Analysis of Initial Velocity Patterns

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One could in principle study the full time course of an enzyme-catalyzed reaction until the equilibrium is reached. This is not practiced because (a) the equations soon become very complex to handle; (b) with reasonable progress of the reaction, inhibition by product(s) sets in; and (c) it is difficult to follow the many, simultaneously changing reactant concentrations. For these reasons experiments are best conducted under steady state and initial velocity conditions. Employing the method reduction approach, data is generated almost always by systematically changing one variable at a time.

Nature of experiments conducted and the information sought from the initial velocity data are as follows:

1. Monitor initial velocity "v" by varying the concentration of one substrate at different fixed concentrations of the others. If the enzyme reaction in question involves single substrate, then there is not much information to be had other than obtaining kinetic constants (V_{max} and K_M).
2. The $v \rightarrow [S]$ data are plotted in the double reciprocal format (Lineweaver-Burk plots), and the patterns are analyzed qualitatively.
3. If the plots are nonlinear, then they suggest that the enzyme under study may exhibit (a) substrate inhibition, the curve being concave upward, or (b) cooperativity in its interaction with its substrates – Concave upward for positive cooperative and concave downward for negative cooperative interactions. We will have more to say on these nonlinear plots later.
4. Gradual changes in the slope and/or intercepts, as a function of the fixed substrate concentration, are noted. It may be recalled that change in slope points to a change in V_{max}/K_M (which in turn reflects on the first-order rate constant). An effect on the intercept is similarly related to V_{max} and the zero-order rate constant.
5. On quantitative analysis of slope and intercept changes, various kinetic constants are evaluated.

N. S. Punekar, *ENZYMES: Catalysis, Kinetics and Mechanisms*,
https://doi.org/10.1007/978-981-13-0785-0_19

Several interesting variations of the initial velocity patterns are possible and are indeed observed. However we will restrict ourselves to some of the more common patterns observed in such studies.

19.1 Intersecting Patterns

This is indicative of sequential combination of the two substrates considered in the study. The equilibria representing such a general case are given below (Fig. 19.1):

Three individual situations commonly encountered could include (a) random, (b) preferred ordered, and (c) ordered addition of *A* and *B*. In an ordered sequential mechanism (the upper path, $E \rightarrow EA \rightarrow EAB \rightarrow$ Products), only *EA* is formed, whereas in the random case (both paths leading to *EAB*, $E \rightarrow EA \rightarrow EAB \rightarrow$ Products as well as $E \rightarrow EB \rightarrow EAB \rightarrow$ Products), both *EA* and *EB* are formed. A general equation derived for a two-substrate sequential case will look like:

$$v = \frac{V_{\max}[A][B]}{K_{iA}K_B + K_A[B] + K_B[A] + [A][B]}$$

This rate equation will be identical, and it cannot distinguish between the ordered and random mechanism. Remember that as long as we reach the same *EAB* complex from the two routes, the equation will be symmetric. This is what is expected of any state function (path-independent property). Therefore, $K_{iA}K_B = K_{iB}K_A$ in the random mechanism. We note that K_{iA} is the kinetic dissociation constant of *A* from *EA* (and K_{iB} for the dissociation of *B* from *EB*) (Frieden 1957).

19.1.1 Determination/Evaluation of Kinetic Constants and Replots

On double reciprocal analysis, one obtains slope and intercept values at different fixed concentrations of *B* (Fig. 19.2). Similar plots can also be obtained with *B* as the varied substrate but at different fixed concentrations of *A*.

