Chapter 4
Enzyme Kinetics: Theory and Practice

Alistair Rogers and Yves Gibon

4.1 Introduction

Enzymes, like all positive catalysts, dramatically increase the rate of a given reaction. Enzyme kinetics is principally concerned with the measurement and mathematical description of this reaction rate and its associated constants. For many steps in metabolism, enzyme kinetic properties have been determined, and this information has been collected and organized in publicly available online databases (www.brenda.uni-koeln.de). In the first section of this chapter, we review the fundamentals of enzyme kinetics and provide an overview of the concepts that will help the metabolic modeler make the best use of this resource. The techniques and methods required to determine kinetic constants from purified enzymes have been covered in detail elsewhere [4, 12] and are not discussed here. In the second section, we will describe recent advances in the high throughput, high sensitivity measurement of enzyme activity, detail the methodology, and discuss the use of high throughput techniques for profiling large numbers of samples and providing a first step in the process of identifying potential regulatory candidates.

4.2 Enzyme Kinetics

In this section, we will review the basics of enzyme kinetics and, using simple examples, mathematically describe enzyme-catalyzed reactions and the derivation of their key constants. However, first we must turn to the mathematical description of chemical reaction kinetics.

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4.2.1 Reaction Rates and Reaction Order

4.2.1.1 First-Order Irreversible Reaction

The simplest possible reaction is the irreversible conversion of substance A to product P (e.g., radioactive decay).

$$\mathrm{A} \xrightarrow{k_1} \mathrm{P} \tag{4.1}$$

The arrow is drawn from A to P to signify that the equilibrium lies far to the right, and the reverse reaction is infinitesimally small. We can define the reaction rate or velocity (v) of the reaction in terms of the time (t)-dependent production of product P. Since formation of P involves the loss of A, we can also define v in terms of the time-dependent consumption of substance A, where [A] and [P] are the concentrations of the substance and product, respectively.

$$v = \frac{\delta[\mathrm{P}]}{\delta t} = -\frac{\delta[\mathrm{A}]}{\delta t} = k_1[\mathrm{A}] \tag{4.2}$$

The transformation of substance A to product P is an independent event and therefore is unaffected by concentration. As substance A is transformed to product P, there is less of substance A to undergo the transformation, and therefore the concentration of substance A will decrease exponentially with time (Fig 4.1A). The rate constant (k_1) of this reaction is proportional to the concentration of A and has the unit s^{-1}.This type of unimolecular reaction is known as a first-order reaction because the rate depends on the first power of the concentration. Integration of Eq. (4.2) from time zero (t_0) to time t gives

$$\ln \frac{[\mathrm{A}]}{[\mathrm{A}]_0} = -k_1 t \tag{4.3}$$

or

$$\frac{[\mathrm{A}]}{[\mathrm{A}]_0} = \mathrm{e}^{-k_1 t} \tag{4.4}$$

where $[\mathrm{A}]_0$ is the starting concentration at t_0. Eq. (4.4) describes how the concentration of A decreases exponentially with time as shown in Fig. 4.1A. When the ln[A] is plotted against time (Fig. 4.1B), a first-order reaction will yield a straight line, where the gradient is equal to $-k_1$.

4.2.1.2 First-Order Reversible Reaction

Few reactions in biochemistry are as simple as the first-order reaction described above. In most cases, reactions are reversible and equilibrium does not lie far to one side.

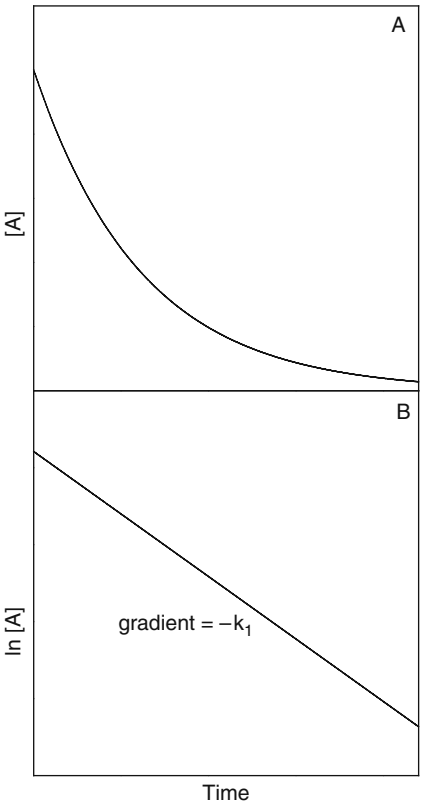


Fig. 4.1 A first-order reaction showing the decrease of substance A over time expressed as the concentration of A ([A], **Panel A**) and in a semi-logarithmic plot (ln[A], **Panel B**)

$$[\mathrm{A}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\mathrm{P}] \tag{4.5}$$

Therefore, the corresponding rate equation is

$$v = -\frac{\delta[\mathrm{A}]}{\delta t} = k_1[\mathrm{A}] - k_{-1}[\mathrm{P}] \tag{4.6}$$

where k_1 and k_{-1} are the rate constants for the first-order, forward and reverse, reactions respectively. Consumption of A will stop when the rates of the forward and reverse reactions are equal and the overall reaction rate is zero, i.e., when a state of equilibrium has been attained ($[\mathrm{A}]_{\mathrm{eq}}$ and $[\mathrm{P}]_{\mathrm{eq}}$ are the substrate concentrations at equilibrium). Note that in catalyzed reactions, the position of equilibrium is not altered by the presence of an enzyme. The effect of a catalyst is to increase the rate at which equilibrium is attained.

$$0 = -k_1[\mathrm{A}]_{\mathrm{eq}} + k_{-1}[\mathrm{P}]_{\mathrm{eq}} \tag{4.7}$$

For this reaction, where the forward and reverse reactions are both first order, the equilibrium constant (K_{eq}) is equal to the ratio of the rate constants for the forward and reverse reactions. For a reaction to precede in the direction of product (P) formation, the equilibrium constant must be large.

$$K_{eq} = \frac{k_1}{k_{-1}} = \frac{[P]_{eq}}{[A]_{eq}} \tag{4.8}$$

4.2.1.3 Second-Order Reaction

In addition to being reversible, most reactions are second order or greater in their complexity. Whenever two reactants come together to form a product, the reaction is considered second order, e.g.,

$$A + B \xrightarrow{k_1} P \tag{4.9}$$

The rate of the above reaction is proportional to the consumption of A and B and to the formation of P. The reaction is described as second order because the rate is proportional to the second power of the concentration; the rate constant k_1 has the unit $s^{-1}\,M^{-1}$.

$$v = -\frac{\delta[A]}{\delta t} = -\frac{\delta[B]}{\delta t} = \frac{\delta[P]}{\delta t} = k_1[A][B] \tag{4.10}$$

Integration of Eq. (4.10) yields an equation where t is dependent on two variables, A and B. To solve this equation, either A or B must be assumed to be constant. Experimentally, this can be accomplished by using a concentration of B that is far in excess of requirements such that only a tiny fraction of B is consumed during the reaction and therefore the concentration can be assumed not to change. The reaction is then considered pseudo-first order.

$$v = k_1[A][B]_0 = k_1'[A] \tag{4.11}$$

Alternatively, when the concentration of both A and B at time zero are the same, i.e., $[A_0] = [B_0]$, Eq. (4.10) can be simplified:

$$v = -\frac{\delta[A]}{\delta t} = k_1[A]^2 \tag{4.12}$$

4.2.2 What Does an Enzyme Do?

Transition state theory suggests that as molecules collide and a reaction takes place, they are momentarily in a strained or less stable state than either the reactants or the products. During this transition state, the potential energy of the activated complex increases, effectively creating an energy barrier between the reactants and

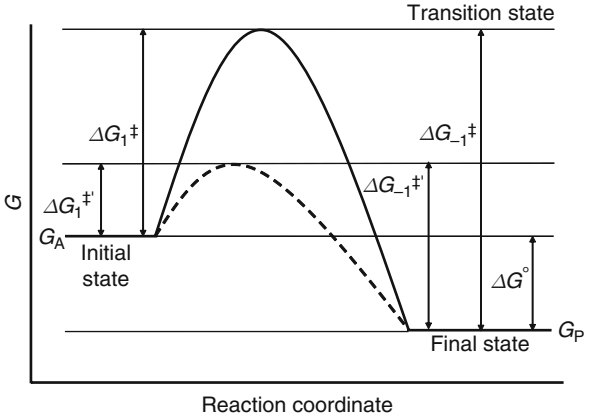


Fig. 4.2 A free energy (G) diagram for a simple reversible exothermic reaction A↔P (*solid and broken lines*). G_A and G_P represent the average free energies per mole for the reactant A and the product P, the initial and final states respectively. The standard state free energy change for the reaction is ΔG°. In order for reactant A to undergo transformation to product P, it must pass through the transition state (indicated at the apex of these plots). The $\Delta G_1^{\ddagger}$ and $\Delta G_1^{\ddagger\prime}$ indicate the energy of activation necessary to make that transition for the uncatalyzed (*solid*) and catalyzed (*broken*) reactions respectively. The energy of activation for the reverse reaction (P→A) is indicated by $\Delta G_{-1}^{\ddagger}$ (uncatalyzed) and $\Delta G_{-1}^{\ddagger\prime}$ (catalyzed)

products (solid line, Fig. 4.2). Products can only be formed when colliding reactants have sufficient energy to overcome this energy barrier. The energy barrier is known as the activation energy ($\Delta G^{\ddagger}$) of a reaction. The greater the activation energy for a given reaction is, the lower the number of effective collisions. The molecular model currently used to explain how an enzyme catalyzes a reaction is the induced-fit hypothesis. In this model, the enzyme binds it's substrate to form an enzyme–substrate complex where the structure of the substrate is distorted and pulled into the transition state conformation. This reduces the energy required for the conversion of a given reactant into a product and increases the rate of a reaction by lowering the energy requirement (broken line, Fig. 4.2) and therefore increasing the number of effective collisions that can result in the formation of the product. In addition, enzymes also promote catalysis by positioning key acidic or basic groups and metal ions in the right position for catalysis. In reality, the free energy diagram for an enzyme-catalyzed reaction is considerably more complicated than the example in Fig. 4.2. Typically an enzyme-catalyzed reaction will involve multiple steps, each with an activation energy that is markedly lower than that for the uncatalyzed reaction.

