Appendix A
Enzyme Kinetics

As a catalyst, an enzyme accelerates the reaction of a substrate but does not affect equilibrium. The increase in the substrate concentration results in increasing the rate of the reaction estimated by the accumulation of the final product. Contrary to common chemical reactions, the rate of the enzymatic process reaches saturation, indicating a complicated mechanism of the conversion. Numerous investigations and discussions about possible mechanisms ended at the turn of the nineteenth and twentieth centuries when the German biochemist L. Michaelis and the Canadian physician M. Menten suggested the simplest empirical description of the dependence of the rate of an enzymatic reaction on the substrate concentration for invertase. Later on, in 1925, the integral form of the Eq. (A.1) was suggested (Keleti 1986).

\[ E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow{k_2} E + P \]  \hspace{1cm} (A.1)

\( E, S \) and \( P \) denote free enzyme, substrate and product molecules, respectively. The \( k_i \) are the rate constants of the elemental steps of the reaction. The rate of the formation of the product \( P \) of reaction (A.1) is expressed by the Michaelis–Menten Eq. (A.2).

\[ v = \frac{dc_P}{dt} = \frac{v_{\text{max}}c_S}{K_m + c_S} \]  \hspace{1cm} (A.2)

The reaction rate \( v \) is commonly determined as a slope of a tangent line drawn to the kinetic curve \( c_S (c_P) - t \) at the reaction beginning \( (t = 0) \) (Fig. A.1).

The Eq. (A.2) follows from the steady-state assumption where the rate of the \( ES \) complex formation is equal to its conversion, so that the concentration of the enzyme-substrate complex remains about constant and time-independent. For general reaction (A.1), rearrangement yields an expression (A.3) for Michaelis constant \( K_m \) (so-called Briggs-Haldane conditions).

\[ K_m = \frac{k_{-1} + k_2}{k_1} \]  \hspace{1cm} (A.3)
If the total enzyme reaction is limited by the breakdown of the ES complex ($k_{-1} \gg k_2$, Michaelis–Menten conditions), the $K_m$ is equal to the dissociation constant $K_S$ of the enzyme-substrate complex. The $K_m$ corresponds also to the substrate concentration giving the reaction rate equal to half of its maximal value ($v = 1/2v_{max}$). In homogeneous conditions, $K_m$ is an upper limit of the substrate concentration that can be determined from the linear piece of calibration curve in $v$–$C_S$ plots.

The Eq. (A.2) can be simplified by the assumption of $K_m \gg C_S$. The rate of an enzymatic reaction linearly depends on the substrate concentration in the range of its low values (A.4).

$$v \approx \frac{v_{max}C_S}{K_m} = \frac{v_{max}}{K_m}C_S$$

(A.4)

This makes it possible to determine the substrate concentration by the rate of its conversion.

The Eq. (A.2) gives a hyperbolic shape of the reaction curve (Fig. A.2), which coincides well enough with most experimental data. The Eq. (A.2) is often transformed to obtain a linear dependence of the experimental parameters that simplify the calculation of the main kinetic parameters of the reaction.

---

**Fig. A.1** Kinetic curve of the accumulation of the product $P$ of enzymatic reaction

**Fig. A.2** The graphs corresponding to the Michaelis–Menten kinetics and its graphical representation on a double reciprocal plot
A double reciprocal plot (Line weaver-Burk plot) is most often used for both the graphical representation of enzyme kinetics and their quantification. Though rather popular, the Line weaver-Burk plot does not provide the most accurate estimation of the $K_m$ and $v_{\text{max}}$ values because of the non-linear transformation of experimental data. Instead, some other approaches can be recommended, e.g., Eadie-Hofstee or Hanes-Wolf plots. In all these methods, the kinetic parameters are determined by the $x$- and $y$-intercepts and the slope of the linear piece of the curve. These values are presented in Table A.1.

