16

Deviations from Linear Kinetics

INTRODUCTION

In the consideration of enzyme kinetics in Chaps. 13 to 15 we examined a variety of systems and formal kinetic mechanisms. In each a common theme has been the development of equations to describe the initial rate behavior of the system. These (with one exception, which is considered again in this chapter) have all been, in the double-reciprocal form, equations describing linear Lineweaver–Burk plots. Although the theory discussed in Chaps. 13 to 15 was developed to describe linear kinetics, there are many instances of experimental observations that do not obey the simple theories. It is the purpose of this chapter to examine some of the types of nonlinear Lineweaver–Burk plots that have been reported and discuss the possible causes for such behavior. The experimental approaches that may be used in attempting to establish which of these various possibilities account for a particular deviation from linearity are also considered.

EXPERIMENTAL OBSERVATIONS

As has become apparent from Chaps. 13 to 15, a Lineweaver–Burk or Michaelis–Menten plot for an enzyme-catalyzed reaction is usually a straight line or a simple rectangular hyperbola, as illustrated in Fig. 16-1. Also shown are two often observed types of deviation from this simple behavior. In the first, the rate of the reaction appears to be more sensitive to changes in the substrate concentration at low substrate concentrations, while in the second the rate appears to be less sensitive to changes in substrate concentration at low substrate concentrations.

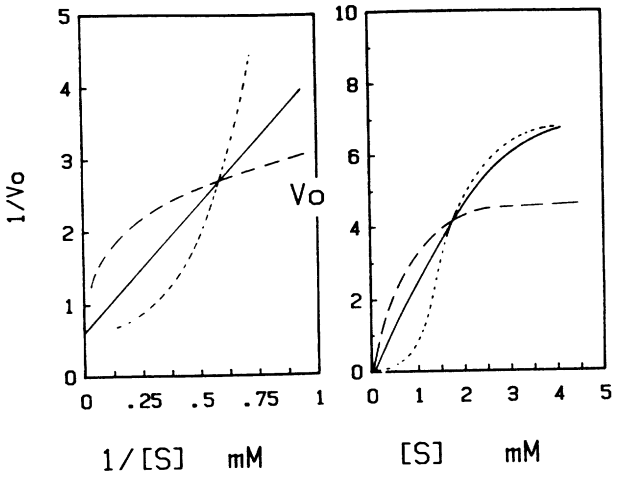

Figure 16-1 Lineweaver–Burk plot and Michaelis–Menten plot showing "normal" behavior (——) and deviations from normal behavior resulting in an increased response to changes in [S] at low concentrations (– – –) or a decreased response to changes in [S] at low concentrations (· · ·).

Another sort of deviation from normal behavior is shown in Fig. 16-2. In this instance as the substrate concentration increases past a certain point, the rate actually decreases, as indicated by the downward curvature of the V versus [S] plot or the sudden upward turn of the Lineweaver–Burk plot as 1/[S] decreases.

The final deviation is given in Fig. 16-3. This superficially resembles one of the cases illustrated in Fig. 16-1, but is distinguished based on the abruptness of the

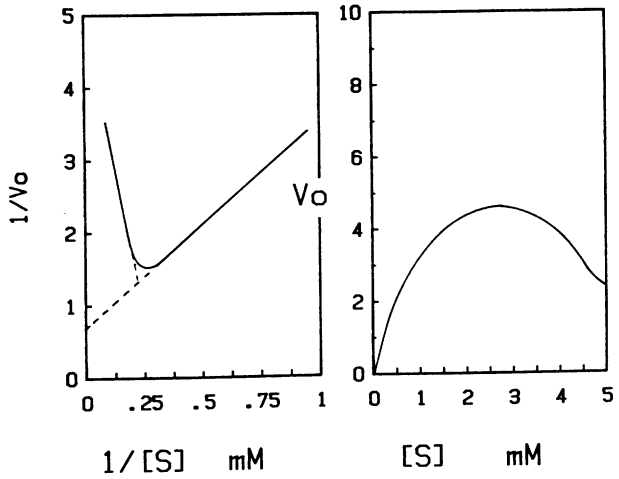

Figure 16-2 Deviations from normal behavior in a Lineweaver–Burk plot or a Michaelis–Menten plot, indicating substrate inhibition at high substrate concentrations.