4.2.3 The Michaelis–Menten Equation

The Michaelis–Menten equation (Eq. 4.26), as presented by Michaelis and Menten and further developed by Briggs and Haldane [6, 34], is fundamentally important to enzyme kinetics. The equation is characterized by two constants: the

Michaelis–Menten constant (K_m) and the indirectly obtained (see Eq. 4.25) catalytic constant, k_{cat}. Although derived from a simple, single-substrate, irreversible reaction, the Michaelis–Menten equation also remains valid for more complex reactions.

The simple conversion of substrate (A) into product (P) catalyzed by the enzyme (E) is described below. As outlined by the induced-fit hypothesis, the first step is substrate binding and the second step is the catalytic step.

$$\mathrm{E+A} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{EA} \xrightarrow{k_2} \mathrm{E+P} \tag{4.13}$$

Following Eq. (4.2), we can define the formation of the product in terms of the dissociation rate (k_2) of the enzyme–substrate complex, commonly denoted as k_{cat}, and the concentration of the enzyme–substrate complex ([EA]).

$$v = k_{cat}[\mathrm{EA}] \tag{4.14}$$

It is assumed that the dissociation rate (k_{cat} in Eq. 4.14 or k_2 in Eq. 4.13) of the enzyme–substrate complex (EA) is slow compared to association (k_1) and redissociation (k_{-1}) reactions and that the reverse reaction (P→A) is negligible. Figure 4.3 shows how the consumption of substrate, the production of product, and the concentration of the free enzyme and the enzyme–substrate complex change over the course of the reaction. During a very brief initial period, the enzyme–substrate complex is

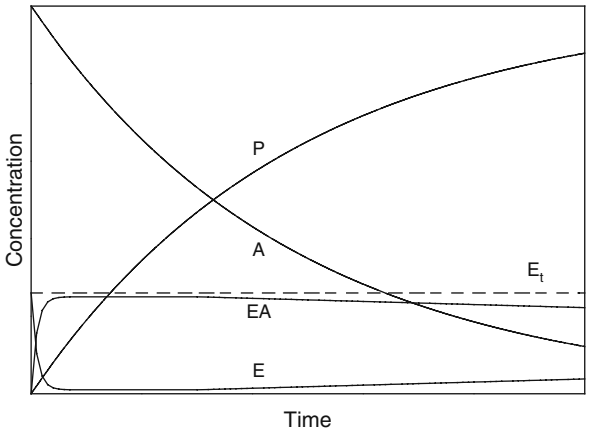


Fig. 4.3 Change in substrate (A), product (P), free enzyme (E), enzyme–substrate complex (EA), and total enzyme (E_t) concentration over time for the simple reaction described in Eq. (4.13). After a very brief initial period, the concentration of the enzyme–substrate complex reaches a steady state in which consumption and formation of the enzyme–substrate complex are balanced. As substrate is consumed, the concentration of the enzyme–substrate complex falls slowly and the concentration of the free enzyme rises. The amounts of enzyme and enzyme–substrate are greatly exaggerated for clarity

formed and reaches a concentration at which its consumption is matched by its formation. The [EA] then remains almost constant for a considerable time; this period is known as the steady state, and it is this steady-state condition that the Michaelis–Menten equation describes. Eventually, the reaction enters a third phase characterized by substrate depletion in which the [EA] gradually falls.

At steady state, the enzyme–substrate concentration is stable, i.e.,

$$\frac{\delta[\mathrm{EA}]}{\delta t} = 0 \tag{4.15}$$

and therefore the formation of the ES complex (association reaction) and the breakdown of the ES complex (the sum of the redissociation and dissociation reactions) are equal.

$$k_1[\mathrm{E}][\mathrm{A}] = k_{-1}[\mathrm{EA}] + k_{\mathrm{cat}}[\mathrm{EA}] \tag{4.16}$$

Rearrangement of Eq. (4.16) yields

$$\frac{k_1[\mathrm{E}][\mathrm{A}]}{k_{-1} + k_{\mathrm{cat}}} = [\mathrm{EA}] \tag{4.17}$$

The three rate constants can now be combined as one term. This new constant, K_{m}, is known as the Michaelis–Menten constant

$$K_{\mathrm{m}} = \frac{k_{-1} + k_{\mathrm{cat}}}{k_1} \tag{4.18}$$

and Eq. (4.17) can be rewritten as

$$\frac{[\mathrm{E}][\mathrm{A}]}{K_{\mathrm{m}}} = [\mathrm{EA}] \tag{4.19}$$

The concentration of enzyme in Eq. (4.19) refers to the unbound enzyme. The amount of free enzyme (E) and enzyme that is bound to the substrate (EA) varies over the course of a reaction, but the total amount of enzyme ($\mathrm{E_t}$) is constant (see Fig. 4.3) such that

$$\mathrm{E} = \mathrm{E_t} - \mathrm{EA} \tag{4.20}$$

Substituting into Eq. (4.19) yields

$$\frac{([\mathrm{E_t}] - [\mathrm{EA}])[\mathrm{A}]}{K_{\mathrm{m}}} = [\mathrm{EA}] \tag{4.21}$$

which can be rearranged to yield

$$\frac{[\mathrm{E_t}][\mathrm{A}]}{K_{\mathrm{m}} + [\mathrm{A}]} = [\mathrm{EA}] \tag{4.22}$$

Substituting into Eq. (4.14) gives

$$v = \frac{k_{cat}[E_t][A]}{K_m + [A]} \tag{4.23}$$

The maximum possible reaction rate (v_{max}) would be achieved when all the available enzyme is bound to the substrate and involved in catalysis, i.e.,

$$[EA] = [E_t] \tag{4.24}$$

Substituting Eq. (4.24) into Eq. (4.14) under conditions of saturating substrate concentration yields

$$v_{max} = k_{cat}[E_t] \tag{4.25}$$

and substituting Eq. (4.25) into Eq. (4.23) yields what is widely recognized as the Michaelis–Menten equation.

$$v = \frac{v_{max}[A]}{K_m + [A]} \tag{4.26}$$

4.2.4 Key Parameters of the Michaelis–Menten Equation

4.2.4.1 K_m (mol.l^{-1})

Assuming a stable pH, temperature, and redox state, the K_m for a given enzyme is constant, and this parameter provides an indication of the binding strength of that enzyme to its substrate. Michaelis–Menten kinetics assumes that k_{cat} is very low when compared to k_1 and k_{-1}. Therefore, following Eq. (4.18), a high K_m indicates that the redissociation rate (k_{-1}) is markedly greater than the association rate and that the enzyme binds the substrate weakly. Conversely, a low K_m indicates a higher affinity for the substrate (E1 in Fig. 4.4). However, as Eq. (4.18) shows, a large K_m could also be the result of very large k_{cat}. Therefore, care should be taken when using K_m as a proxy for the dissociation equilibrium constant of the enzyme–substrate complex.

4.2.4.2 k_{cat} (s^{-1})

The k_{cat}, also thought of as the turnover number of the enzyme, is a measure of the maximum catalytic production of the product under saturating substrate conditions per unit time per unit enzyme. The larger the value of k_{cat}, the more rapidly catalytic events occur. Values for k_{cat} differ markedly, e.g., 2.5 s^{-1} for rubisco (EC 4.1.1.39) with CO_2 as a substrate to c. 1,150 s^{-1} for fumarase (EC 4.2.1.2) with fumarate as a substrate [13, 49].

