate and pyruvate compete

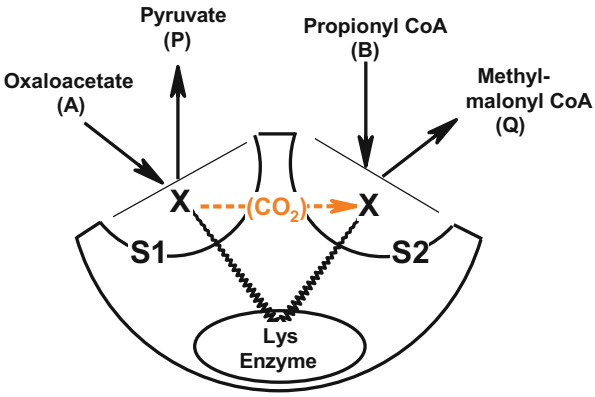

Fig. 28.5 Schematic representation of the transcarboxylase reaction. Oxaloacetate (*A*) and pyruvate (*P*) interact with site S1 while the thioester pair (propionyl CoA and methylmalonyl CoA, shown as *B* and *Q*, respectively) interacts at the S2 site. The swinging arm with biotin (*X*) shuttles the carboxyl group from S1 to S2

for the other site. Because of this, the product inhibition patterns are reverse of the patterns for the classical ping-pong mechanism.

In multisite ping-pong mechanisms, the group to be transferred is moved from one site to the other. This movement may involve a swinging arm attached to the enzyme such as biotin (transcarboxylase), lipoic acid (pyruvate dehydrogenase complex), phosphopantetheine (fatty acid synthase), or a protein channel connecting the two sites (glutamate synthase).

28.2 Assigning Kinetic Mechanisms: An Action Plan

Various lines of kinetic experimentation (presented in earlier sections) provide different bits of information on the overall enzyme mechanism. The tools range from steady-state kinetics to isotopic analysis to tracking enzyme intermediates. Obviously, no single line of practical enquiry provides all the details needed to set down the complete mechanistic scheme. It is therefore essential to decide when to undertake which type of experiment(s) – studying a new enzyme – requires an action plan. A logical sequence of collecting data is thus an important part of this experimental strategy. An organized decision tree capturing this mental process is outlined below (Fig. 28.6).

It is much more demanding to distinguish among the possible sequential mechanisms (Leskovac et al. 2004). Isotope exchange study is powerful enough to tell the subtle differences between various sequential Schemes. A parallel initial velocity pattern quickly leads one to ping-pong mechanism, but the caveat of "how parallel is parallel" requires considerable care. The apparently parallel initial velocity pattern for brain hexokinase (with glucose as substrate) became unambiguously intersecting with fructose as alternate substrate. Collecting confirmatory evidence adequately settles such issues however.

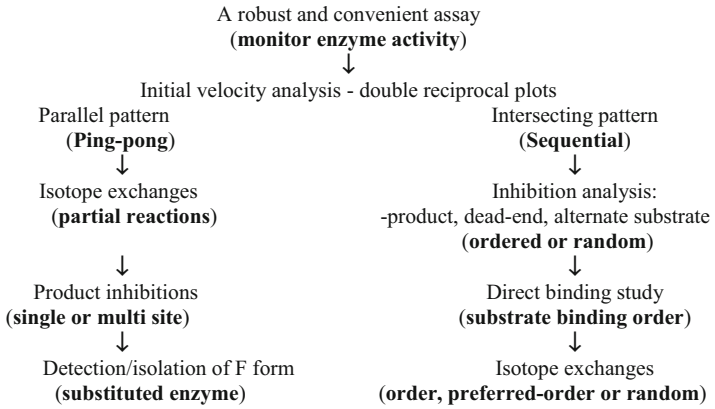

Fig. 28.6 Flow chart outlining the mental process leading to enzyme kinetic mechanism from experiments. Conclusions reached after each data generated is given in parenthesis in bold

28.3 Practical Relevance of Enzyme Kinetics

Apart from the intellectual satisfaction of having understood the inner workings of a remarkable catalyst, there are many direct benefits of a detailed kinetic analysis. This aspect of enzyme kinetic mechanism is often underappreciated by many. We will enumerate three important areas where the knowledge of enzyme kinetics directly becomes relevant.

28.3.1 Affinity Chromatography and Protein Purification

Moderate affinities encountered with substrates, products, or their analogs hinder their use as ligands for affinity purification. The intrinsic ligand affinity may be further compromised upon chemical cross-linking for immobilization on the matrix. Transition-state analogs offer useful affinity ligands; they often exhibit tighter binding and therefore are promising candidates. They can also be used as eluents for conventional substrate affinity chromatography.

Creatine kinase was conveniently purified with one such strategy. The transition state of creatine kinase involves a flat phosphate intermediate (see Fig. 28.4 in Chap. 32). Nitrate ion (NO_3^-) mimics this flat phosphate moiety. This knowledge was used to purify creatine kinase by immobilizing it on ADP column. The enzyme binds tightly to this column in the presence of creatine and NO_3^- – a tight transition-state complex is formed. The bound enzyme could be released selectively by adding free ADP to compete with the column bound ADP.