In addition to steady-state kinetics, the $K_m$ value can be derived from the progress curve indicating temporal changes of the substrate concentration. In this case, the Eq. (A.2) is transformed to (A.5), or, in an integral form, to (A.6).

$$v = -\frac{dc_S}{dt} = \frac{v_{\text{max}}c_S}{K_m + c_S} \quad \text{(A.5)}$$

$$K_m \ln \frac{c_S^0}{c_S} + \left( c_S^0 - c_S^t \right) = v_{\text{max}}t, \quad \text{(A.6)}$$

in which $c_S^0$ and $c_S^t$ are the substrate concentration at the time points 0 (zero) and $t$, respectively. For graphical presentation, the Eq. (A.6) is rearranged into (A.7) (Taylor 2002).

$$\frac{1}{t} \ln \frac{c_S^0}{c_S^t} = \left( \frac{c_S^0 - c_S^t}{K_m t} \right) + \frac{v_{\text{max}}}{K_m}, \quad \text{(A.7)}$$

**Table A.1** Linearization of the experimental data and determination of kinetic parameters of the enzyme reaction

<table>
<thead>
<tr>
<th>Method</th>
<th>Linearization equation</th>
<th>Slope</th>
<th>Axes intercepts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lineweaver-Burk plot</td>
<td>$\frac{1}{c_S} - \frac{1}{v_{\text{max}}}$</td>
<td>$K_m$</td>
<td>$\frac{1}{v_{\text{max}}}$</td>
</tr>
<tr>
<td>Eadie-Hofstee plot</td>
<td>$\frac{1}{v} - \frac{v}{c_S}$</td>
<td>$-\frac{1}{K_m}$</td>
<td>$v_{\text{max}}$</td>
</tr>
<tr>
<td>Hanes-Wolf plot</td>
<td>$c_S - c_S^t$</td>
<td>$\frac{1}{v_{\text{max}}}$</td>
<td>$-K_m$</td>
</tr>
</tbody>
</table>

![Fig. A.3 Determination of kinetic parameters $v_{\text{max}}$ and $K_m$ from a progressive curve](image)
The kinetic parameters of the reaction are determined from the straight line in the plots $t^{-1} \ln \left( \frac{c_0^n}{c_S^n} \right)$ versus $\frac{[c_0^n - c_S^n]}{t}$ (Fig. A.3).

All the equations describing the Michaelis–Menten kinetics are based on the simplest scheme of the reaction with a single substrate and a single active site in the enzyme molecule that interact in the 1:1 ratio. Multi-substrate reactions, as well as allosteric enzymes, often exhibit a formally similar behavior, at least within a limited range of substrate concentration. This allows calculating the kinetic parameters called an effective (or experimental) Michaelis constant and maximal reaction rate. They can be expressed by a combination of the constant rates of elemental stages, which is more complicated than that in Eq. (A.3).
Appendix B
Inhibition Kinetics

Formal kinetics offers rather simple and reliable protocols for establishing the mechanism of inhibition. As other kinetic approaches, they are valid for a certain set of primary conditions (concentrations of a substrate, inhibitor and enzyme active sites). Changes in the conditions of the experiments as well as immobilization of an enzyme can shift the relative rate of the stages of the complex reaction scheme so that the criteria of the mechanisms would be not valid or contradict with the chemical backgrounds of the process. Nevertheless, kinetic analysis of inhibition is very useful to establish the limits of the inhibitor concentrations that are determined by appropriate enzymes in ideal conditions. The mechanism of inhibition determines the requirements of the optimal conditions of inhibition detection as the substrate concentration and necessity in intermediate incubation and/or washing immobilized enzyme preparations.

An irreversible inhibition can be monitored by the relative decay of enzyme activity. For the inhibitor concentration significantly exceeding that of the enzyme active site, the Aldridge Eq. (B.1) is used (Aldridge 1950).

\[ \ln \frac{v_0}{v_t} = k_{II} c_I \tau \]  \hspace{1cm} (B.1)

Here, \(v_0\) and \(v_t\) are the rates of enzymatic reaction prior to and after the incubation step, \(c_I\) is the inhibitor concentration, and \(\tau\) is the incubation time. The \(k_{II}\), bimolecular inhibition constant, describes the efficiency of the enzyme-inhibitor interaction. This depends on the nature of the reactants and reaction conditions but not on the quantity of the enzyme and inhibitor as such. The bimolecular inhibition constant can be expressed by a combination of rate constants of various steps of the reaction.

Reversible inhibitors exert a great variety of interaction mechanisms, all of which refer to the true equilibria of the stages with enzyme participation (Keleti 1986). For Michaelis-Menten conditions (one \(ES\) complex, 1:1 interaction of an enzyme active site and substrate molecule) the general scheme of reversible inhibition can be presented as follows (B.2):
The scheme takes into account the formation of an enzyme-inhibitor complex and a triple enzyme-substrate-inhibitor $E-S-I$ complex. Contrary to irreversible inhibition, such complexes retain their possibility of participating in the formation of the final product $P$. This implies that the inclusion of an inhibitor does not fully block the active site that can interact with a substrate even though the efficiency of such an interaction decreases. Empirical coefficients $\alpha$ and $\beta$ account for the influence of an inhibitor on the target reaction of the substrate conversion. Here, capital letters $K_I$ and $\alpha K_I$ correspond to equilibrium constants, whereas lower case letters $k_1, k_{-1}, k_2, \alpha k_{-1},$ and $\beta k_2$ involve the rate constants of the appropriate stage of the reaction.