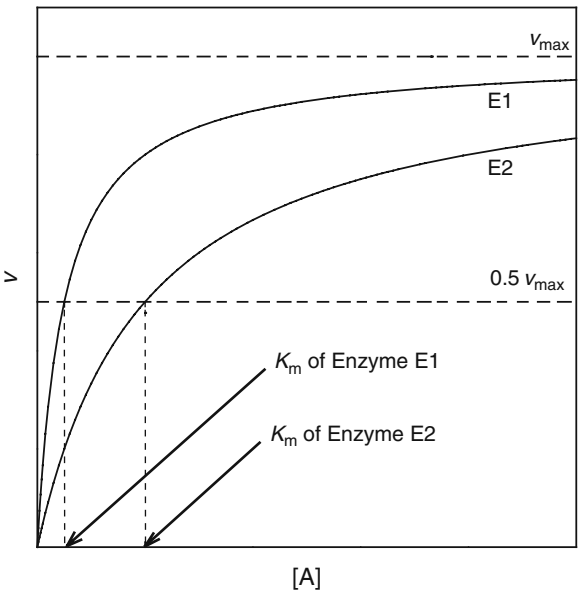


Fig. 4.4 Change in velocity (v) with the concentration of substrate A ([A]) for the reaction shown by Eq. (4.13) catalyzed by two enzymes E1 and E2. The substrate concentration at the point at which the reaction has half its maximum velocity (0.5 v_{max}) is equal to the K_m. Enzyme E2 has a K_m four times greater than enzyme E1 but the same v_{max}

4.2.4.3 Enzyme Efficiency (s^{-1} $(mol.l^{-1})^{-1}$)

The ratio of k_{cat}/K_m is defined as the catalytic efficiency and can be taken as a measure of substrate specificity. When the k_{cat} is markedly greater than k_{-1}, the catalytic process is extremely fast and the efficiency of the enzyme depends on its ability to bind the substrate. Based on the laws of diffusion, the upper limit for such rates, as determined by the frequency of collisions between the substrate and the enzyme, is between 10^8 and 10^9. Some enzymes actually have efficiencies that approach this range, indicating that they have near-perfect efficiency, e.g., fumarase, 2.3×10^8 $s^{-1}(mol.l^{-1})^{-1}$ [13, 50].

4.2.4.4 v_{max}

The v_{max} is the maximum velocity that an enzyme could achieve. The measurement is theoretical because at given time, it would require all enzyme molecules to be tightly bound to their substrates. As shown in Fig. 4.4, v_{max} is approached at high substrate concentration but never reached.

4.2.5 Graphical Determination of Michaelis–Menten Parameters

Since the Michaelis–Menten parameters provide useful information for the network modeler, we need to consider the methods used to estimate K_m, k_{cat}, and v_{max}. There are a number of practical approaches to measuring reaction rates (see Section 4.3). Briefly, we need some way to follow the consumption of substrate or formation

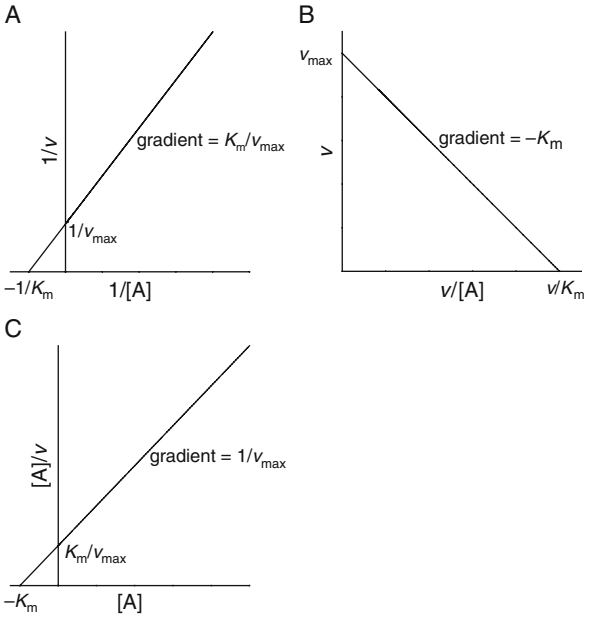


Fig. 4.5 Linear representations of the Michaelis–Menten equation (Eq. 4.26). Lineweaver–Burk (**A**), Eadie–Hofstee (**B**), and Hanes (**C**) plots. The intercepts with the *x*- and *y*-axis and the gradient can be used to determine K_m and v_{max}

of product over time. We could simply mix enzyme with substrate and follow the formation of product in a progress reaction (e.g., Fig. 4.3) or conduct several experiments at multiple substrate concentrations and measure initial velocity at each substrate concentration (e.g., Fig. 4.4). However, the graphical evaluation of nonlinear plots to obtain Michaelis–Menten parameters relies on accurate curve fitting. The problems associated with evaluating enzyme kinetics using a nonlinear plot can be avoided by using one of the three common linearization methods to obtain estimates for K_m and v_{max} (Fig. 4.5). However, these methods are not without problems either. Errors in the determination of v at low substrate concentration are greatly magnified in Lineweaver–Burke and Eadie–Hofstee plots and to a lesser extent in Hanes plots. Despite this disadvantage, and in contrast to nonlinear plots, changes in enzyme kinetics, for example, due to the action of an inhibitor, are readily apparent on linear plots (see Fig. 4.6). Clearly, selection of a linear or nonlinear plot should be based on an understanding of the sources of error in the experiment and consistent with the goal of that experiment.

4.2.6 Multisubstrate Reactions

Most biochemical reactions are not simple-, single-substrate reactions, but typically involve two or three substrates that combine to release multiple products. However, the Michaelis–Menten equation is robust and remains valid as reaction complexity increases. When an enzyme binds two or more substrates, the order of the biochemical steps determines the mechanism of the reaction. Below we have detailed the

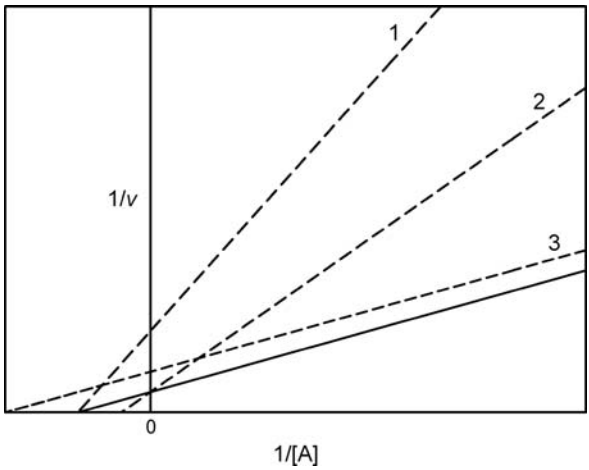
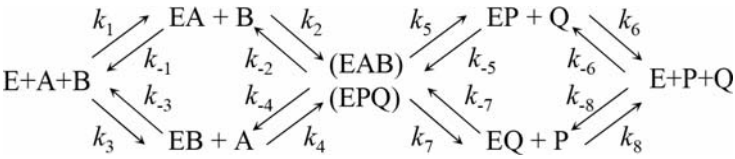


Fig. 4.6 A Lineweaver–Burke plot of an uninhibited enzyme (*solid line*) and the same enzyme in the presence of noncompetitive inhibitor (*plot 1*), competitive inhibitor (*plot 2*), and an uncompetitive inhibitor (*plot 3*). The point where the plots meet the *x*-axis indicates $-1/K_m$, and the intercept with the *y*-axis indicates $1/v_{max}$

three major classes of mechanisms for the reaction where two substrates (A and B) react to yield two products (P and Q). Full derivation of the rate equations for these reactions and discussion of more complex mechanisms is covered elsewhere [4, 12] and is beyond the scope of this chapter.

4.2.6.1 Random Substrate Binding

In its simplest form, this mechanism assumes independent binding of substrates and products. Either substrate A or B can be bound first and either product P or Q released first; binding of the first substrate is independent of the second substrate. The catalytic reactions occur in central complexes and are shown here in parentheses to distinguish them from intermediate complexes that are capable of binding substrates. The phosphorylation of glucose by ATP, catalyzed by hexokinase, is an example of a random-ordered mechanism, although there is tendency for glucose to bind first.



(4.27)

4.2.6.2 Ordered Substrate Binding

In some cases, one substrate must bind first before the second substrate is able to bind effectively. This mechanism is frequently observed in dehydrogenase reactions where NAD^+ acts as a second substrate.

$$\begin{aligned} &\mathrm{E} + \mathrm{A} + \mathrm{B} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{EA} + \mathrm{B} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} (\mathrm{EAB} - \mathrm{EPQ}) \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \\ &\mathrm{EQ} + \mathrm{P} \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} \mathrm{E} + \mathrm{P} + \mathrm{Q} \end{aligned} \tag{4.28}$$

4.2.6.3 The Ping-Pong Mechanism

In this mechanism, enzyme E binds substrate A and then releases product P. An intermediate form of enzyme E (E*), which often carries a fragment of substrate A, then binds substrate B. Finally, product Q is released, and the enzyme is returned to its original form (E). Aminotransferases use this mechanism, e.g., aspartate aminotransferase catalyses the ping-pong transfer of an amino group from aspartate to 2-oxoglutarate to form oxaloacetate and glutamate.

$$\begin{aligned} &\mathrm{E} + \mathrm{A} + \mathrm{B} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (\mathrm{EA} - \mathrm{E}^{*}\mathrm{P}) + \mathrm{B} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{E}^{*} + \mathrm{P} + \mathrm{B} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \\ &(\mathrm{E}^{*}\mathrm{B}\text{–}\mathrm{EQ}) + \mathrm{P} \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} \mathrm{E} + \mathrm{P} + \mathrm{Q} \end{aligned} \tag{4.29}$$

4.2.7 Regulation

The catalytic capacity for a given process in a cell can be regulated at many levels of biological organization. Coarse control is provided by the regulation of the transcription of genes that encode the enzymatic machinery. Here, we outline the major mechanisms by which the activity of functional enzymes can be altered by fine control mechanisms and show how these mechanisms impact enzyme kinetics.

