ENZYMES

INTRODUCTION

Enzymes are proteins that enhance (or accelerate) chemical reactions. This process is called catalysis and enzymes thus catalyze chemical reactions. In enzymatic reactions, the molecules present at the beginning of the reaction are called substrates. Enzymes convert substrates into different molecules, called products. All processes in nature require enzymes in order to occur at significant rates. Enzymes are selective for their substrates and therefore catalyze only a few reactions from among many possibilities.

Like all catalysts, enzymes work by lowering the activation energy for a reaction. This is illustrated in Fig. 1.

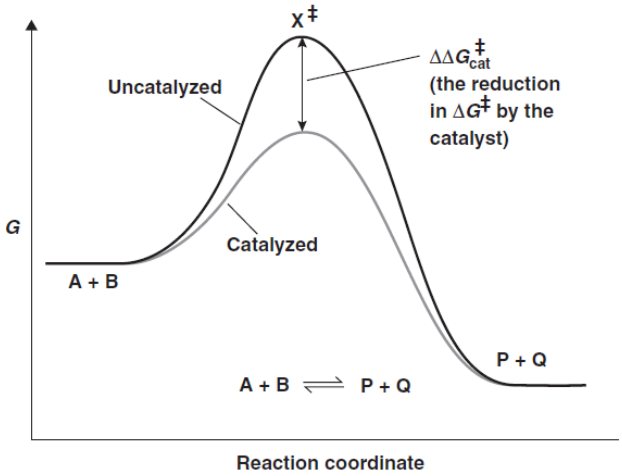

Fig. 1. Lowering the activation energy of a reaction.

Catalysts, like enzymes, act by lowering the energy difference between the reactants (A, B) and the transition state. This lowers the activation barrier for the reaction, allowing it to proceed more rapidly.

Since lowering of the kinetic barrier also accelerates the reverse reaction, the equilibrium of the reaction remains unchanged. As with all catalysts, enzymes are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other catalysts by being much more specific. Although almost all enzymes are proteins, not all biochemical catalysts are enzymes, since some RNA molecules called ribozymes also catalyze reactions.

CLASSIFICATION OF ENZYMES

Enzymes can be classified by the kind of chemical reaction catalyzed. Officially, six groups of enzymes have been classified:

- **Oxidoreductases**: catalyze oxidation/reduction reactions which generally involve the transfer of electrons. Examples are oxidases or dehydrogenases.

- **Transferases**: transfer a functional group (e.g. a methyl or phosphate group) and these generally involve the transfer of a radical. Examples are: transglycosidases, e.g. of monosaccharides; transphosphorylases and phosphomutases, e.g. of a phosphate group; transaminases, e.g. of an amino group; transmethylases, e.g. of a methyl group; and transacetylases, e.g. of an acetyl group.
- **Hydrolases**: catalyze the hydrolysis of various bonds. The hydrolase reaction generally involves addition or removal of water. Examples are: hydrolases, including esterases, carbohydrases, nucleases, deaminases, amidases and proteases; hydrases such as fumarase, enolase, aconitase and carbonic anhydrase.
- **Lyases**: cleave various bonds by means other than hydrolysis and oxidation. This reaction involves the splitting or forming a C=C bond. Examples are desmolases.
- **Isomerases**: catalyze isomerization changes within a single molecule and involve changing the geometry or structure of a molecule. An example is glucose-isomerase.
- **Ligases**: join two molecules with covalent bonds.

Chemistry of enzymes

Enzymes are generally globular proteins, having a size range from just over 60 to more than 2500 amino acids, that is, a MW of ±6000–250 000. The activities of enzymes are determined by their three-dimensional structure.7 Most enzymes are much larger than the substrates they act on. It is therefore even more remarkable that only a small part of the enzyme molecule is directly involved in catalysis. This small section is called the active site and this site usually contains not more than a few (3–4) amino acids which are directly involved in the catalytic process. The substrate is normally bound by the enzyme in close proximity to, or even in, the active site.

