Approaches to Kinetic Mechanism: An Overview

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The order of addition of substrates to and release of products from the enzyme active site, along with establishment of relative rates of various events, defines the kinetic mechanism. These mechanisms fall into two broad groups – those where the full complement of substrates have to assemble on the enzyme active site before the reaction occurs are termed "sequential mechanisms." In the other category, a product (s) is released between additions of two substrates and are called "ping–pong mechanisms." In this category, substitution on the enzyme active site groups occurs and hence is also known as double displacement mechanism. Study of these mechanisms is best approached by rigorous experimental design where data is collected by systematically varying one parameter at a time. This method of reduction is in full display in enzyme kinetics. In fact, elucidating enzyme kinetic mechanisms offers the best example of how scientific hypotheses are tested. Elements of scientific method in sequence include problem recognition → collation of available information → hypothesis building → experimentation → reasoning and deduction → and refining the hypothesis. These steps are iterated in arriving at an enzyme mechanism. Finally when experiments and measurements agree with the theory, truth is secured. In practical enzyme kinetics, this exercise translates into following steps (Table 18.1) for the elucidation of mechanisms.

A very important aspect of kinetic mechanism elucidation is that a given set of data, at times, may fit/describe more than one unique kinetic scheme. For instance, all the available kinetic evidence could not distinguish between a S_N1 (carbonium ion formation) mechanism or a covalent catalysis (involving the active site carboxylate). The S_N1 mechanism predicts retention of stereochemical configuration (at C1 of the glycosidic sugar) in the product, whereas covalent catalysis (via the acylal) passing through two Walden inversions also predicts a retention. Often other methods (other than steady-state kinetics!) may have to be resorted to in such cases. More recent MALDI-TOF evidence for a lysozyme-acylal favors the covalent catalysis mechanism. Resolution of kinetic equivalence invariably requires more incisive experimentation to cleanly distinguish between different possibilities. If

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Table 18.1 Steps to kinetic mechanism elucidation

1. Write a minimal mechanism based on available information
2. Experimentally obtain kinetic parameters/constants involved
3. Build a probable mechanism through diagnostic experimentation involving:
(a) A study of initial velocities
(b) Use of different inhibitions (product, dead end, substrate, alternate substrate)
(c) Isotopic studies (both, exchange analysis and isotope effects)
(d) pH-dependence studies
This is a desirable order for experimentation but one need not necessarily be rigid
4. Review the mechanism by reasoning and deduction
5. Confirm and/or refine the mechanism by designing more experiments

Table 18.2 Nature of information obtained from experiments

Experimental approach	Nature of information obtained
1. Pre-steady-state kinetics	Detection of enzyme complexes/intermediates, rate-limiting k values
2. Variation of [S]; analysis of initial velocity patterns	Kinetic constants; sequence of complexes; binding order
3. Inhibition analyses; variation of [P], [I], etc.	Active site definition; sequence of complexes; binding order
4. Substrate/product structures	Map of the active site and geometry
5. Isotope exchange study	Partial reactions; distinction between mechanisms
6. Isotope effects	Individual rate constants; chemical mechanism; *TS* structure
7. pH variation and kinetics	Relevant pKas for catalysis and/or binding; nature of functional groups

there are no unique testable differences between rival mechanisms, then it may never be possible to resolve the kinetic ambiguity (the so-called black box).

18.1 Which Study Gives What Kind of Information?

All those ligand (substrate, inhibitor, activator, etc.) interactions which result in altered enzyme reaction rates may be exploited to understand the kinetic mechanism. Enzyme-ligand interactions that are kinetically silent are of no consequence to this study. For example, a molecule may bind to the enzyme without changing any of its kinetic properties. Such binding may be potentially useful in enzyme purification and/or stability studies but is useless in defining the kinetic mechanism. The nature of mechanistic information that can be gleaned from various kinetic studies is summarized in the Table 18.2 below.

Of these, pre-steady-state kinetics was introduced in an earlier section (Chap. 11). The remaining approaches form the subject matter of subsequent chapters and will be discussed in greater details.

18.2 Two Thumb Rules

There are two component activities to any kinetic study – one qualitative and the other quantitative. From a systematic analysis of the kinetic data by different plots and replots and slope and/or intercept changes, various kinetic constants like V_{max}, k_{cat}, K_M, K_{iA}, K_P, etc. are evaluated. At the qualitative level, inspection of slopes and intercepts of a double-reciprocal plot is very informative. We are thus looking at the two limiting cases – sub-saturating (slope) and saturating (intercept) concentrations of the varied substrate. Any change in slope (which is K_M/V_{max}) points to a change in the first-order rate constant. An effect on the intercept ($1/V_{max}$) similarly reflects on the zero-order rate constant (see Chap. 15 in Part II). The presence of an inhibitor brings about an increase in the magnitude of intercept, slope, or both. On the other hand, increasing substrate (other than the one whose saturation is being studied) concentration leads to lower intercept, slope, or both (Fig. 18.1).