4.2.7.1 Enzyme Inhibition

Here, we define inhibition as a reduction in enzyme activity through the binding of an inhibitor to a catalytic or regulatory site on the enzyme, or in the case of uncompetitive inhibition, to the enzyme–substrate complex. Inhibition can be reversible or irreversible. Irreversible inhibition nearly always involves the covalent binding of a toxic substance that permanently disables the enzyme. This type of inhibition does not play a role in the fine control of enzyme activity and is not discussed further. In contrast, reversible inhibition involves the noncovalent binding of an inhibitor to the enzyme which results in a temporary reduction in enzyme activity. Inhibitors differ in the mechanism by which they decrease enzyme activity. There are three basic mechanisms of inhibition – competitive, noncompetitive, and uncompetitive inhibition – and these are outlined below using simple examples. The reality is more complex and typically reactions involve mixed and partial mechanisms comprised of these three component mechanisms [4, 12].

4.2.7.2 Competitive Inhibition

A competitive inhibitor is usually a close analogue of the substrate. It binds at the catalytic site but does not undergo catalysis. A competitive inhibitor wastes the enzyme's time by occupying the catalytic site and preventing catalysis. Or put another way, the presence of an inhibitor decreases the ability of the enzyme to bind with its substrate. The reaction scheme below details the mechanism. Here, k_1 and k_{-1} are the rates for the association and redissociation reactions for the enzyme–substrate complex (see Eq. 4.13), and k_2 and k_{-2} are the rates for the association and redissociation reactions between the enzyme and inhibitor (I).

$$\begin{array}{ccccc} \mathrm{E} + \mathrm{A} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & \mathrm{EA} & \xrightarrow{k_{cat}} & \mathrm{E} + \mathrm{P} \\ + & & & & \\ \mathrm{I} & & & & \\ {\scriptstyle k_{-2}}\uparrow\downarrow{\scriptstyle k_2} & & & & \\ \mathrm{EI} & & & & \end{array} \tag{4.30}$$

At steady state, the enzyme–inhibitor concentration is stable; so following Eqs 4.15–4.18, the association and redissociation rate constants for the enzyme–inhibitor complex can be combined in one term K_i, the dissociation constant for inhibitor, and following Eq. (4.19) can be expressed as follows:

$$K_\mathrm{i} = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]} \tag{4.31}$$

Since some enzyme is bound to the inhibitor, the equation describing the total amount of enzyme has an extra term and Eq. (4.20) becomes

$$\mathrm{E} = \mathrm{E_t} - \mathrm{EA} - \mathrm{EI}. \tag{4.32}$$

The resulting rate equation becomes

$$v = \frac{k_\mathrm{cat}[\mathrm{E}]_\mathrm{t}[\mathrm{A}]}{K_\mathrm{m}\left(1 + \dfrac{[\mathrm{I}]}{K_\mathrm{i}}\right) + [\mathrm{A}]} \tag{4.33}$$

and following Eq. (4.25),

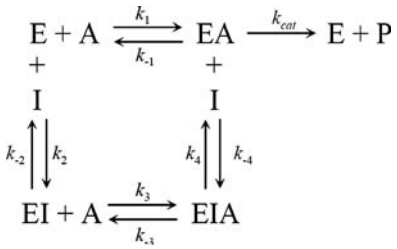
$$v = \frac{v_\mathrm{max}[\mathrm{A}]}{K_\mathrm{m}\left(1 + \dfrac{[\mathrm{I}]}{K_\mathrm{i}}\right) + [\mathrm{A}]} \tag{4.34}$$

As can be seen from Eq. (4.34), an increase in the concentration of a competitive inhibitor will increase the apparent K_m of the enzyme. However, since an infinite substrate concentration will exclude the competitive inhibitor, there is no effect

on v_{max}. The effect of competitive inhibition is readily apparent on a Lineweaver–Burke plot (Fig. 4.6, plot 2).

4.2.7.3 Noncompetitive Inhibition

A noncompetitive inhibitor does not bind to the catalytic site but binds to a second site on the enzyme and acts by reducing the turnover rate of the reaction. The reaction scheme (Eq. 4.35) details the mechanism for a noncompetitive inhibitor. Consider the simplest example of a noncompetitive inhibitor. Here, the binding of the inhibitor and substrate is completely independent, and binding of the inhibitor results in total inhibition of the catalytic step. In this simple case, the association and disassociation rates k_1 and k_{-1} are identical to k_3 and k_{-3} (i.e., K_m), and similarly, k_2 and k_{-2} are equal to k_4 and k_{-4} (i.e., K_i)



(4.35)

The apparent k_{cat} for this simple example is given by

$$k_{cat}^{app} = \frac{k_{cat}}{\left(1 + \frac{[I]}{K_i}\right)} \tag{4.36}$$

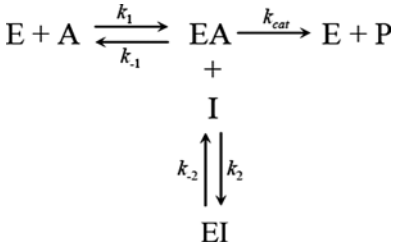
and the resulting rate equation is

$$v = \frac{k_{cat}^{app}[E]_t[A]}{K_m + [A]} \tag{4.37}$$

As can be seen from the rate equation, a simple noncompetitive inhibitor will not alter the K_m but will reduce the apparent k_{cat} as inhibitor concentration increases (Fig. 4.6 plot 1).

4.2.7.4 Uncompetitive Inhibition

An uncompetitive inhibitor does not bind to the enzyme but only the enzyme–substrate complex. Consider the simple example where binding of the uncompetitive inhibitor to the enzyme–substrate complex prevents catalysis (Eq. 4.38):



(4.38)

The rate equation is

$$v = \frac{v_{\max}[\mathrm{A}]}{K_{\mathrm{m}} + [\mathrm{A}]\left(1 + \dfrac{[\mathrm{I}]}{K_{\mathrm{i}}}\right)} \tag{4.39}$$

Sequestration of the enzyme–substrate complex by the inhibitor will reduce the apparent k_{cat} because the inhibited enzyme is less catalytically effective. Apparent $v_{\max}$ is reduced (and apparent K_{m} increased) because binding of the inhibitor cannot be prevented by increasing the substrate concentration (Fig 4.6, plot 3).

4.2.7.5 Substrate and Product Inhibition

The activity of enzymes can also be regulated by their substrates and products. Substrate inhibition, also know as substrate surplus inhibition, occurs when a second substrate molecule acts as an uncompetitive inhibitor binding to the enzyme–substrate complex to form an enzyme–substrate–substrate complex. This mechanism is the same as for uncompetitive inhibition, but here, the inhibitor is replaced by the second substrate molecule. In reversible reactions, the buildup of product can theoretically competitively inhibit the forward reaction by competing with the substrate for the active site. However, since the equilibrium constant is often large, favoring product formation, this type of inhibition is typically negligible. However, the product of a reaction can also behave as a noncompetitive or uncompetitive inhibitor. The mechanisms for these types of inhibition have been described above for the action of inhibitors.

The regulation of enzyme activity by its immediate substrate and or product is not sufficient to allow regulation of complex metabolic pathways with shared substrates. Effective regulation must include the inhibition and activation of enzyme activity by molecules that are distinct from the substrates and products of the regulated rate. These molecules are usually produced by reactions that are multiple biochemical steps away from the regulated enzyme. Allosteric regulation allows this type of control.

4.2.7.6 Allosteric Enzymes

Allosteric enzymes exhibit cooperativity in their substrate binding and regulation of their active site through the binding of a ligand to a second regulatory site. These two

traits make allosteric enzymes particularly good at controlling flux through a given metabolic chokepoint when compared to enzymes with classic Michaelis–Menten kinetics. Indeed, classic Michaelis–Menten enzymes require an 81-fold increase in substrate concentration to increase reaction rate from 10% to 90% of the maximal velocity [12].