Specificity of enzymes

One of the most relevant and also intriguing properties of enzymes is their specificity. Some enzymes exhibit absolute specificity. This means that these enzymes catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group. In general, there are four distinct types of specificity:

- **Absolute specificity**: highly specific enzymes catalyze only one reaction.
- **Group specificity**: group specific enzymes act only on molecules that have specific functional groups, such as amino, phosphate or methyl groups.
- **Linkage specificity**: such enzymes act on chemical bonds of certain nature, regardless of the rest of the molecular structure.
- **Stereochemical specificity**: stereospecific enzymes act only on a particular steric or optical isomer and not on their isomeric counterparts.

The specificity of enzymes is determined by complementary shape, charge, hydrophilic/hydrophobic characteristics of the substrates and their three-dimensional organization. The three-dimensional interaction has been described in various interaction models. The two most relevant are described.

1. 'Lock and key' model

Emil Fischer suggested as early as 1894 that enzyme specificity was caused by specific complementary geometric shapes of both the enzyme and the substrate (Fig. 2). Thanks to these shapes, enzyme and substrate would fit exactly into one another. This is often referred to as 'the lock and key' model. However, while this model explains enzyme specificity, it fails to explain the stabilization of the transition state that enzymes achieve. The 'lock and key' model has proven inaccurate and the induced fit model is the most currently accepted model for enzyme–substrate–coenzyme interaction.

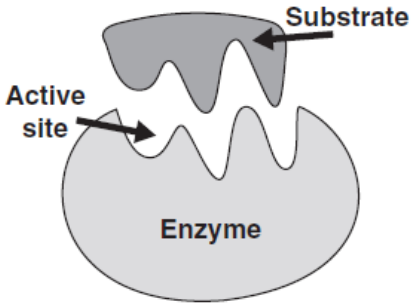

Fig. 2. Complementary geometric shapes.

2. Induced fit model

In 1958, Koshland suggested a modification to the lock and key model: since enzymes are rather flexible structures, the active site is continuously reshaped by interactions with the substrate as the substrate interacts with the enzyme. As a result, the substrate does not simply bind to a rigid active site; the amino acid side chains which make up the active site are moulded into the precise positions that enable the enzyme to perform its catalytic function. In some cases, such as glycosidases, the substrate molecule also changes shape slightly as it enters the active site. The active site continues to change until the substrate is completely bound, at which point the final shape and charge are determined.

MECHANISMS OF ENZYMES

Enzymes can act in several ways, whereby each enzyme lowers the energy needed for the reaction to occur or to proceed. These mechanisms are described briefly as follows:

- Lowering the activation energy by creating an environment in which the transition state is stabilized. This can be achieved by binding and thus stabilizing the transition-state conformation of the substrate/product molecules.
- Lowering the energy of the transition state, but without distorting the substrate, by creating an environment with the opposite charge distribution to that of the transition state.
- Providing an alternative pathway. For example, temporarily reacting with the substrate to form an intermediate enzyme–substrate (ES) complex, which would be impossible in the absence of the enzyme.
- Reducing the reaction entropy change by bringing substrates together in the correct orientation to react. Considering an energy effect (_$H‡$) alone overlooks this effect.

The enzyme–substrate complex

A theory to explain the catalytic action of enzymes was proposed by Arrhenius at the end of the nineteenth century. He proposed that the substrate and enzyme formed some intermediate transition state which is known as the ES complex. This can be schematically represented as shown in equation (1).

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \qquad \text{----- Eq. 1}$$

The existence of intermediate ES complexes has been demonstrated in the laboratory, for example, using catalase and a hydrogen peroxide derivative.

From this intermediate ES complex, the reaction proceeds with the formation of the product(s) and the enzyme then returns to its original form after the reaction is concluded.

Chemical equilibrium

Many chemical reactions do not go to true completion. Enzyme-catalyzed reactions do not form an exception to that chemical ‘law’, which is due to the reversibility of most (enzyme catalyzed) reactions.

Equilibrium is a sort of steady-state condition which is reached when the forward reaction rates equal the backward rates. Enzyme activity studies are always based on the principle of equilibrium reactions.

ENZYME KINETICS

Enzyme kinetics is a fundamental way of describing, predicting and calculating how enzymes bind substrates, turn these into products and also how fast and efficiently this is happening.

