Enzyme Inhibition Analyses

20

Enzymes are delicate protein catalysts with subtle conformational flexibilities. This makes them vulnerable, and a number of environmental conditions and/or ligands could bring about decline in the net catalytic activity. An enzyme may be irreversibly killed (inactivation by high temperature, extremes of pH, nonaqueous solvent, etc.) or inhibited by ligands that bind to them. Inhibitors are usually small molecular weight ligands that bring about a decrease in the rate of enzyme-catalyzed reaction. For a molecule to act as an inhibitor, it must physically interact with the enzyme. Interactions with the enzyme that do not affect its catalytic activity (that are kinetically silent) are of no inhibitory consequence. For example, a molecule may bind to the enzyme without changing any of its kinetic properties. Although such ligands may serve as potential baits in enzyme purification but are useless in study of kinetic mechanisms.

A study of enzyme inhibition provides powerful insights into their reaction mechanisms. Utility of such an inhibitor kinetic analysis with detailed description is covered in the subsequent sections. We can classify inhibitors based on their chemical nature and also the unique features of inhibition exhibited by them. The nature of enzyme inhibition may be reversible or irreversible. Some more common forms of inhibition and terminology are given in the table below (Table 20.1).

20.1 Reversible Versus Irreversible Inhibition

Reversible inhibitors are excellent tools to study enzyme kinetic mechanisms. It is important to establish the reversible nature of inhibition before embarking on its use to study enzyme mechanisms. A diagnostic test of reversibility is to physically separate E and I from their complex and show full recovery of the added enzyme

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Table 20.1 Common inhibitor types encountered

Enzyme inhibition category		Nomenclature
Irreversible	*Active site directed*	Affinity labels
		Suicide substrates
		Tight binding
Reversible	*Site of binding*	Isosteric
		Allosteric
	Extent of inhibition	Partial
		Complete
	Ligand types	Product
		Substrate
		Dead end
	Kinetic features[a]	Competitive
		Uncompetitive
		Noncompetitive

[a]Each one of these may further be grouped as linear, hyperbolic, or parabolic with respect to their slope and/or intercept changes

activity. Dialysis, ultrafiltration, and gel filtration chromatography are useful techniques in separating enzyme molecules from their small molecular weight inhibitors. These techniques may not be able to differentiate between tight-binding inhibition and true irreversible inactivation. In such cases one can look for the release of the original inhibitor molecule after denaturing the enzyme protein. An additional approach is to study the effect of inhibitor on $v \rightarrow [E_t]$ curve for the enzyme (also refer to Chap. 12 in Part II). Increasing concentrations of the enzyme are incubated in the absence or presence of a fixed concentration of the inhibitor. Subsequently the enzyme activity remaining is measured in each case (Fig. 20.1).

An irreversible inhibitor would stoichiometrically (and depending on the rate of inactivation) inactivate and titrate out enzyme molecules. The active enzyme molecules remaining however will be kinetically indistinguishable from the native enzyme molecules. Therefore the curve will be parallel to the control $v \rightarrow [E_t]$ curve but will not pass through origin. The point of intersection on the X-axis represents the amount of enzyme irreversibly inactivated by the concentration of the inhibitor used. In the presence of a reversible inhibitor, all the enzyme molecules will be active but are kinetically less efficient. Hence the corresponding $v \rightarrow [E_t]$ curve will have a lower slope but still passes through the origin. This is a simple and quick way to establish the reversible nature of an inhibitor. A word of caution – the enzyme–inhibitor incubation conditions – should be carefully chosen; significant enzyme inactivation has to occur in the given time of incubation with an irreversible inhibitor.

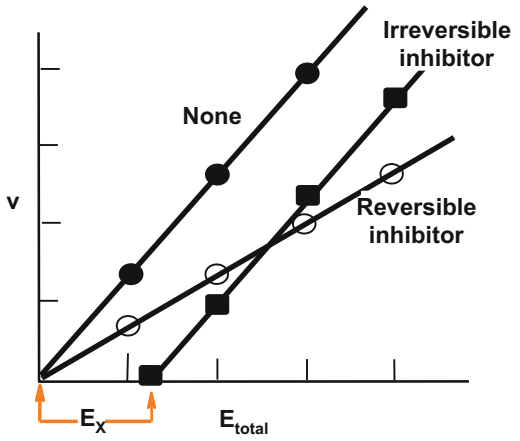

Fig. 20.1 Influence of an inhibitor on the enzyme concentration versus initial velocity curve. Amount of enzyme (E_x) titrated by the irreversible inhibitor is shown

20.2 Partial Versus Complete Inhibition

A simple inhibition experiment involves monitoring rates of an enzyme-catalyzed reaction in the presence of increasing concentrations of inhibitor. The resultant $v \rightarrow [I]$ curve is nonlinear and asymptotic to X-axis. At saturating values of a complete inhibitor, the enzyme activity tends to zero (Fig. 20.2, panel A). On the other hand, the enzyme activity plateaus to a nonzero limiting value by increasing concentrations of a partial competitive inhibitor (Whiteley 2000a). However due to the nonlinear, asymptotic nature of such curves and for reasons of experimental feasibility (unable to achieve very high concentration of the inhibitor in practice), it is difficult to determine the limiting value.