4.2.7.7 Cooperativity (Homoallostery)

In enzymes with multiple binding sites, cooperative substrate binding describes the phenomenon whereby the binding of the first substrate molecule impacts the ability of the subsequent substrate molecules to bind. In the case of positive cooperativity, binding of the first substrate molecule enhances the ability of the following molecules to bind. An enzyme exhibiting positive cooperativity will appear to have a large K_m at low substrate concentration, but as the substrate concentration rises, the K_m will decrease and the substrate will be bound more readily. Figure 4.7 shows an example of positive cooperativity (plot 3). The physiological advantage of the sigmoidal kinetics is that enzyme activity can be increased more markedly within a narrow range of substrate concentration (gray area Fig. 4.7) when compared to a normal hyperbolic kinetic response (plot 1). The enzyme with positive cooperativity is much more responsive to changes in substrate concentration and can also better maintain a substrate concentration at or below a given threshold. In negative cooperativity, the binding of the first substrate interferes with the occupation of the second site. This can be advantageous when an enzyme needs to respond to a wide

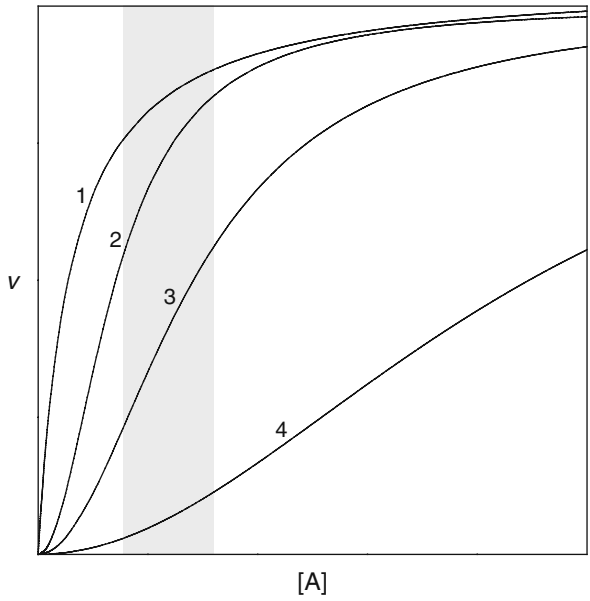


Fig. 4.7 Change in velocity (v) with substrate concentration ([A]) for an enzyme with normal binding (*plot 1*) and positive cooperative binding (*plot 3*) in the presence of an allosteric activator (*plot 2*) and an allosteric inhibitor (*plot 4*). The *gray area* indicates a hypothetical physiological range for this enzyme

range of substrate concentrations. An enzyme with negative cooperativity will be activated by a low concentration of substrate but will not saturate until the substrate concentration is extremely high.

There are two main models that attempt to describe how the enzyme changes its affinity for substrate with cooperative binding [28, 35]. These models share the concept that the subunits of the enzyme can exist in both a tense state (T-state), where substrate binding is weak, and a relaxed state (R-state), where substrate binding is strong, and that the initial binding of the substrate to the T-state enzyme shifts more subunits/enzyme molecules to the R-state, where the substrate can bind more readily.

The Hill equation (Eq. 4.40) describes the fraction of binding sites filled (r) by n molecules of substrate A, where K_d is the dissociation constant for A. The Hill coefficient derived from the gradient of a log-transformed plot of Eq. (4.40) indicates the degree and direction of cooperativity. An enzyme with classic hyperbolic binding behavior has a Hill coefficient of 1; enzymes with positive cooperativity have a Hill coefficient >1; and enzymes with negative cooperativity have a Hill coefficient <1.

$$r = \frac{n[\mathrm{A}]^{\mathrm{n}}}{K_{\mathrm{d}} + [\mathrm{A}]^{\mathrm{n}}} \tag{4.40}$$

4.2.7.8 Heteroallostery

The second major component of allosteric enzymes is the control of enzyme activity by heteroallosteric effectors (inhibitors or activators). Since allosteric enzymes exhibit cooperativity and can be considered to exist in two states, the T-state and R-state, it follows that an effector that can alter the balance between the T and R states will be able to effect the kinetics. Allosteric inhibitors bind to the subunit and stabilize its T-state. Therefore, a greater substrate concentration is required to compensate for the shift of equilibrium toward the T-state. Activators shift the equilibrium toward the R-state by acting as a cooperative ligand. Figure 4.7 shows the effect of an allosteric inhibitor (plot 4) and activator (plot 2) on the sigmoidal kinetics of an allosteric enzyme.

4.3 Measurement of Enzyme Activity

Enzyme activities have been measured for more than a century in the frame of a wide range of applications ranging from fundamental approaches to industrial applications, from biochemistry to medical diagnostics, and assessment of food quality. The importance of characterizing the catalytic properties of individual enzymes is self-evident to biochemists. Traditionally, enzymes have been purified from individual organisms or tissues and subjected to various in vitro experiments in order to study the corresponding reaction mechanisms (e.g., [3, 5, 22, 33]), and eventually

determine their constants (K_m, K_i, k_{cat}, v_{max}); such data can now be found in databases, e.g., BRENDA (www.brenda.uni-koeln.de/).

The next step is to integrate catalytic properties with data describing the structure of proteins, from sequence data to crystal structures. The understanding of structure–function relationships indeed represents one of the major aims in biology [23]. Recent progress in molecular techniques has enabled the design of alterations in the structure of enzymes, via site-directed mutagenesis [43] or tilling [44], and in cases combined with heterologous expression systems [1] which can provide new insight into structure–function relationships. Such approaches are generally focused on a few targets and do not usually involve high throughput techniques.

Another major aim in biology is to link the properties of macromolecules with phenotypes. Variation in the properties of enzymes can indeed have important consequences on metabolism, also on plant form and function. Variation in the sequence of a given structural gene may affect the properties of the corresponding enzyme, and depending or not on growth conditions, affect the phenotype. For example, the introgression of a regulatory subunit of ADP-glucose pyrophosphorylase, from a wild tomato species into a cultivated tomato, has been found to stabilize the active protein and thus maintain a higher activity of this key enzyme in starch synthesis. As a consequence, developing fruits accumulate more starch, which results in the release of more soluble sugars in ripening fruits [38]. Another striking example is the effect of an apoplastic invertase introgressed from another wild tomato species. Its higher affinity for sucrose, due to a single nucleotide polymorphism, results in a higher content of soluble organic compounds in the ripe fruits [15]. Both examples show that variations in properties of enzymes can dramatically affect phenotypes. We can thus predict that many such relationships will be found by screening genotypes (natural populations or mutants) for alterations in the properties of key enzymes.

In addition, environmental parameters like light, temperature, or nutrient availability can influence enzyme activities, via transcriptional, posttranscriptional, posttranslational, or allosteric regulations. Several enzymes, like nitrate reductase [26] and ADP-glucose pyrophosphorylase [1, 25, 51], have been intensively investigated, revealing highly complex regulations, but only a few studies haven been undertaken in a more systematic way. These studies showed, for example, that diurnal changes in transcript levels were not reflected at the level of the activities of the encoded enzymes in leaves of *Arabidopsis* [16], but were generally integrated over time, leading to semi-stable metabolic phenotypes [17, 36, 45, 52].

Finally, the properties and response of enzymes need be understood in their physiological context. In other words, kinetic properties, usually determined in vitro on isolated enzymes, need to be linked to pathway kinetics. Modeling metabolic networks will benefit from the accumulation of data dealing with variations in the levels and catalytic properties of enzymes associated with given genotypes and/or precise growth conditions. Furthermore, high throughput approaches will be crucial to access such data, especially in natural communities where diurnal, seasonal, spatial, and climatic variability requires extensive sampling. We will thus emphasize

methodologies dedicated to the determination of activities in complex samples, as they typically represent the first step in the identification of regulatory candidates.

4.3.1 Methodology

Enzyme activity is determined by measuring the amount of product formed or substrate consumed under known conditions of temperature, pH, and substrate concentration. In general, the initial rate, defined as the slope of the tangent to the progress curve at time 0, is determined. It is important to keep in mind that activities express velocities, not concentrations or amounts of molecules. By definition, the determination of a given enzyme activity thus requires *unique* physical and chemical conditions. In consequence and due to the fact that enzymes catalyze very diverse reactions (dehydrogenations, transfers, isomerizations, etc.), the profiling of various enzyme activities implies the application of a wide range of principles. It is therefore at the opposite of "true" profiling approaches, in which a class of molecules (transcripts, metabolites, proteins) sharing similar physical and chemical properties is being analyzed. Below we have detailed some of the key concepts and advances that have made high throughput enzyme analysis possible.

4.3.1.1 Quantification Techniques

Various principles allow the quantification of changes in the concentrations of substrates or products of enzymatic reactions. The most widely used principles are UV-visible spectrophotometry [2], fluorimetry, [19, 20] and, to a lesser extent, luminometry [10, 11, 14]. Spectrophotometric methods benefit from the fact that many reactions involve directly or indirectly the oxidized and reduced forms of NAD(P), the reduced forms absorbing specifically at 340 nm. NAD(P)H can also be determined in a fluorimeter, with a much higher sensitivity [24]. Furthermore, various fluorogenic substrates reacting with a wide range of enzymes are commercially available. The luminometric method involving luciferase and its substrate luciferin is very often used to measure ATP, ADP, and AMP [11], giving access to the quantification of ATP-dependent reactions [10]. Such methodology can benefit from a multiparallel setup (microplates, microfluidic systems) which is well suited for high throughput.

Radioactivity is used when specificity and/or sensitivity cannot be achieved with conventional methods. A typical application is the determination of the incorporation of $^{14}CO_2$ in the carboxylation reaction catalyzed by rubisco (EC 4.1.1.39; [30]). The throughput of such methods is generally low, and their use is increasingly affected by constraints on the use of radioactivity due to increased regulation of environmental health and safety.

Mass spectrometry methods are increasingly developed for the determination of enzyme activities [21]. One major advantage is the possibility to check and quantify almost every type of molecule, given substrates and products can be easily separated

and/or have different masses. This technology however requires expensive equipment and considerable expertise.

Electrochemical detection [27] can also be applied to biochemistry, as for example, amperometry which consists of the determination of electrical currents with electrodes eventually coated with enzymes catalyzing ion-producing reactions. The use of such biosensors remains limited and requires sophisticated equipment. This technology is however amenable to high throughput, for example, when combined with microfluidics [41, 54].