Early last century, a quantitative theory of enzyme kinetics was proposed, but the experimental data were not useful since the logarithmic pH-scale was not known yet. This scale

was introduced a little later15 together with the concept of buffering. Later on, when these early experiments were repeated, the equations were confirmed and referred to as (Henri–) Michaelis–Menten kinetics. This work was further developed and resulted in kinetic equations which are still in use.

The model for enzyme action, as first elucidated by Michaelis and Menten, suggests the binding of free enzyme to the reactant forming an enzyme-reactant complex. This complex undergoes a transformation, releasing product and free enzyme. This is schematically shown in equation (2).

$$ES \xrightarrow{K_2} E + P \qquad \text{----- Eq. 2}$$

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P \qquad \text{----- Eq. 3}$$

When reactions (1) and (2) are combined into reaction (3), a model for enzyme catalysis is obtained. First, the enzyme (*E*) and substrate (*S*) come together to form an ES complex; the reaction occurs by which the substrate is converted into the product of the reaction and then the ES complex is broken apart, yielding enzyme (*E*) plus product (*P*).

The Michaelis–Menten model assumes that only a negligible amount of ES complex reverts to reactants (i.e. $K_1 \gg K_{-1}$ in equation (1)). The rate of formation of product (shown in equation (4)) can be determined from equation (2) in the mechanism written above:

$$\text{Rate of formation of product is } K_2\,[S] \qquad \text{----- Eq. 4}$$

and the rate of formation of the intermediate ES (equation (5)) can be determined from equations (1) and (2) in the mechanism written above:

$$\text{Rate of formation of } ES = K_1[E][S] - (K_2 + K_1)[ES] \qquad \text{----- Eq. 5}$$

Using the steady-state approximation, that is, the assumption that the concentration of intermediates (ES) stays constant while the concentrations of reactants and products change, the equation for the rate of formation of the product can be calculated as follows:

$$\frac{\delta[P]}{\delta t} = \frac{K_2[E_0][S]}{[S] + K_m} \qquad \text{----- Eq. 6}$$

Here $[E_0]$ is the initial concentration of free enzyme, $[S]$ is the substrate concentration and K_m is a constant specific to a given enzyme known as the Michaelis–Menten constant. The value

of K_m relates to the rate constants shown in equations (1) and (2), as given by the following equation:

$$K_m = \frac{K_{-1} + K_2}{K_1} \qquad \text{----- Eq. 7}$$

The Michaelis–Menten constant (K_m) is very important, because it can be determined experimentally and describes the catalytic power of an enzyme. K_m can also be used to predict the rate of an enzyme-catalyzed reaction when the starting conditions (enzyme and substrate concentration) are known.

The enzyme reactions take place in two stages. In the first, the substrate binds reversibly to the enzyme, forming the ES complex. In the second reaction, the enzyme catalyzes the chemical step and releases the product.

Enzymes can catalyze up to several million reactions per second. Enzyme rates depend on solution conditions and substrate concentration. Conditions that denature the protein reduce or eliminate enzyme activity. Such conditions are high temperature, extreme pH or high salt concentrations. Raising substrate concentration tends to increase activity. To find the maximum speed of an enzymatic reaction, the substrate concentration is increased until a constant rate of product formation is seen. Saturation happens because, as substrate concentration increases, more and more of the free enzyme is converted into the substrate bound ES form. At the maximum velocity (V_{max}) of the enzyme, all the enzyme active sites are bound to substrate, and the amount of ES complex is the same as the total amount of enzyme. However, V_{max} is only one kinetic constant of enzymes. The amount of substrate needed to achieve a given rate of reaction is also important. This is given by the Michaelis–Menten constant (K_m), which is the substrate concentration required for an enzyme to reach one-half of its maximum velocity. Each enzyme has a characteristic K_m for a given substrate, and this can show how tight the binding of the substrate is to the enzyme. Another useful constant is k_{cat}, which is the number of substrate molecules handled by one active site per second.

The efficiency of an enzyme can be expressed in terms of k_{cat}/K_m. This is also called the specificity constant and incorporates the rate constants for all steps in the reaction. Because the specificity constant reflects both affinity and catalytic ability, it is useful for comparing different enzymes against each other, or the same enzyme with different substrates. The theoretical maximum for the specificity constant is called the diffusion limit and is about 10^8–10^9 (M^{-1} s^{-1}). At this point, every collision of the enzyme with its substrate will result in catalysis, and the rate of product formation is not limited by the reaction rate but by the diffusion rate. Enzymes with this property are called catalytically perfect or kinetically perfect.