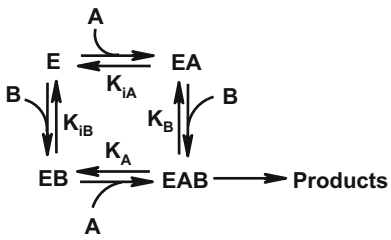

Fig. 19.1 Equilibria representing sequential interaction of substrates in a bi-reactant mechanism

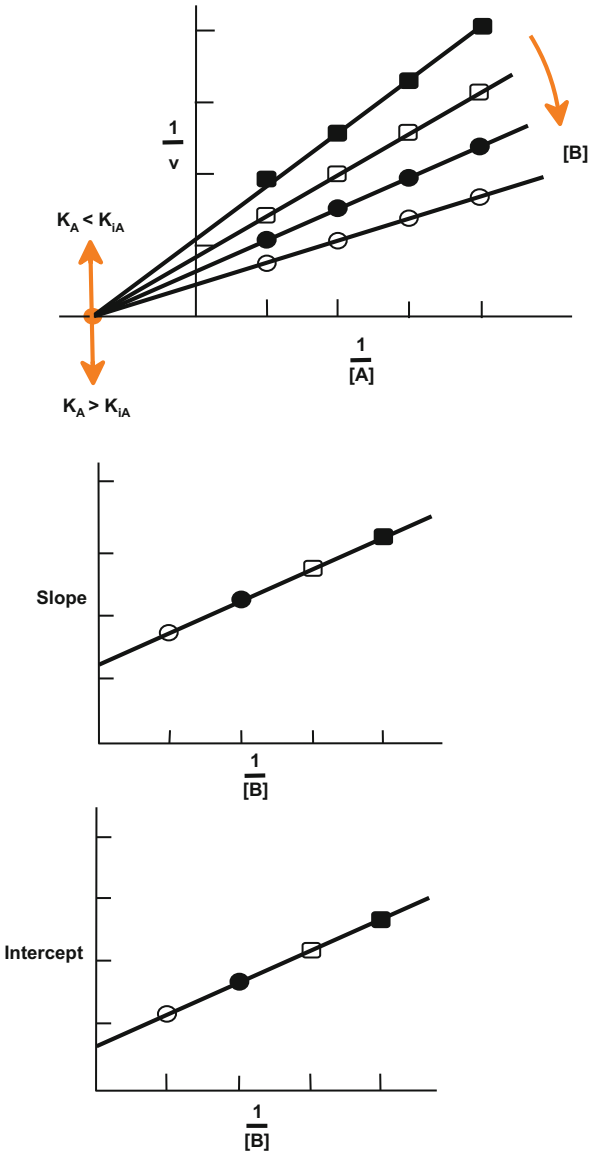

Fig. 19.2 Double reciprocal plot for sequential mechanism with *A* as the varied substrate. Replots of slope→1/[*B*] and intercept→1/[*B*] are shown below

They are further evaluated from the following relationships:

$$\text{slope} = \frac{K_A}{V_{max}} + \frac{K_{iA}K_B}{V_{max}[B]}$$
$$\text{intercept} = \frac{1}{V_{max}} + \frac{K_B}{V_{max}[B]}$$

A replot of intercept→1/[*B*] is linear with (a) reciprocal of intercept giving V_{max} and (b) dividing slope by intercept gives K_B. Substituting these values in the slope→1/[*B*] equation, we can extract values of K_A and K_{iA}. Although useful, this is not of course the best way to evaluate these constants; one should use statistical fits to the entire data set, and user-friendly software/programs are available for the purpose.

19.1.2 Interpretation

Intersecting Lineweaver–Burk patterns arise because both slope (first-order rate) and intercept (zero-order rate) change as a function of different fixed [*B*] values. Note that *A* and *B* bind two different enzyme forms at equilibrium, and hence both slope and intercepts change. These are indicative of sequential mechanisms, but we cannot distinguish various subsets ranging from "random" to "ordered" by initial velocity pattern analysis alone. We need to perform other types of experiments to get there. These include (a) direct substrate-binding experiments with radiolabels, e.g., LDH (from bovine heart muscle) binds NAD^+ (E-NAD^+ forms) but not lactate (E-lactate does not form); it binds lactate only in the presence of NAD^+. Ordered addition of substrates is indicated – NAD^+ followed by lactate; (b) direct monitoring of binary and/or ternary complexes by MALDI-TOF; (c) ordered versus random binding through product inhibition and isotope exchange studies (Chap. 26; Isotope exchanges at equilibrium and Chap. 28; From kinetic data to mechanism and back).