The scheme (B.2) is described by the Eq. (B.3). However, its application to real cases of inhibition is complicated by many parameters.

The rate of enzymatic reaction in the absence and the presence of an inhibitor is denoted as $v_i$ and $v_0$, respectively. The $K_m$ value also corresponds to the zero concentration of the reversible inhibitor. Usually the $\alpha > 1$ and $\beta < 1$ values are typical for reversible inhibitors.

Although the reaction scheme (B.2) involves accurate development, some simplified cases are popular. They are classified in accordance with relation between various parameters and are called competitive, non-competitive and uncompetitive inhibition (Leskovac 2004).

For competitive inhibition, $\alpha \to \infty$. This results in the following reaction scheme (B.4).

The inhibitor and substrate both compete for the same active site of an enzyme and the enzyme-inhibitor complex cannot catalyze the substrate conversion. For such a reaction, the following equation can be given for the reaction rate (B.5):

The comparison of the Eq. (B.5) with the Michaelis-Menten equation makes it possible to conclude that the presence of competitive inhibitors increases the experimental $K_m$ value proportionally to the inhibitor concentration. Meanwhile, the formal expression of the dependencies remains the same in the competitive
inhibition and cannot be confirmed by the kinetics analysis. An example of appropriate linear curves is given in Fig. B.1.

The maximal rate of the reaction, $v_{\text{max}}$, does not depend on the concentration of the competitive inhibitor. It corresponds to the saturation of enzyme-active sites with a substrate when all the inhibitor molecules are supplanted by a substrate.

It should be noted that competitive inhibition describes the interaction of a fully irreversible inhibitor with an enzyme active site in the presence of the substrate.

Non-competitive inhibition ($\alpha = 1$, $\beta = 0$) corresponds to the case where the substrate and inhibitor do not interfere with each other in enzyme binding, and the interaction with an inhibitor affects the affinity of an enzyme towards a substrate but not the reactivity of the $ES$ complex. In this case, the general scheme (B.2) is reduced to (B.6).

$$E + S \rightleftharpoons E-S \rightarrow E + P \quad + I \quad \downarrow \quad + I \quad \downarrow \quad E-I + S \rightleftharpoons E-S-I \quad (B.6)$$

From the theoretical point of view, there are three possible mechanisms of non-competitive inhibition:

- inhibitor and substrate binding sites of the enzyme are different;
- inhibitor and substrate bind to the same binding site but via different functional groups; or
- inhibitor does not bind with an active site of an enzyme but affects the protein confirmation. This changes the charge distribution and/or acid-base properties of functional groups involved in the ES complex formation.

In non-competitive inhibition, the $K_m$ constant remains the same as in the absence of the inhibitor, but the maximal reaction rate decays proportionally to the inhibitor concentration (B.7).

$$v_i = \frac{v_{\text{max}}}{1 + c_I/K'_I} \frac{c_S}{K_m + c_S} \quad (B.7)$$
The appropriate graphic illustrations of non-competitive inhibition are presented in Fig. B.2.

Uncompetitive inhibition \((\alpha = \beta; \alpha, \beta < 1)\) assumes the interaction of an inhibitor with the \(E–S\) complex, but not with a free enzyme active site \((B.8)\). In this case, both \(K_m\) and \(v_{\text{max}}\) experience a change, so that the curves in the Line weaver-Burk plot obtained for various inhibitor concentrations form parallel lines (Fig. B.3). The appropriate equation is given in (B.9).

\[
v_i = \frac{v_{\text{max}}}{\left(1 + c_I/K_I''\right)K_m\left(1 + c_I/K_I''\right) + c_S}
\]

The Eq. (B.9) can be used for the estimation of the inhibition constant and confirmation of the mechanism of uncompetitive inhibition. It can be shown that the \(v_{\text{max}}\) and \(K_m\) change proportionally and to a degree that depends on the inhibitor concentration and inhibition constant \((B.10)\).