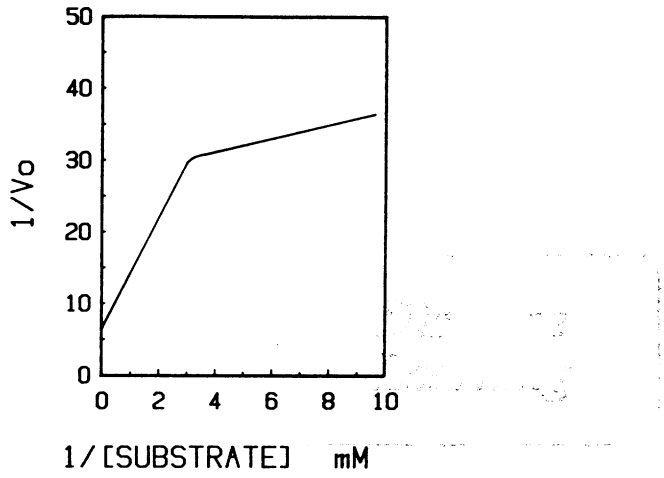

Figure 16-3 Lineweaver–Burk plot illustrating sharp transitions between apparently linear phases.

change in slope. In Fig. 16-1 the line is represented as essentially a continuous curve, while in Fig. 16-3 there is a sharper transition between two (or more) apparently linear regions.

POSSIBLE EXPLANATIONS

For each of the deviations in Figs. 16-1 to 16-3 there are at least two possible explanations, including substrate inhibition, substrate activation, the existence of isoenzymes, cooperative kinetics, and the existence of a steady-state random-order kinetic mechanism. The issue is further complicated by the fact that any given enzyme may display more than one of these phenomena, giving rise to quite complex Lineweaver–Burk or V versus [S] plots. Although in some systems it is possible that complex Lineweaver–Burk plots result from the experimental use of very wide, nonphysiological ranges of substrate concentrations, the complexity of an enzyme's kinetic behavior is more often related to its regulatory properties.

Substrate Inhibition

Substrate inhibition is the term used to describe behavior of the sort illustrated in Fig. 16-2, where at some particular substrate concentration the rate of the reaction actually decreases as the concentration of the substrate is further increased. If this type of kinetic behavior is observed, special precautions must be taken when initial rate parameters are being determined. The various substrate concentration ranges employed must fall outside the range where the onset of substrate inhibition occurs. Careful inspection of Fig. 16-2 shows that the deviation from linearity actually occurs