Coordinates for the point of intersection depend upon the relative values of K_A and K_{iA}. The X-axis coordinate corresponds to $-1/K_{iA}$ (which is $-1/K_{iB}$ when analyzing for *B*), while the ordinate intercept value is $\frac{1}{v} = \frac{1}{V_{max}}\left(1 - \frac{K_A}{K_{iA}}\right)$ (which is $= \frac{1}{V_{max}}\left(1 - \frac{K_B}{K_{iB}}\right)$ when *B* is studied). How the expressions for these coordinates are obtained may be found in the appendix to this chapter.

The position of the crossover point depends on the relative magnitudes of K_A and K_{iA} (the kinetic dissociation constant of *A* from *EA*). These are illustrated in the Fig. 19.2. For example,

$K_A = K_{iA}$ then the lines intersect on X-axis
$K_A < K_{iA}$ then the lines intersect above X-axis
$K_A > K_{iA}$ then the lines intersect below X-axis

There are examples (like Mg-ATP binding to creatine kinase) where $K_{iA} >> K_A$. This indicates a tighter binding of A to the enzyme when *B* is bound and is termed synergistic binding. Clearly, the K_{iA} (the kinetic dissociation constant of *A* from

EA) may not be a simple dissociation constant but may contain additional rate constants – such as for enzyme conformational change – within it.

19.2 Parallel Patterns

Parallel initial velocity patterns are suggestive of a ping-pong (double displacement) mechanism. The equilibria representing such a general case are given below (Fig. 19.3):

Two situations commonly encountered include (a) single-site ping-pong and (b) multisite ping-pong mechanisms. In a two-substrate ping-pong mechanism, no ternary complex (*EAB*) is formed. A general equation derived for a two-substrate ping-pong case will look like

$$v = \frac{V_{\max}[A][B]}{K_A[B] + K_B[A] + [A][B]}$$

Notice that no K_{iA} term appears in the denominator of this rate equation (compare with the expression for sequential mechanism above). This is same as putting $K_{iA} = 0$ and is consistent with the absence of *EAB* from of the enzyme and an absence of slope effect in the double reciprocal plots. Now the question is – what may appear parallel – is it really parallel? Here we should quickly note that K_{iA} may have a very small value; and if so, it becomes quite difficult from measured data to decide whether the slope is really changing or not. The lines may appear parallel but actually intersect far away to the left of the origin – this is what happens with brain hexokinase with glucose as substrate. We are thus relying on quantitative information to answer a qualitative question. While it is easy to conclude that a set of lines intersect (i.e., $K_{iA} \neq 0$), we need additional lines of evidence to ensure that a pattern is genuinely parallel (i.e., $K_{iA} = 0$). For instance, D-amino acid oxidase showed "almost" parallel lines in initial velocity analysis. It was originally thought

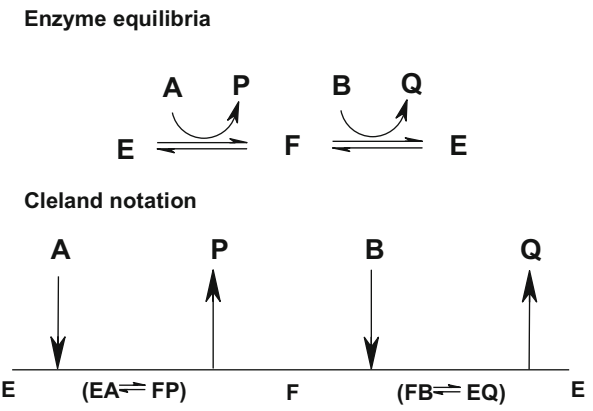

Fig. 19.3 Equilibria representing a bi-reactant ping-pong mechanism

that the imino acid (oxidized amino acid that leads to pyruvate formation) is released from E-$FADH_2$ prior to O_2 binding. Subsequently, pyruvate was found to compete with alanine. This product inhibition pattern clearly showed the reaction to be sequential and not ping-pong. We will revisit the question "How parallel is parallel?" while dealing with reversible enzyme inhibition analysis (Chap. 22; Reversible inhibitions).