In plants, fluorimetric and luminometric methods are difficult to use, due to the presence in extracts of high levels of various compounds interfering at almost every wavelength, e.g., pigments or polyphenols. Without careful fractionation, such compounds will quench the emitted signals, even when present at low concentrations, leading to an underestimation of the actual activities. This is a pity because most recent technological developments in enzymology rely on fluorimetry, in particular, microfluidic chips [40]. In consequence, the microplate[1] format still offers the best compromise between cost and throughput. Microplates can be used manually but are amenable to high throughput applications. Due to the wide range of applications and equipment available nowadays, microplates have almost completely replaced cuvette-based applications. Although microplate readers also exploit the Beer–Lambert law [7], there might be some confusion. In a cuvette photometer, the absorbance (OD for optical density) is defined as follows:

$$\mathrm{OD} = \varepsilon.\mathrm{c}.l \tag{4.41}$$

where ε is the extinction coefficient of the substance being measured, c is its concentration, and l, the length of the optical path (generally 1 cm, see Fig. 4.8). In a cuvette, the light path is constant and OD varies with concentration. In a microplate reader, the light path is vertical and dependent on the volume (V) of the solution being measured. Where r is the radius of a well,

$$l = \frac{\mathrm{V}}{\pi \mathrm{r}^2} \tag{4.42}$$

since,

$$\mathrm{c} = \frac{\mathrm{n}}{\mathrm{V}} \tag{4.43}$$

OD will be proportional to the amount (moles) of absorbing molecules (n) and will be independent of the light path, i.e., in Fig. 4.8 the OD for wells B and C will be the same. Thus giving:

[1]This format was invented in the early 1950s by the Hungarian G. Takatsky and became popular during the late 1970s with the ELISA application, that's probably the reason why so many researchers call microplates "elisa plates."

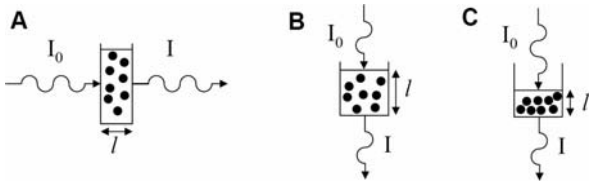


Fig. 4.8 Incident (I_0) and transmitted (I) light in a cuvette (**A**) and two microplate wells (**B** and **C**). The light path (l) is constant in the cuvette (typically 1 cm) but varies in the microplate well. Wells (**B**) and (**C**) have the same amount of analyte (*black dots*) but different concentrations. However, the amount of transmitted light (I) will be the same

$$\mathrm{OD} = \varepsilon.\mathrm{n}.\frac{1}{\pi \mathrm{r}^2} \tag{4.44}$$

It is worth noting that the well radius in microplates varies with manufacturer and model and care should be taken to select a plate with a flat bottomed cylindrical well. In addition, the presence of a meniscus in the well can affect this relationship, especially when using low volumes.

4.3.1.2 Continuous and Discontinuous Assays

In a continuous assay, the progress of the reaction is monitored directly in a recorder. This is only possible when changes in either a product or a substrate can be monitored in real time, as is the case with highly active dehydrogenases. In discontinuous assays, the reaction is stopped after fixed time intervals and a product is measured with a second specific reaction. In routine measurements, only two time points may be measured but linearity has to be checked to ensure that the supply of substrates and cofactors has not been depleted.

4.3.1.3 Sensitivity

The sensitivity of an assay can be defined as the smallest quantity that can be determined significantly. When activities are measured in raw extracts, it is also convenient to express it as the smallest amount of biological material that can be assayed. The theoretical detection limit of a standard filter-based photometric microplate reader is 0.001 which represents 0.06 nmol of NADH at 340 nm. In practice, due to experimental noise, the detection limit is much higher, at least 10 times when performing endpoint measurements. Highly sensitive assays allow the determination of activities that are present at very low levels but increased sensitivity also means that interferences can be removed, or significantly reduced by dilution.

A range of highly sensitive methods dedicated to the determination of enzyme activities are available commercially, but as mentioned above, most of them are not suitable for plant extracts, as they rely on fluorimetry or luminometry, an alternative is the use of cycling assays (Fig. 4.9).

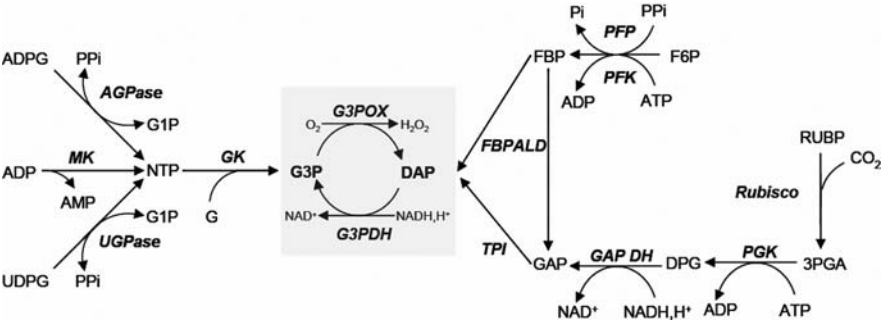


Fig. 4.9 Examples of assay principles based on the glycerol-3-phosphate cycling. Each enzyme activity (represented in *bold italics*) can be determined by adding coupling enzymes and metabolites downstream of its relevant product. After stopping the reaction, the product is determined using the cycling system (*highlighted*), directly or after conversion into either G3P or DAP. The principle is that the net rate of the cycle is a pseudo-zero-order reaction whose rate (δ[analyte measured]/$\delta t = \delta$[precursor of this analyte]/δt) depends on the initial concentration of G3P and/or DAP being determined. Quantification is achieved by measuring the rate of NADH consumption at 340 nm, and by comparison with a standard curve, in which different concentrations of the G3P and/or DAP are added in the presence of pseudo-extract. Abbreviations: *Metabolites* 3PGA, 3-phosphoglycerate; ADPG, ADP-glucose; DAP, dihydroxyacetone phosphate; DPG, 1,3-diphosphoglycerate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; G, glycerol; G1P, glucose-1-phosphate; G3P, glycerol-3-phosphate; GAP, glyceradehyde-3-phosphate; NTP, nucleotide triphosphate; PPi, pyrophosphate; RUBP, ribulose-1,5-bisphosphate; UDPG, UDP-glucose. *Enzymes* AGPase, ADPG pyrophosphorylase; FBPALD, FBP aldolase; GAPDH, NAD-GAP dehydrogenase; GK, glycerokinase; G3PDH, G3P dehydrogenase; G3POX, G3P oxidase; MK, myokinase; PFK, ATP-phosphofructokinase; PGK, phosphoglycerokinase; PFP, PPi-phosphofructokinase; rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; TPI, triose-phosphate isomerase; UGPase, UDPG pyrophosphorylase

Cycling assays are less prone to interferences coming from raw extracts as they can be used with standard microplate photometers and provide a 100–10,000 increase in sensitivity compared to direct or endpoint spectrophotometric methods. Cycling assays were developed by Warburg et al. [53] and made popular by the efforts of Lowry et al. [31, 32]. However, these assays are time consuming and tedious when used in cuvettes. Cycling assays prove to be much easier in microplates [18] and can be adapted for the determination of a number of enzyme activities via discontinuous assays [16]. A wide range of reactions can be measured provided they can be coupled to the production or consumption of NAD(H), NADP(H), glycerol-3-phosphate, dihydroxyacetone phosphate, or nucleotide triphosphates (Fig. 4.9).

4.3.1.4 Coupling Reactions

The majority of enzyme activities cannot be monitored directly. One or more coupled reactions are needed to convert a product of the enzyme reaction being measured into a quantifiable product. For example, phosphoglucose isomerase can be

assayed by coupling the production of glucose-6-phosphate to NADPH production, using glucose-6-phosphate dehydrogenase, as shown below:

$$\text{F6P} \xrightarrow{PGI} \text{G6P} \xrightarrow{G6PDH} \text{NADPH} \tag{4.45}$$

Abbreviations: F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PGI, phosphoglucose isomerase (EC5.3.1.9); G6PDH, glucose-6-phosphate dehydrogenase (EC1.1.1.49)

Coupling reactions may also be used when the primary reaction has an unfavorable equilibrium constant, e.g., malate dehydrogenase in its forward direction

$$\begin{aligned} &\text{Malate} + \text{NAD}^+ \xleftrightarrow{\text{MDH}} \text{Oxaloacetate} + \text{NADH} + \text{H}^+ \\ &K_{eq} = 5.94 \text{ x} 10^{-13} \end{aligned} \tag{4.46}$$

Abbreviation: MDH, malate dehydrogenase (EC 1.1.1.37). The value for K_{eq} is from Outlaw and Manchester [37].

The addition of citrate synthase and acetyl coenzyme A will consume oxaloacetate and thus displace the equilibrium of the primary reaction:

$$\begin{aligned} &\text{Oxaloacetate} + \text{Acetyl coenzyme A} \xrightarrow{CS} \\ &\text{Citrate} + \text{Coenzyme A} \end{aligned} \tag{4.47}$$

Abbreviation: CS, citrate synthase (EC 2.3.3.1).