\[
\frac{(v_{\text{max}})_{\text{exp}}}{(v_{\text{max}})_{c_I=0}} = \frac{(K_m)_{\text{exp}}}{(K_m)_{c_I=0}} = \frac{\alpha(K_I' + c_I)}{\alpha K_m + c_I}
\]
Mixed inhibition ($\alpha, \beta \neq 1$) describes the dependence of the enzymatic activity on substrate/inhibition concentration as a superposition of the special cases described above. Thus, the changes in the reaction rate in many cases can be presented as a combination of competitive and non-competitive inhibition (B.11) with $K_I$ and $K'_I$ corresponding to the inhibition constants describing competitive and non-competitive inhibition, respectively. The total inhibition can be expressed in this case by the so-called reduced inhibition constant $\bar{K}_I$ (B.12).

\[
\frac{1}{v_i} = \frac{K_m}{v_{\text{max}}c_S} \left(1 + \frac{c_I}{K_I}\right) + \frac{1}{v_{\text{max}}} \left(1 + \frac{c_I}{K'_I}\right)
\]

(B.11)

\[
\frac{v_0}{v_i} = \frac{K_m(1 + c_I/K_I) + c_S(1 + c_I/K'_I)}{K_m}
\]

\[
\bar{K}_I = \frac{K_I K'_I}{K_I + K'_I}
\]

(B.12)

In all the mechanisms of reversible inhibition, the increase in the inhibition constant decreases the sensitivity of an enzyme towards an inhibitor. The value of a constant corresponds to the inhibition concentration resulting in a 50 % decrease of the rate of the enzymatic reaction. This rule makes it possible to compare the relative strength of inhibitors that react with an enzyme by different reaction paths. For the same reasons, other empirical estimates based on simplified experiments involve a reduced number of measurements. Thus for simple cases, $I_{50}$ (concentration of an inhibitor yielding a 50 % decrease in the rate of enzymatic reaction) represents the $K_I$ value. To some extent, this refers to other similar variables like $I_{50}$. The only, but serious, limitation of such empirical characteristics is that they do not provide any information on the possible mechanism of inhibition.

For “true” inhibition constants, the conclusions about possible mechanisms are made together with the inhibition constants’ calculation from a series of experiments with varied concentrations of the substrate/inhibitor. As could be seen from Figs. B.1–B.3, the x- and y-intercepts, as well as line concurrence points, are of most importance. The appropriate examples are given for the Line weaver-Burk plot but can be easily extended to other linearization methods mentioned above (see Table B.1).

<table>
<thead>
<tr>
<th>Inhibition mechanism</th>
<th>Relative decay of the rate of enzymatic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purely competitive</td>
<td>$\frac{v_0 - v_i}{v_i} = \frac{K_a}{K_a(K_a + c_S)} c_I$</td>
</tr>
<tr>
<td>Purely non-competitive</td>
<td>$\frac{v_0 - v_i}{v_i} = \frac{c_I}{K'_I}$</td>
</tr>
<tr>
<td>Purely uncompetitive</td>
<td>$\frac{v_0 - v_i}{v_i} = \frac{c_I}{2K'_I(K'_I + c_S)} c_I$</td>
</tr>
<tr>
<td>Mixed</td>
<td>$\frac{v_0 - v_i}{v_i} = \frac{2K_a + c_S}{2K'_I(K'_I + c_S)} c_I$</td>
</tr>
</tbody>
</table>
The description of the mechanisms of reversible inhibition presented here is to some extent the idealized presentation of much more complicated reactions. Some of the restrictions on the enzyme/substrate/inhibitor interactions specifying a particular reaction scheme can be non-obligatory. Thus, instead of the full inactivation of some intermediates, they can decrease their reactivity. For this reason, apart from the “pure”, or “complete” mechanisms presented above, partially non-reversible, uncompetitive, etc., mechanisms are denoted. They are certainly more complicated in formal kinetics than those presented here but do not change the general criteria and empirical rules necessary for establishing the specificity of inhibitor action.

The relation between the $K_m$ and $c_S$ is the principal criterion of the substrate influence on the inhibition measurement. For example, competitive inhibition offers the reciprocal dependence of the inhibition degree and substrate concentration for $c_S \gg K_m$. For this case,

$$\frac{v_0 - v_i}{v_i} = \frac{K_m c_I}{K_I c_S} \quad (B.13)$$

so that the lower the substrate concentration, the lower the inhibitor concentration corresponding to the same inhibition degree. However, the opposite relation ($c_S \ll K_m$)

$$\frac{v_0 - v_i}{v_i} = \frac{c_I}{K_I} \quad (B.14)$$

makes the inhibition independent of the substrate concentration similarly to non-competitive inhibition and uncompetitive inhibition in the case of $c_S \gg K_m$.

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