Fractional inhibition analysis is a convenient tool to distinguish between partial and complete inhibitors (Whiteley 2000b). For this, a relative quantity termed fractional inhibition (denoted i) is defined as shown.

$$i = 1 - \frac{v_i}{v} = \frac{v - v_i}{v}$$

Here v_i is the inhibited rate in the presence of inhibitor. If the inhibition is complete, then i will take a value of unity (because at saturating concentration of the inhibitor, v_i will be zero). For a partial inhibitor, however, v_i will never reach zero, and i will be always less than one. The inhibition data is plotted as $1/i \rightarrow 1/[I]$ to obtain a linear plot (this is analogous to the Lineweaver–Burk treatment of $v \rightarrow [S]$ plot!). The Y-axis intercept of such a double reciprocal plot is unity for a complete competitive inhibitor, whereas it will be greater than one for partial inhibitors (Fig. 20.2; panel B).

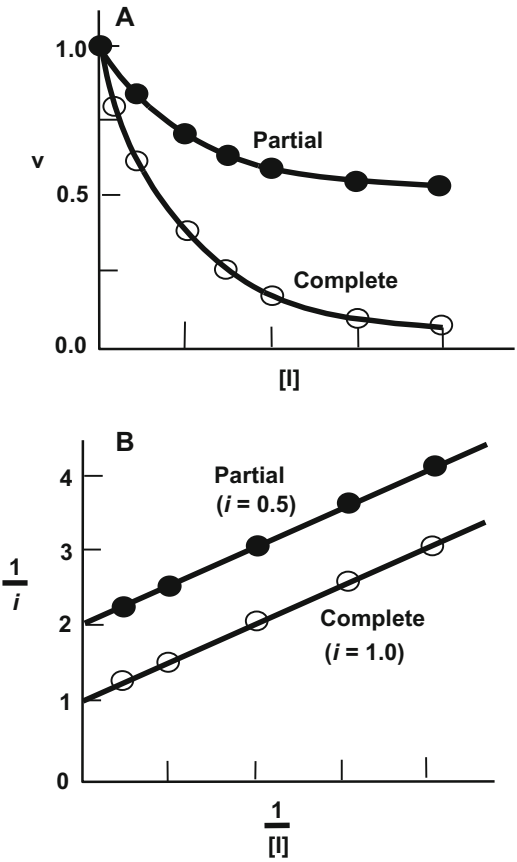

Fig. 20.2 Fractional inhibition analysis. A relative value of velocity is plotted in the $v \rightarrow [I]$ plot (panel A). A double reciprocal plot of $1/i \rightarrow 1/[I]$ is shown in panel B

20.3 Other Inhibitor Types

Inhibitors are often structurally related to the product(s) or substrate(s) of that enzyme. It is easy to appreciate that molecules structurally similar to a substrate/product can occupy the same space (pocket!) at the enzyme active site. Such inhibitors are called *isosteric* inhibitors. In some metabolic pathways, a terminal metabolite without any chemical analogy/reactivity to an earlier step is a powerful inhibitor of its own synthesis (Part V, Chap. 37; Regulation of enzyme activity). Obviously such structurally unrelated molecule cannot occupy the isosteric enzyme active site but inhibits by binding to a site distinct from the active site. Such inhibitors are called *allosteric* inhibitors, and the site where they bind is called an allosteric site. Binding of an inhibitor at the allosteric site is communicated to the active site through the protein matrix – as a conformational change.

Product of the enzyme reaction can act as an inhibitor. Product inhibition may result due to reversal of the forward reaction since it will be the substrate for the reaction backward. This mode of action will obviously be possible only if the full complement of products is present in the assay. In a multiproduct reaction, however, presence of a single product may lead to inhibited rates by playing musical chair with substrates. Let us consider an example to illustrate these modes of inhibition. Lactate dehydrogenase catalyzes the following reversible reaction.

$$\textbf{Pyruvate} + \textbf{NADH} + \textbf{H}^{+} \rightleftarrows \textbf{Lactate} + \textbf{NAD}^{+}$$

In the presence of pyruvate and NADH, only the forward reaction occurs. If the assay also contains lactate and NAD^+, then the reverse reaction also becomes significant. The net forward rate will then be reduced (inhibition is seen) because of a certain backward reaction rate. In this sense product inhibition is a result of reversal of the reaction. However, presence of NAD^+ alone can inhibit this reaction. Since there is no reversal of the reaction possible (because lactate is missing from the assay!), inhibition by NAD^+ occurs because it can displace NADH from the enzyme active site and prevents E. pyruvate.NADH complex formation. Logically, because of their structural similarity, NAD^+ and NADH are expected to compete for the same active site pocket on the enzyme. We note that E.pyruvate.NAD^+ complex, if at all formed, is not productive. Such a combination is termed dead-end complex – implying that this enzyme form is not on the normal reaction path of the catalytic turnover.