A number of theoretical studies have been undertaken to model and optimize coupled enzyme assays [2, 46]. Coupled assays are valid if the velocity of the coupling system equals the velocity of the reaction of interest. Thus, an efficient coupling is only possible if steady-state concentrations of the product of the primary reaction are much smaller than the corresponding K_{m} [46]. The fact that a coupling reaction may increase the time lag required to reach steady state has also to be taken into account. Equations can be used to optimize the concentrations of the coupling enzymes, generally in order to reduce costs or to avoid interfering reactions. It is however recommended to test a range of concentrations of coupling enzymes and to check the duration of the lag phase for each of them.

4.3.1.5 Interferences with Other Components of the Extract

The use of raw extracts to determine kinetic properties of enzymes is subject to various interferences. Undesired substrates of reactions under investigation, including coupling reactions, can lead to underestimations or overestimations of actual activities, especially when non-saturating conditions are being used. Specific or non-specific inhibitors or activators may also interact with the reactions under study. Another possible source of error is the presence of numerous enzymes in the extract, as some of them may react with constituents of the assay.

Running blanks is a way to retrieve interferences; it is particularly useful when an enzyme yielding a common product is present. A typical example is given by the assay for glutamine synthetase, an enzyme involved in nitrogen assimilation and in photorespiration [39]:

$$\text{Glu} + NH_4^+ + \text{ATP} \xrightarrow{GS} \text{Gln} + \text{ADP} + \text{Pi} \tag{4.48}$$

Abbreviations: Glu, glutamate; ATP, adenosine triphosphate; Gln, glutamine; ADP, adenosine diphosphate; Pi, orthophosphate; GS, glutamine synthetase (EC 6.3.1.2).

Pyruvate kinase and lactate dehydrogenase are then used as coupling enzymes, to convert the ADP into NAD^+:

$$\begin{aligned} &\text{PEP} + \text{ADP} \xrightarrow{PK} \text{Pyruvate} + \text{ATP} \\ &\text{Pyruvate} + \text{NADH}, \text{H}^+ \xrightarrow{LDH} \text{Lactate} + \text{NAD}^+ \end{aligned} \tag{4.49}$$

Abbreviations: PEP, phosphoenolpyruvate; PK, pyruvate kinase (EC 2.7.1.40); $NADH,H^+$, reduced nicotinamide adenine dinucleotide; NAD+, oxidized nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase (EC 1.1.1.27).

In the presence of AMP (generally present as a contaminant of commercial preparations of ATP), adenylate kinase from the extracts will also yield ADP:

$$\text{ATP} + \text{AMP} \xrightarrow{\text{AK}} \text{ADP} + \text{ADP} \tag{4.50}$$

Abbreviations: AMP, adenosine monophosphate; AK, adenylate kinase (EC 2.7.4.3).

Thus, the coupling system will measure the activities of both glutamine synthetase and adenylate kinase. It will then be useful to run a blank without ammonium (or conversely glutamate). However, the effect of ammonium (or glutamate) on the activity of adenylate kinase has to be checked.

Therefore, it is sometimes useful to add specific inhibitors to block interfering enzymes. P^1,P^5-di (adenosine-5′)-pentaphosphate is a strong inhibitor of adenylate kinase [29] and can be included into the assay mixture for the determination of glutamine synthetase. A blank without one of the substrates is however still necessary, as the inhibition of the interfering activity may be incomplete.

Another way to diminish interferences from the extracts is to dilute them until interferences become negligible. As mentioned above, purification steps including desalting of extracts are time consuming and may provoke losses of activities. Nevertheless, the fact that many enzymes become unstable when they are diluted [42] has to be taken into account. Dilution experiments can be performed in order to determine the optimal dilution of the extract in the assay. Interestingly, various enzymes from leaves of various species could be measured at strong dilutions without losses in activity ([16] and unpublished results). When stopped assays are used,

linearity with time should always be checked, as well as the recovery of the product of the enzyme under investigation. This is best achieved by spiking the extraction buffer with various amounts of the product, below and above the range expected to be produced by the enzyme. Alternatively, extracts under study can be mixed with an extract with a known activity.

4.3.2 Logistics

Building a microplate-based platform enabling the determination of dozens of enzyme activities in parallel requires several points to be taken into account.

4.3.2.1 Type of Assay

Stopped assays are usually preferred to continuous assays, as they provide several major advantages. First, they offer more flexibility because the determination of the products of the enzymatic reactions under study can be performed separately, while the continuous assays require as many temperature-controlled photometers as enzymes being measured. Incubators, including automated hotels, where microplates can be incubated at predetermined temperatures for set periods of time before reactions are stopped, are indeed less expensive than photometers and can easily be included as part of an automated pipetting station. Secondly, stopped assays can provide a much higher sensitivity, when products of enzymes are being measured with kinetic or fluorimetric methods. This allows routine determination of enzymes with low activities from raw extracts, without the need for sophisticated time-consuming purification and concentration procedures. Thirdly, the first step of stopped assays can be performed with low volumes (e.g., 20 µl in 96-well microplates), while for optical reasons, continuous assays require a minimal volume (e.g., 100 µl in 96-well flat bottom microplates). This can lead to a substantial lowering of the costs, assuming the reagents used for the determination of the products are less expensive than those used in the first step. The major disadvantage of stopped assays is that they require more pipetting steps, which implies that they are more time consuming and more prone to error. Time and error can however be considerably reduced when using electronic multichannel pipettes or liquid handling robots. The use of continuous assays will be usually restricted to enzymes with high activities and those requiring inexpensive reagents, like triose phosphate isomerase, malate dehydrogenases, or phosphoglucomutase.

4.3.2.2 Reagents

As by definition, each enzyme activity requires unique conditions to be determined; a multiparallel platform will require a large variety of reagents, which implies well-organized logistics. Typically, microplates have to be prepared in advance, so that enzyme reactions can be started right after extracts have been prepared. However, assay mixes are generally stable for only a few hours, so that they

have to be prepared right before starting extractions. It is very useful to build a "bank" of reagents that can be organized as ready-to-use kits. Whenever possible, stock solutions should be prepared in advance and stored at adequate temperatures (e.g., −80°C when containing enzymes and/or coenzymes). Pipetting schemes used for the preparation of assay mixes should be kept as simple as possible in order to decrease the time needed, for example, by adjusting concentrations of reagents in such a way that only a few, easy to manage, pipetting volumes will be used.

Another important issue is that more and more reagents are no longer commercially available, probably due to the fact that many enzyme-based analytical procedures used to determine metabolites have been replaced by mass spectrometry-based methods. For example, yeast glycerokinase, used as a coupling enzyme in a range of assays measuring ATP- or UTP-producing enzymes and exploiting the glycerol-3-phosphate cycling system [16, 18], cannot be replaced by its homologues from bacteria, as these have a much weaker affinity for ATP and do not react with UTP. Heterologous expression systems can be used to produce such enzymes, but imply extra costs and can be time consuming. Substrates like xylulose-5-phosphate or sedoheptulose-1,7-bisphosphate, necessary for the determination of important enzymes from the Calvin cycle or the oxidative pentose phosphate pathway, also became unavailable recently. In these cases, skills in organic chemistry will be welcome. Alternatively, private or public laboratories can produce such compounds on demand, as relevant protocols are often available, but this is usually very expensive.

4.3.2.3 Sample Handling

Samples are prepared by quenching tissues into liquid nitrogen immediately after harvesting. If labile posttranslational modifications are studied, sampling should happen within seconds. Furthermore, several enzymes that are regulated via light-dependent redox mechanism, like fructose-1,6-bisphosphase (EC 3.1.3.11;[9]), may activate/deactivate very quickly. It is then crucial to plunge the tissues into liquid nitrogen in the light. Samples should always be stored at –80°C and processed at very low temperatures (grinding and weighing of aliquots for analysis) until extraction.

4.3.2.4 Preparation of Extracts

The optimal dilution varies from enzyme to enzyme, in large part because enzymes from various pathways cover 4–5 orders of magnitude in terms of activity. Thus, depending on the enzymes being measured, it will be necessary to achieve several dilutions of the extracts. This is best achieved when extracts are prepared in 96-well format.

4.3.2.5 Stability of Enzymes

Many enzymes are not stable once extracted and do not resist a freezing/thawing cycle, even in the presence of glycerol. The assay must therefore be performed

as quickly as possible once the extracts have been prepared. This implies that a compromise has to be found between number of extractions and number of enzymes to be measured.

4.3.2.6 Temperature

Kinetic properties of enzymes vary with temperature, it is thus important to keep it constant, by using incubators and/or temperature controlled photometers.

4.3.2.7 Timing

Time management is essential for conducting stopped assays. When several enzymes are assayed in parallel in many extracts, manual timing becomes very difficult. This can be simplified by using the automated timing available in standard programs driving liquid handling robots.

4.3.2.8 Automation

The need to process more samples faster is a continuing trend in academic and industrial research. The wide adoption of microplates in laboratory routines has significantly influenced the development of a huge diversity of labware and automation solutions. Almost everything dealing with enzyme activities can now be processed with the help of robots in this format, from preparation of samples to detection. Depending on needs and means, the best balance between man and machine has nevertheless to be found in the jungle of laboratory robotics.