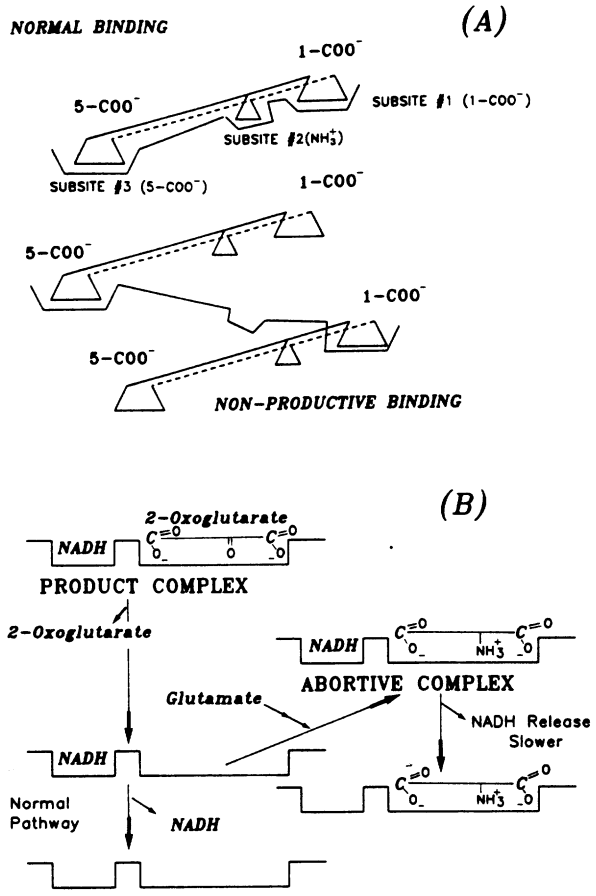

Figure 16-4 Hypothetical case of alternative substrate binding modes that can lead to substrate inhibition: (A) nonproductive binding of glutamate to a hypothetical binding site; (B) glutamate is illustrated forming an abortive complex in the glutamate dehydrogenase reaction once 2-oxoglutarate has vacated the binding site.

before the velocity starts to decrease. There are several possible causes for substrate inhibition.

Nonproductive Substrate Binding. The binding site for the substrate is usually made up of several subsites, each of which recognizes a specific structural feature of the substrate. If as the substrate concentration is increased, it is possible for these various subsites to interact with different substrate molecules (as outlined for a hypothetical case in Fig. 16-4), the resultant enzyme is catalytically inert, and the substrate binding is said to be nonproductive. The nonproductive nature of the binding results from the fact that unless all the required interactions of the various substrate moieties are made with their appropriate enzyme subsites, the catalytic groups on the enzyme and the reactive regions of the substrate cannot be correctly aligned.

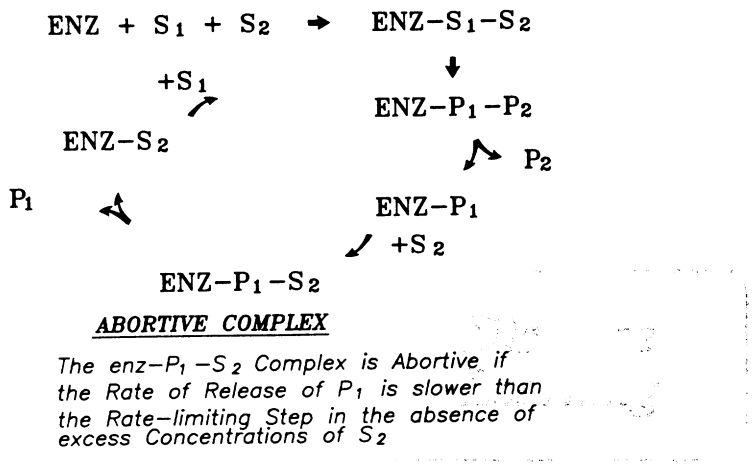

Figure 16-5 Abortive complex formation in a multisubstrate enzyme.

Abortive Complex Formation. In a multisubstrate enzyme system one of the substrates frequently resembles one of the products. Depending on the order of product release during the reaction cycle, it may be possible to form an enzyme complex that contains both some substrates and some products. A possible outline of this instance is illustrated in Fig. 16-5.

If the E-P-S complex releases product more slowly than the normal E-P complex, the presence of S in the E-P-S complex *may* result in a slowing of the overall reaction. In such an instance the E-P-S complex is said to be an abortive complex.

Even if product release in an E-P-S complex is slower than that from the E-P complex, substrate inhibition is not necessarily the result. The overall rate of the reaction (as measured by V_0) is decreased only if the rate of release of product from E-P is normally rate limiting or becomes rate limiting when E-P-S is formed. The appearance of substrate inhibition in, for example, a Lineweaver–Burk plot, is only apparent if the substrate has a lower affinity for E-P than it has for E. If substrate has the same affinity for E-P and E, an equal proportion of E-P-S is formed at all levels of saturation and the Lineweaver–Burk plot is linear.