The difficulty of concluding whether a set of lines are parallel (in double reciprocal plots) may be sorted by employing Woolf–Hanes plot (see Chap. 17). For a ping-pong reaction, plots of $[A]/v \rightarrow [A]$ at various fixed $[B]$ values will converge on $[A]/v$ axis. Such a convergence (and any deviation from the common point of interaction) is readily recognized.

19.2.1 Determination/Evaluation of Kinetic Constants and Replots

The double reciprocal form of the above equation will be.

$$\frac{1}{v} = \frac{K_A}{V_{max}} \frac{1}{[A]} + \frac{1}{V_{max}} \left(1 + \frac{K_B}{[B]}\right)$$

Upon inspection and analysis, one obtains no change in slope (as expected with a parallel set of lines), while the intercept values at different fixed concentrations of B do change (Fig. 19.4).

They are evaluated from the following relationships:

$$\text{slope} = \frac{K_A}{V_{max}}$$

$$\text{intercept} = \frac{1}{V_{max}} + \frac{K_B}{V_{max}[B]}$$

A replot of intercept$\rightarrow 1/[B]$ is linear with (a) reciprocal of intercept giving V_{max} and (b) slope divided by intercept gives K_B. Substituting for V_{max} in the slope$\rightarrow 1/[B]$ equation, we can assign a value for K_A. The best way of course is to evaluate these constants by suitable statistical fits to the entire data set.

19.2.2 Interpretation

Parallel patterns (in a two-substrate case) are normally indicative of a ping-pong mechanism. Consider the two-substrate two-product classical ping-pong mechanism (Fig. 19.3 above). Employing the thumb rules listed earlier (see Chap. 18; Approaches to kinetic mechanism – An overview), the following predictions on the initial velocity patterns can be made (Table 19.1).

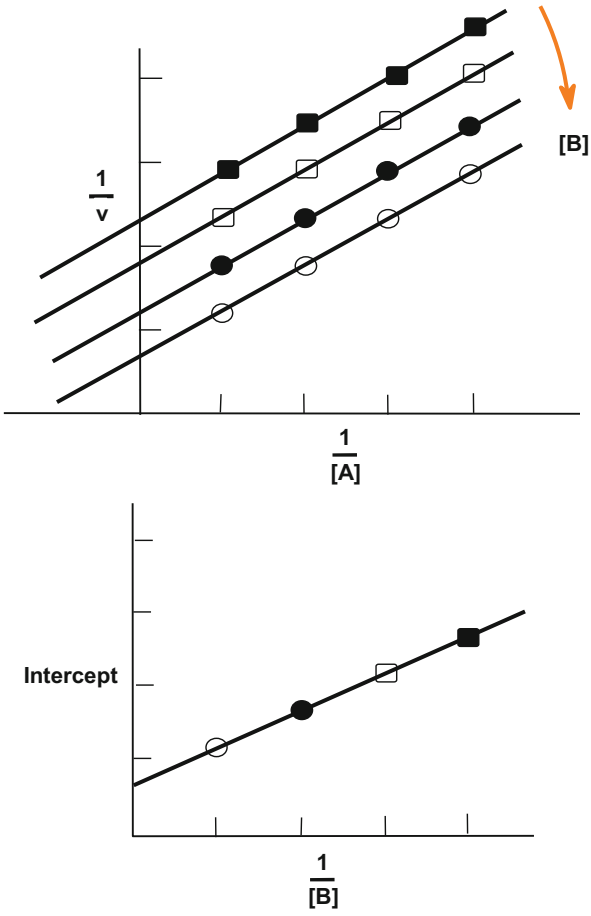

Fig. 19.4 Double reciprocal plot for a ping-pong mechanism with *A* as the varied substrate. Replots of intercept→1/[*B*] is shown below

Table 19.1 Expected initial velocity patterns for a two-substrate ping-pong mechanism