Based on the desired throughput, and assuming the labor of 2–4 people, we can roughly estimate the needs:

- Low throughput, below 500 activity determinations a week, robotics is not an absolute requirement. Standard microplate equipment including multichannel pipettes and a spectrophotometer might be adequate.
- Mid throughput, between 500 and 50,000, at least one liquid handling robot is needed, ideally a 96-channel robot equipped with a gripper to transport microplates, a shaker, temperature control, and several microplate readers. Pipetting robots working in the range of 0.5–50 μl are the most adequate; in addition to the throughput, they usually provide a very good accuracy at low volumes. A cryogenic grinding/weighing robot (Labman, Stokesley, UK) may also be very useful to process samples prior to extraction, as these steps are highly time consuming. A laboratory information management system may also be implemented in order to decrease time and error in calculations.
- High throughput, above 50,000, requires fully automated solutions. A high degree of sophistication will be required to include steps such as centrifugation, adhering, or removing adhesive lids, integration of microplate readers, and so on, implying an exponential increase of the costs. A further consequence is a strong decrease in flexibility.

4.3.3 Determination of Enzyme Properties in Raw Extracts

The purification of enzymes from living organisms, even partial, is a time-consuming process eventually leading to losses in activity and/or alterations in the actual catalytic properties. In consequence, its use is generally restricted to detailed biochemical studies. Highly purified enzymes are needed to determine important kinetic parameters like K_m and k_{cat}, to search for inhibitors or activators, or to obtain crystals.

If k_{cat} is known, it is then theoretically possible to evaluate enzyme concentrations in complex extracts. This is however biased by possible changes due to isoform composition or to posttranslational regulation events. This is also not a priority, as advances in proteomics are likely to become more adequate for such purpose and able to deal with a much larger number of analytes in parallel [8]. Apparent activities of enzymes should therefore be considered as integrating various levels of regulation, each of them being potentially subject to environmental or genetic inputs.

We believe that the collection of large sets of activity data obtained from various genotypes, organs, or tissues and from various growth conditions could be useful to modeling scientists. It would therefore be advantageous to determine the activities in standardized conditions, like temperature, pH (depending on the subcellular compartmentation and assuming that most enzymes from a compartment will have similar pH optima), or buffers. Metadata consisting in precise documentations of the assay condition for each enzyme should also be documented.

4.3.3.1 Measurement of Total Activity

When assay conditions are optimized in such a way that a given activity from a raw extract is maximized, we will consider that v_{total} is being measured. Under conditions at which enzymes are by far more diluted than their substrates, most of them obey the law of Michaelis–Menten. As a consequence and assuming that assay conditions, including concentrations of substrates, are kept nearly constant, rates of reactions will be dependent solely on the enzyme concentration, due to the establishment of pseudo-zero-order reactions (see Section 4.2.1). Thus, measurement of v_{total} is an estimate of the amount of enzyme present.

4.3.3.2 Measurement of Apparent Kinetic Constants

Various linearization methods have been established to determine such constants (see Section 4.2.5 and Fig. 4.5), but as previously stated [12], computer-based methods should be preferred, assuming some understanding of the underlying calculations. In particular, the structure of the experimental error may drive the choice of the method being used, as each method handles the error in a different way (see Section 4.2.5). It is possible to determine kinetic constants in raw extracts that are close to kinetic data obtained with purified enzymes and that can be found in literature or in databases [47, 48]. As shown in Fig. 4.10, the affinity of rubisco for ribulose-1,5-bisphosphate and its total activity were determined by fitting the Michaelis–Menten

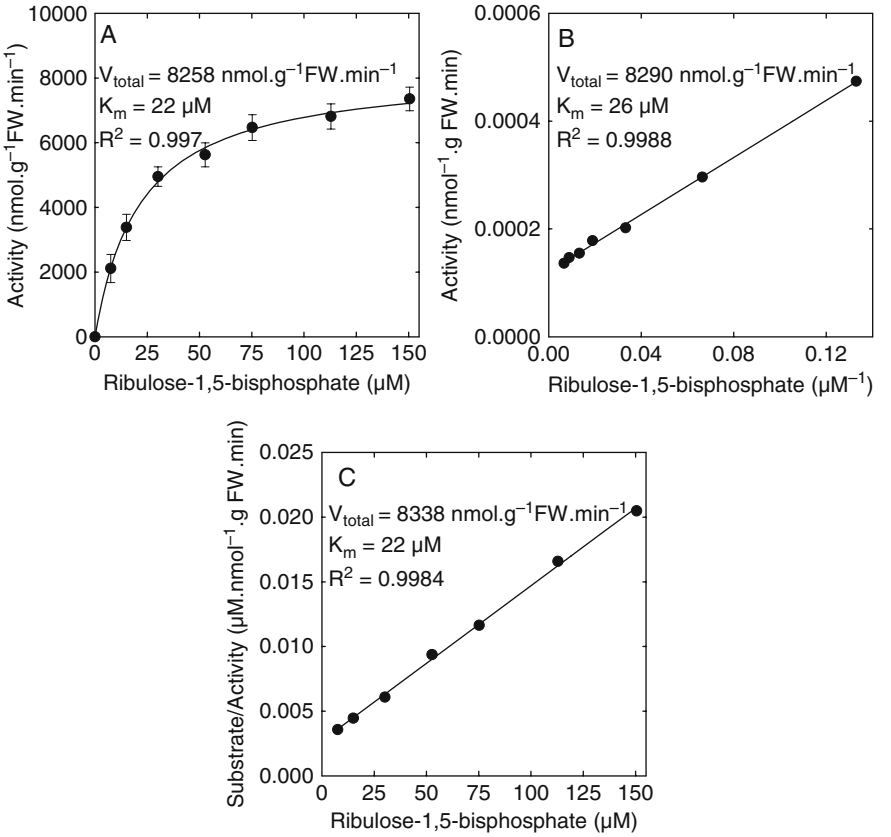


Fig. 4.10 Change in velocity with the concentration of ribulose-1,5-bisphosphate for the reaction catalyzed by ribulose-1,5-carboxylase/oxygenase (EC 4.1.1.39) and determination of K_m and V_{total} with hyperbola fitting (**A**), Lineweaver and Burk (**B**), and Hanes (**C**) methods. Data are expressed as means ± SD ($n = 6$). The fitting of the hyperbola was achieved using the Sigma Plot software [48]

equation (Eq. 4.26) and by using the methods of Lineweaver and Burk, and Hanes. Values obtained with the three methods were very similar, with the exception of the apparent K_m for ribulose-1,5-bisphosphate which was found to be higher when using the method of Lineweaver and Burk, probably due the overweighting of the 2 points obtained with the two lowest substrate concentration (see Section 4.2.5). The apparent K_m or $K_{0.5}$ for ribulose-1,5-bisphosphate was found to have a value of about 20 µM, which is close to values obtained with the purified enzyme from various species of higher plants (http://www.brenda.uni-koeln.de/).

Kinetic properties obtained with raw extracts should always be considered with caution as various sources of error are possible. Effectors present in the extracts may inhibit or activate the enzyme under study and thus lead to erroneous results. Artifacts may also result from the destabilizing effect of the dilution of the substrate,

especially when the enzyme is already highly diluted [42]. Such an effect can lead to an erroneous interpretation, as the dose–response curve may have a sigmoid shape and thus evoke cooperativity (see Section 4.2.7). It may therefore be useful to repeat measurements with different concentrations of both extract and substrate. Furthermore, the use of K_m might be misleading and should be replaced with apparent K_m or more generally $K_{0.5}$, i.e., the condition at which the enzyme reaches 50% of its total velocity in the extract.

Inhibition types and constants may also be determined using raw extracts. Therefore, a large number of determinations have to be performed, i.e., various substrate concentrations at various inhibitor concentrations. Such determinations are probably prone to error, due to the complexity of raw extracts. If alterations in inhibition constants are to be searched across a large range of genotypes and/or growth conditions, it seems more adequate to determine the $K_{0.5}$ corresponding to the inhibitors or activators under study first, by using the same approach described above.

It is important to note that both low and high concentrations of substrate should be used anyway. For example, competitive inhibitors would not exert any visible effect at high substrate concentrations. Once $K_{0.5}$ has been determined in conditions that are satisfactory in terms of accuracy and reproducibility, a high throughput screen can be designed in "saturating" and "half saturating" conditions. Any shift in the ratio between $v_{0.5}$ and v_{total} would indicate a possible variation in the properties of the enzyme under study.

4.4 Conclusion

Enzyme activity integrates information from several levels of biological organization and in that respect the information is perhaps more valuable than relying on assumptions made from, for example gene transcript abundance alone, and unlike metabolite or gene transcript data, enzyme activity also provides information about flux, which is key to understanding metabolic networks. However, parallel determination of, for example, gene transcript abundance, metabolite and protein levels in conjunction with enzyme activity will provide rich data sets where integration of information is likely to be of greater value than the sum of the parts.

Traditional methods for analyzing enzyme activity are laborious and not comparable to the high throughput "omics" approaches currently being used to investigate the levels of gene transcripts, proteins, and metabolites. The enzyme analysis platform described here is a step toward the type of high throughput tool that we have become familiar with in the "omics" arena. However, because the biochemistry of enzyme activity analysis is considerably more complex than other high throughput technologies, true high throughput profiling on the scale of genomics is unlikely. Nevertheless, high throughput enzyme activity analysis is now a reality.

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