Substrate Activation

A downward-curving Lineweaver–Burk plot, as shown in Fig. 16-1A or 16-3, indicates that as the substrate concentration increases, the rate of the reaction is disproportionally faster than is expected by simple extrapolation of the plot obtained at low substrate concentrations; this phenomenom is referred to as *substrate activation.* Such data could easily be obtained in an enzyme that contains a regulatory binding site for the substrate. If this site binds the substrate with lower affinity than the active site but after binding causes an activation of the reaction catalyzed by the

active site, the resultant Lineweaver–Burk plot resembles the case we are considering. As the substrate concentration is raised, more of the regulatory site is bound and the enzyme is activated to a greater degree than at low substrate concentrations. If the regulatory site and the active site have equal affinities for substrate, linear Lineweaver–Burk plots result since both sites are saturated at the same rate.

Isoenzymes

At a number of points in this book we have discussed the existence of isoenzymes and briefly considered the fact that they can often be electrophoretically separated. It is possible, however, that in the course of protein purification and characterization, the existence of isoenzymes escapes detection. In such a case, if the isoenzymes have different substrate affinities, nonlinear Lineweaver–Burk plots are expected. At low substrate concentrations the isoenzyme having the higher affinity (i.e., lower K_m) preferentially binds substrate and catalyzes the reaction. As the substrate concentration is raised, the isoenzyme with the higher affinity becomes saturated at lower substrate concentrations than the other isoenzyme. The V versus [S] plot at low substrate concentrations predominantly reflects the K_m of the isoenzyme having the higher affinity. At higher substrate concentrations the plot reflects predominantly the K_m of the isoenzyme with the lower affinity. In such a simple case with two isoenzymes, the Lineweaver–Burk plot shows a curvature, as illustrated in Fig. 16-1A. If the K_m values of the isoenzymes are sufficiently separated, the plot has a much sharper discontinuity and resembles the Lineweaver–Burk plot of Fig. 16-3. In the more complex situation of many isoenzymes all having different K_m values, the Lineweaver–Burk plot tends toward a more continuous curve.

A very important point that must be brought up in this context is the definition of an isoenzyme. As already indicated, isoenzymes are often thought of as being readily separable from one another. This implies that they differ in some biochemical or biophysical way, resulting in altered physical properties, but it is possible that they differ only in conformation (and thus do not have distinct physical differences). In many instances isoenzymes are multi-subunit, made up of varying proportions of different isoenzyme subunits. It is also possible that some multi-subunit enzymes, while they may contain chemically identical subunits, do not have them in the same conformations (i.e., they are conformationally asymmetric). Such a molecule clearly does not fall into the general concept of an isoenzyme. The type of kinetic behavior such an oligomeric protein might show is, however, little different from a mixture of isoenzymes. If the subunits behave independently within the system but are inherently asymmetric in terms of their ability to bind substrate, nonlinear Lineweaver–Burk plots of exactly the same types as would be expected for mixtures of isoenzymes are observed. As emphasized in Chap. 21, this is particularly important in light of the possibility that the observed nonlinear Lineweaver–Burk plots may be the result of cooperative interactions within an oligomer.

Cooperative Kinetics

Although in the section on isoenzymes the possible effects of oligomeric proteins were discussed, none of the explanations for nonlinear kinetics considered so far *require* the enzyme to contain subunits. The two types of deviations illustrated in Figs. 16-1 and 16-3 can also be explained by some sort of interaction between subunits in an oligomeric protein. The downward curvature of Figs. 16-1 and 16-3 can be the result of allosteric interactions between subunits, leading to decreased affinity for substrate as the saturation of the enzyme increases. The deviation shown in Fig. 16-1 can also result from subunit interactions, which lead to increased affinity for substrate as the saturation of the oligomer is increased. The various allosteric models that can generate these sorts of nonlinear kinetics are examined in detail in Chap. 21.