Substrate varied	Fixed substrate at	*Enzyme parameter affected*		Initial velocity pattern
		Intercept ($1/V_{max}$)	Slope (K_M/V_{max})	
A	$B = \infty$	Yes	No	Parallel
A	$B = K_B$	Yes	No	Parallel
B	$A = \infty$	Yes	No	Parallel
B	$A = K_A$	Yes	No	Parallel

$B = \infty$ implies saturating [*B*]; $B = K_B$ implies subsaturating [*B*]

These predictions are based on an understanding of different enzyme forms and their equilibria. *A* binds to *E* and *B* binds the *F* form of the enzyme; the two forms are not reversibly connected as product *P* release is irreversible when $[P] = 0$. The arguments for the situation where [*A*] is varied are as follows:

(a) At low [A] we are looking at slope effect (first-order rate), and any level of [B] cannot affect the interaction of A with E; conversion of $E \rightarrow F$ is rate limiting and not $F \rightarrow E$, hence no slope effect in double reciprocal plots.
(b) At $A = \infty$ we are looking at intercept effect (zero-order rate), and all enzyme is captured into EA form; because $F \rightarrow E$ becomes rate limiting, any level of [B] affects the overall reaction rate and intercept is affected.
(c) Summing up the results of a and b above, only intercept (zero-order rate) of the double reciprocal plot changes as a function of different fixed [B] values; we obtain parallel set of lines as a consequence.
(d) E and F enzyme forms get reversibly connected when finite levels of P are present. At low [A], now F can go back by binding with P or go forward on interacting with B. Therefore varying [B] will affect the rate (and slope). With $A = \infty$ situation, B affects the rate as before (in b above). The net result is an intersecting pattern.

Thus a parallel pattern turns intersecting (and a slope effect is introduced!) if one of the products (say P) is deliberately included in the experiment. Such a change is diagnostic of P release interrupting the sequential addition of A and B (scheme for the ping-pong mechanism above). Addition and presence of P establish a reversible path between the two forms of the enzyme to which A and B bind, respectively. Notice that initial velocity measurements in the presence of added P can be made by estimating the other product Q.

It is not necessary that parallel patterns always mean a ping-pong mechanism. There are occasions where one obtains parallel patterns, but the mechanism is not ping-pong: (a) In a three-substrate fully ordered sequential mechanism, intersecting $A \rightarrow C$ pattern is observed when [B] is subsaturating. This pattern turns parallel with saturating levels of B. This is actually diagnostic of B being the middle substrate. The slope effect disappears because saturating B introduces an irreversible step (by forcing all the enzyme forward to EAB form) in between the additions of A and C. Saccharopine dehydrogenase is an example of this kind with lysine as the middle substrate. Similar study with a three-substrate partly ordered sequential mechanism (first substrate ordered, e.g., ATP citrate lyase) gives two parallel patterns. No parallel patterns are observed in the case of fully random or C-ordered three-substrate mechanisms. Three-substrate ping-pong mechanisms generally give at least two parallel patterns. A detailed analysis of all such cases may be found in Viola and Cleland (1982). (b) In a Theorell–Chance mechanism, $A \rightarrow B$ pattern appears parallel (see below).

19.3 Few Unique Variations

The relative magnitudes of various kinetic constants in a given mechanism, sometimes lead to interesting examples. In such cases the patterns appear distinct from the two typical cases described in A and B above. We will look at two unusual variations of initial velocity patterns in this section.

A two-substrate ordered mechanism where the addition of *A* is at equilibrium is called an equilibrium-ordered mechanism. As expected of a sequential mechanism, both patterns are intersecting. However two unusual features arise due to the equilibrium addition of *A*. (a) The slope replot of $1/v \rightarrow 1/\mathrm{A}$ pattern passes through origin indicating that $1/K_\mathrm{A}$ tends to infinity (i.e., $K_\mathrm{A} \approx 0$). Since A gets trapped on the enzyme to form *EAB* complex at infinite [*B*], the corresponding double reciprocal plot is a horizontal line (zero slope). (b) For the same reason, $1/v \rightarrow 1/B$ pattern intersects on the Y-axis (saturating [*B*]) giving no intercept effect. Both these features, diagnostic of an equilibrium-ordered mechanism, also define the first substrate to add – that is *A*.