A careful interpretation of slope and intercept changes is at the heart of understanding and postulating a kinetic mechanism. Let us therefore attempt to make some generalizations on how this can be done. We have seen earlier (derivation of rate equations) that along the reaction path, few distinct kinetically significant enzyme forms may occur. The total enzyme ($[E]_t$) is distributed into these forms depending on the extant equilibria (and/or steady state). A corresponding rate equation was derived from this description by evaluating for the fraction of $[E]_t$ present as *ES* (the productive complex). Factors (such as substrate, inhibitor, activator, or pH) that perturb this equilibrium result in a redistribution of $[E]_t$ into various enzyme forms. Any consequent change in the concentration of *ES* leads to change in reaction velocity. Intuitively, we can therefore predict how the slopes and intercepts of Lineweaver–Burk plots are affected by any substance (substrate, product, etc.) based on the distribution of enzyme forms and their equilibria. The converse of this exercise is of course of great practical value – we can set up appropriate equilibria from the slope and intercept effects caused by any substance. This is the crux of enzyme kinetic mechanism. From the expected redistribution of $[E]_t$ into different enzyme forms, two thumb rules may be stated here. These rules were framed by the famous enzymologist WW Cleland, to predict slope and intercept effects for product and dead-end inhibitors. However we generalize and extend them to any substance that binds and perturbs the enzyme equilibrium (of $[E]_t$ distribution).

Rule I. A ligand (substrate, product, or inhibitor) affects the intercept (zero-order rate) of the double-reciprocal plot when it combines reversibly with an enzyme form other than that with which the varied substrate combines.

Rule II. The slope (first-order rate) of the double-reciprocal plot is affected when (a) the ligand and the varied substrate combine reversibly with the same enzyme form or (b) the ligand and varied substrate bind reversibly to two different enzyme forms that are connected by a series of reversible steps along the reaction path.

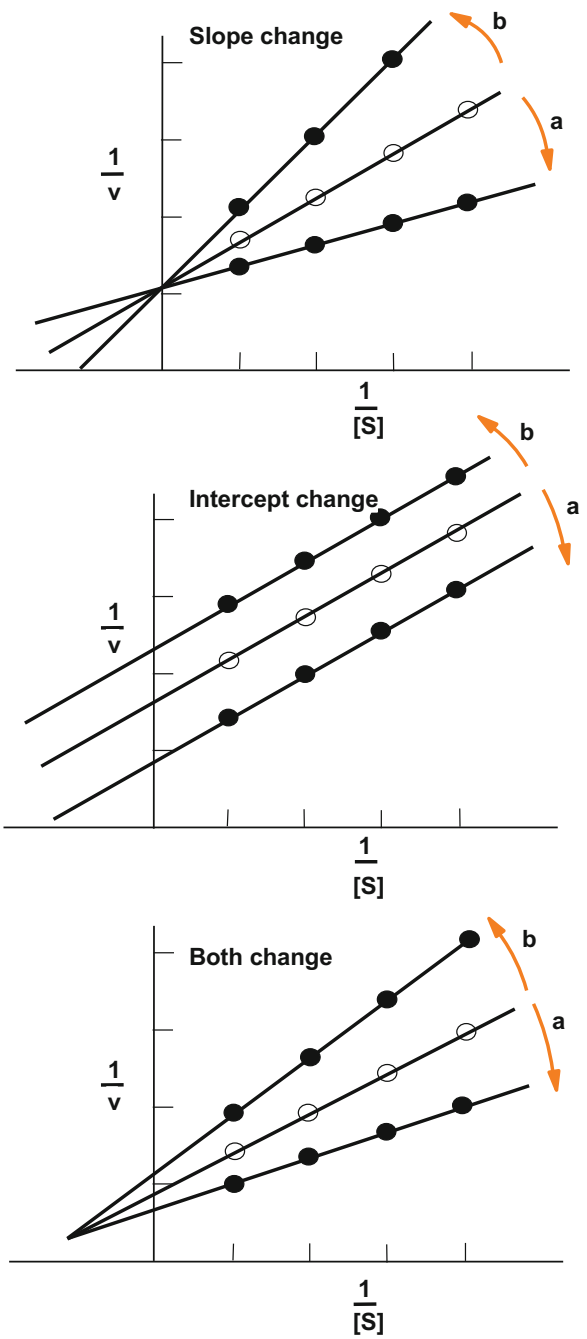

Fig. 18.1 Possible changes in the slope and intercept of double-reciprocal plots. Direction of change with increasing concentrations of (*a*) other substrate, activator, etc. and (*b*) product, inhibitor, etc. is indicated by respective arrows

It goes without saying that once the slope and intercept effects have been independently predicted, they can be combined to generate the whole picture (pattern). The rule II.b above requires that we know whether two enzyme forms, along the reaction path, are reversibly connected or not. Seeking such reversible connectivity with dead-end inhibitors (Chap. 20 Enzyme Inhibition Analysis) is tricky since they do not form the part of normal reaction sequence. A step becomes irreversible when it involves (i) addition of substrate at saturation ($[S] \rightarrow \infty$), (ii) release of product under initial velocity conditions ($[P] = 0$), or (iii) some irreversible chemical event (with a large negative ΔG) like CO_2 release, oxidation of an aldehyde to acid or aromatization of a ring, etc. Logical connections between (a) prediction of slope and intercept effects from a given kinetic scheme and (b) arriving at a kinetic mechanism from experimentally observed slope and intercept effects are better understood with suitable examples. A few case studies are therefore included in a concluding section of the elucidation of kinetic mechanism (Chap. 28; From kinetic data to mechanism and back).