Steady-State, Random-Order Mechanisms

All of the mechanisms considered to explain nonlinear kinetic data invoke either heterogeneity (of enzyme molecules or of binding sites) or subunit interactions in an oligomeric protein. It is possible, however, for a single-subunit enzyme, with no isoenzymes or regulatory binding sites, to exhibit the deviations of either type shown in Fig. 16-1. As discussed in some detail in Chap. 14, a multi-substrate enzyme that follows a steady-state, random-order addition of substrates can, depending on the relative magnitudes of various rate constants in the mechanism, give rise to an initial rate equation that predicts nonlinear Lineweaver–Burk plots. Such nonlinearities can be of either sort illustrated in Fig. 16-1.

EXPERIMENTAL APPROACHES TO DISTINGUISH AMONG POSSIBLE EXPLANATIONS

With such a diversity of possible explanations for nonlinear Lineweaver–Burk plots, experimental distinction among the alternatives can prove difficult. Table 16-1 summarizes the three basic types of deviation found and the possible explanations of each.

Although as shown in Table 16-1 there are at least two possibilities for each type of deviation, certain experimental approaches permit identification of the appropriate one. The simplest first step in distinguishing between them is to examine the substrate binding to the protein. A variety of techniques that can be used to study substrate binding are described in detail in Chap. 18. If nonlinear binding is also observed, the simple kinetic explanation of the existence of a steady-state, random-order mechanism can be eliminated, as in this case, simple, hyperbolic saturation to one binding site per enzyme molecule results. In the case of cooperative kinetics or isoenzymes, multiple classes of substrate binding to the active site are observed. These involve only the active-site binding, and since multiple classes of sites can exist by the same ligand being

TABLE 16-1 Deviations from linear kinetics

Type of deviation	Figures	Possible explanations
Downward curvature	16-1, 16-3	Substrate activation, isoenzymes, cooperative kinetics, steady state, random order
Upward curvature	16-1	Cooperative kinetics, steady state, random order
Substrate inhibition	16-2	Nonproductive binding, abortive complex formation

able to bind at a regulatory site as well as the active site, it is necessary to show that the observed "heterogeneity" results from active-site binding only. Accurate determination of the stoichiometry of binding is essential and it is helpful if a co-substrate can be shown to affect the affinity of all the putative active binding sites. In substrate activation it is possible to demonstrate binding of the substrate to its regulatory site in addition to the active site. As with the heterogenous classes of binding sites just discussed, the stoichiometry of binding must also be determined. Where substrate can bind to a second, nonactive site per molecule (or subunit), the total stoichiometry is twice that obtained in the previous case, where only active-site binding occurs. In both instances nonlinear binding is observed, emphasizing the requirement for stoichiometry determinations to distinguish between these possibilities. Binding studies are also useful in choosing between the possible explanations of substrate inhibition. For nonproductive binding a second binding site per subunit becomes apparent at high substrate concentrations. Abortive complex formation requires that the release of product from the E-P-S complex is slower (and becomes rate limiting) compared to release from the E-P complex. Such decreased "off"-velocity constants are often associated with decreased dissociation constants for the product ($K_d = k_-/k_+$: where k_- is the "off"-velocity constant and k_+ is the "on"-velocity constant), and it is possible to demonstrate in binding studies that the E-P-S complex is more stable than the E-P complex. It is possible that k_- and k_+ for the product are affected equally and thus a change in k_- is not reflected in K_d. The change in k_- still results in an overall slowing of the reaction in this case and must be demonstrated directly using rapid reaction techniques, which are discussed in Chap. 18.

Binding studies are an important contributor in understanding the basis of nonlinear kinetics. Binding studies, however, cannot discriminate between cooperative kinetics and isoenzymes as a possible explanation for observed downward curvature in a Lineweaver–Burk plot. The distinction between these two alternatives is examined further in Chap. 21.