The second example is Theorell–Chance mechanism. Here sequential ordered addition of *A* and *B* occurs, while the central complexes (*EAB*↔*EPQ*) are found in insignificant levels. At any subsaturating level of [*A*], *B* takes it quickly forward to release *P*. Because of this apparent irreversibility, the slope effect is quite small, and the $A \rightarrow B$ pattern looks nearly parallel. However if we run the reaction in the slow reverse direction (with *P* and *Q* as substrates), the pattern definitely intersects. Alcohol dehydrogenase from horse liver follows Theorell–Chance mechanism.

Because parallel patterns could appear outside of ping-pong mechanisms, further proof of ping-pong mechanism will come from demonstration of (a) relevant partial reactions and (b) the substituted form (the *F* form) of the enzyme. Partial reactions are best evidenced through isotope exchange studies (Chap. 26; Isotope exchanges at equilibrium). Substituted (*F* form) of enzyme is often characterized by isolating and/or trapping it. We will have more to say on these forms with respect to their chemical mechanisms (in Part IV).

Appendix

Coordinates for the point of intersection in the Lineweaver–Burk plots for sequential mechanism.

These coordinates can be readily evaluated from the general rate equation above by a bit of tedious algebra. Consider the double reciprocal form of the above equation for sequential mechanism.

$$\frac{1}{v} = \left[\frac{K_\mathrm{A}}{V_\mathrm{max}}\left(1 + \frac{K_\mathrm{iA}K_\mathrm{B}}{K_\mathrm{A}[B]}\right)\right]\frac{1}{[A]} + \frac{1}{V_\mathrm{max}}\left(1 + \frac{K_\mathrm{B}}{[B]}\right)$$

At two different values of [*B*], i.e., $[B]_1$ and $[B]_2$, we obtain the same $1/v$ only at the crossover point (point of intersection). We can thus equate the two rates and simplify the equation:

$$\frac{K_A}{V_{max}}\left(1+\frac{K_{iA}K_B}{K_A[B]_1}\right)\frac{1}{[A]}+\frac{1}{V_{max}}\left(1+\frac{K_B}{[B]_1}\right)$$
$$=\frac{K_A}{V_{max}}\left(1+\frac{K_{iA}K_B}{K_A[B]_2}\right)\frac{1}{[A]}+\frac{1}{V_{max}}\left(1+\frac{K_B}{[B]_2}\right)$$

And therefore $\left(\frac{K_{iA}}{[A]}+1\right)\left(\frac{1}{[B]_1}-\frac{1}{[B]_2}\right)=0$

Since $\left(\frac{1}{[B]_1}-\frac{1}{[B]_2}\right)$ term cannot be zero by experimental design, we have $\frac{K_{iA}}{[A]}+1=0$ and hence $\frac{1}{[A]}=-\frac{1}{K_{iA}}$. At the crossover point, therefore $1/[A]$ corresponds to $-1/K_{iA}$ (accordingly for B it is $-1/K_{iB}$). We can now substitute $-1/K_{iA}$ for $1/[A]$ in the equation above and simplifying.

$$\frac{1}{v}=-\left[\frac{K_A}{V_{max}}\left(1+\frac{K_{iA}K_B}{K_A[B]}\right)\right]\frac{1}{K_{iA}}+\frac{1}{V_{max}}\left(1+\frac{K_B}{[B]}\right)$$
$$\frac{1}{v}=\frac{1}{V_{max}}-\frac{K_A}{K_{iA}}\frac{1}{V_{max}}$$
$$\frac{1}{v}=\frac{1}{V_{max}}-\frac{K_A}{K_{iA}}\frac{1}{V_{max}}=\frac{1}{V_{max}}\left(1-\frac{K_A}{K_{iA}}\right)$$

In a similar manner, we can obtain the ordinate intercept in the case of B.

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