Secondly, prior knowledge of kinetic mechanism permits judicious development of binding/elution strategies in enzyme purification. Enzyme interaction with a substrate analog affinity matrix can be strengthened by suitably including another

substrate in the binding buffer. This may be achieved on the matrix itself, either by exploiting a synergistic binding or through kinetically locking the ternary (*EAB*) complex. For instance, glutamate dehydrogenase binds poorly to NAD-Sepharose; inclusion of glutarate (substrate mimic and a dead-end inhibitor) in the developing buffer retards the enzyme significantly on this column. Bound enzyme may be released simply by excluding the second ligand (e.g., glutarate) from the elution medium. Enzyme mechanism and substrate analogs can be used to arrive at best conditions for retardation/binding/elution from substrate analog affinity matrices. Such kinetic lock-in strategies hold much promise in large-scale enzyme purifications.

28.3.2 Dissection of Metabolism

Specific enzyme inhibition serves a very valuable tool in teasing out metabolism. Competitive inhibition of succinate dehydrogenase by malonate is a classic example in the discovery of citric acid cycle. Attempts to define the glycolytic sequence and mitochondrial electron transport chain also made use of specific inhibitors. Inhibition of an enzyme in vivo may be seen as a metabolic cross-over at that step of metabolic pathway.

Starting from kinetic properties of individual enzymes, it may be possible to reconstruct the characteristics of an entire pathway. Capturing metabolic complexity and structure through such a bottom-up approach is one of the objectives of "systems biology." Lastly, the kinetic characterization of the key enzymes around a metabolic branch point provides some indications of relative in vivo flux to competing pathways. Everything else being equal, the enzyme with lower K_M for the common substrate (metabolite) dictates the pathway direction (refer to Chap. 38 In vitro versus in vivo – Concept and consequences, in Part V). For an example, arginase and nitric oxide synthase compete for the cellular pool of arginine; the knowledge of their inhibition kinetics was exploited to design specific inhibitors and augment flux through nitric oxide synthase.

The disadvantage of a competitive inhibitor is that higher concentrations of the substrate can nullify its effect. Noncompetitive inhibitors could be more effective as they cannot be overwhelmed by more substrate.

28.3.3 Enzyme–Targeted Drugs in Medicine

Enzymes are catalysts that make and break specific covalent chemical bonds. They bind as well as catalyze – while other protein classes including receptors, transporters, antibodies, and even DNA only bind – thereby offering unique features for drug design. A number of enzyme targets have been exploited and the corresponding inhibitors are in the market as drugs (Alexander et al. 2017). Many of the drugs are natural compounds whose molecular basis of action was clarified ex post facto. With excellent understanding of enzyme action over time, rational drug

Table 28.4 Examples of enzyme-targeted therapy

Enzyme target	Drug example	Mode of inhibition	End use
Dihydropteroate synthase	Sulfanilamide	Competitive	Antibacterial
Dihydrofolate reductase	Methotrexate	Tight binding	Leukemia
Cyclooxygenase	Ibuprofen	Tight binding	Anti-inflammatory
HMG CoA reductase	Atorvastatin	Substrate analog	Hypercholesteremia
Xanthine oxidase	Allopurinol	Mechanism based	Gout
α-Amylase	Acarbose	Transition state	Diabetes
Angiotensin-converting enzyme	Captopril	Transition state	Hypertension
HIV retropepsin	Saquinavir	Transition state	AIDS
Alanine racemase	D-Cycloserine	Covalent PLP adduct	Tuberculosis
Tracylglycerol lipase	Orlistat	Covalent adduct	Obesity
D-Ala-D-Ala carboxypeptidase	β-Lactams	Covalent adduct	Antibacterial

design has arrived. Experimental access to transition-state features of a target enzyme is an excellent opportunity to design potent drugs. We have already come across some cases of tight binding and suicide enzyme inhibitors in use (Chap. 21). Few more representative examples of successful drugs, their target enzyme, and the nature of inhibition are given in Table 28.4. Enzyme activators have not had much practical success so far. Activators of glucokinase may yet find applications in the future diabetes therapy.

Captopril was the first rationally designed enzyme-targeted drug. It may be viewed as a transition-state inhibitor of angiotensin-converting enzyme (ACE; peptidyl dipeptidase A). Similarly HIV retropepsin inhibitors were developed through detailed kinetic knowledge and a variety of structure-assisted drug design techniques. Saquinavir contains a hydroxyethylamine isostere moiety and functions as a transition-state analog. It is quite clear from the current state of art that rational drug design through a thorough understanding of enzyme kinetics, transition state, and active site structure is here to stay. It has overtaken the traditional serendipity-based screening approach in pharmaceutical industry (De Cesco et al. 2017).

Finally, enzyme targets are also valuable in other applications. Few organophosphorus compounds inhibiting acetyl cholinesterase are in the market as insecticides/pesticides. Three successful herbicides act via potent inhibition of their respective target plant enzymes. Phosphinothricin is activated on phosphorylation by glutamine synthetase to a tight-binding inhibitor which mimics its transition state. N-Phosphonomethyl glycine (glyphosate) acts as an herbicide by preventing aromatic amino acid biosynthesis in plants (Boocock and Coggins 1983). It acts as an uncompetitive inhibitor and is thought to resemble the transition state of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) belonging to the shikimate pathway. Branched chain amino acid biosynthesis is blocked by sulfonylureas. Sulfonylurea herbicides inhibit in a time-dependent manner, bear no

resemblance to acetolactate synthase (the target enzyme in plants) substrates, and are noncompetitive inhibitors. The first two of the above herbicides are interesting molecules in that they contain a direct C–P bind in them. In all the three cases, genes expressing inhibitor resistance have been isolated. These herbicides in conjunction with their resistance genes have found direct use in developing genetically modified crops.

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