The dead-end combination (exemplified for NAD^+ with lactate dehydrogenase above) may also occur with substrate/product analogs that are not substrates/products themselves for the enzyme. Such inhibitors are typical *dead-end* inhibitors; their complexes with the enzyme do not form the part of normal reaction sequence – hence no catalysis occurs. Examples of some dead-end inhibitors and their target enzymes are shown in Fig. 20.3.

Dead-end inhibitors are excellent tools in the study of enzyme kinetic mechanism. Apart from initial velocity analysis and product inhibition (discussed above), one could use dead-end inhibitors to help deduce kinetic mechanisms. The thumb rules to predict dead-end inhibition patterns are similar to those employed for product inhibitions (Chap. 18; Approaches to kinetic mechanism – An overview) but with one exception. Being dead-end inhibitors, they cannot bring about the partial reversal of the reaction; hence they give more number of uncompetitive inhibition patterns.

Alternate products or substrates can be viewed as inhibitors of the enzyme reaction with their normal counterparts. In rare instances, substrate itself acts as an inhibitor at higher concentrations. These cases and their relevance to the study of enzyme mechanisms will be discussed a little later (Chap. 23; Alternate substrate (product) interactions). Reversible inhibitors, especially the product and dead-end inhibitors, provide valuable insights to establish enzyme kinetic mechanisms.

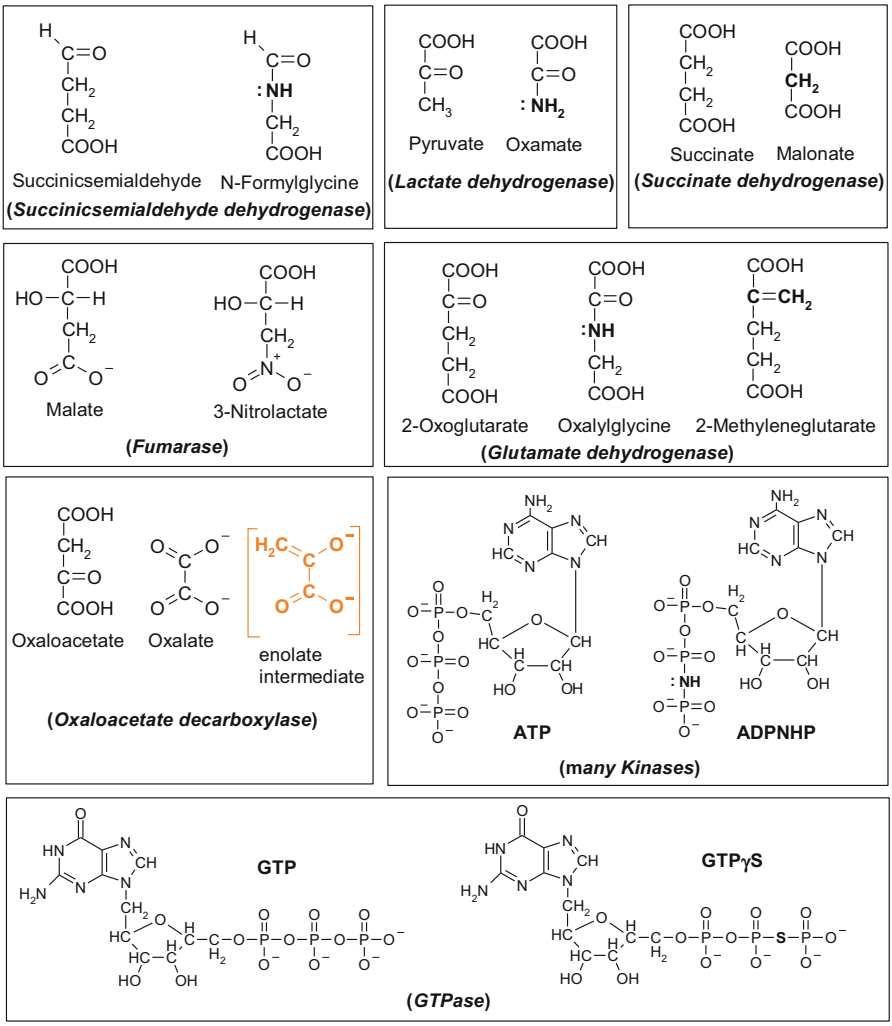

Fig. 20.3 Structures of few dead-end inhibitors. The enzyme inhibited and the corresponding substrates which they mimic are shown. Key structural differences are shown in bold

References

Whiteley CG (2000a) Enzyme kinetics: partial and complete competitive inhibition. Biochem Educ 28:144–147

Whiteley CG (2000b) Mechanistic and kinetic studies of inhibition of enzymes. Cell Biochem Biophys 33:217–225