Factors affecting Enzyme Activity

Knowledge of basic enzyme kinetic theory is important in order to understand the basic enzymatic reaction mechanism and to select methods for enzyme analysis. Several factors affect the rate at which enzymatic reactions proceed, such as temperature, pH, enzyme concentration, substrate concentration and the presence of any inhibitors or activators.

1. Enzyme concentration

In order to study the effect of increasing the enzyme concentration upon the reaction rate, the substrate must be present in an excess amount; that is, the reaction must be independent of the substrate concentration. Any change in the amount of product formed over a specified period of time will be dependent upon the level of enzyme present. These reactions are said to be 'zero order' because the rates are independent of substrate concentration and are equal to some constant *k*. The formation of product proceeds at a rate which is linear with time. The addition of more substrate does not serve to increase the rate. In zero-order kinetics, allowing the assay to run for double time results in double the amount of product.

The amount of enzyme present in a reaction is measured by the activity it catalyzes. The relationship between activity and concentration is affected by many factors such as temperature, pH, etc. Highest enzyme activity is generally measured when substrate concentration is unlimiting.

2. Substrate concentration

It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, the reaction velocity will increase until it reaches a maximum. After this point, increases in substrate concentration will not increase the velocity. It is theorized that when this maximum velocity is reached, all of the available enzyme is converted to ES, the ES complex. Michaelis constants have been determined for many of the commonly used enzymes. The size of *K*m tells us several things about a particular enzyme:

i. A small K_m indicates that the enzyme requires only a small amount of substrate to become saturated. Hence, the maximum velocity is reached at relatively low substrate concentrations.
ii. A large K_m indicates the need for high substrate concentrations to achieve maximum reaction velocity.
iii. The substrate with the lowest *K*m upon which the enzyme acts as a catalyst is frequently assumed to be enzyme's natural substrate, though this is not true for all enzymes.

3. Allostery

Allostery or allosteric regulation is the regulation of an enzyme or other protein by binding an effector molecule at the protein's allosteric site. The allosteric site is a site other than the active site of the enzyme. Effectors that enhance the enzyme's activity are referred to as allosteric activators, whereas those that decrease the protein's activity are called allosteric inhibitors.

Following this mechanism, allosteric inhibition is a form of non-competitive inhibition. The term allostery comes from the Greek allos, 'other', and stereos, 'space', referring to the regulatory site of an allosteric protein being separate from its active site. Allosteric regulation is a natural example of feedback control.

4. Cofactors

Many enzymes require the presence of other compounds, called cofactors, which are needed in order to demonstrate their catalytic activity. This entire active complex is referred to as the holoenzyme; that is, the apoenzyme (protein portion) plus the cofactor (coenzyme, prosthetic group or metal-ion-activator) together is called the holoenzyme.

A cofactor may be:

i. a coenzyme – a non-protein organic substance which is dialyzable, thermostable and loosely attached to the protein part;
ii. a prosthetic group – an organic substance which is dialyzable and thermostable which is firmly attached to the protein or apoenzyme portion; and
iii. a metal-ion-activator – these include K^{+}, Fe^{++}, Fe^{+++}, Cu^{++}, Co^{++}, Zn^{++}, Mn^{++}, Mg++, Ca^{++} and Mo^{+++}.

Enzymes usually bind these cofactors in or close by the active site. Furthermore, some enzymes can additionally bind other molecules than substrates or cofactors and these are often molecules inhibiting the enzyme or are interfering with the catalytic process. In this way the enzymatic reaction can be regulated by the cell. Some enzymes do not need any additional components to show full activity. However, others require non-protein molecules called cofactors to be bound for activity. Cofactors can be either inorganic (e.g. metal ions or iron–sulphur clusters) or organic compounds, (e.g. flavin or haem). Organic cofactors can be either prosthetic groups, which are tightly bound to an enzyme, or coenzymes, which are released from the enzyme's active site during the reaction. Coenzymes include NADH, NADPH and ATP. These molecules act to transfer chemical groups between enzymes.

The tightly bound cofactors are usually found in the active site and are involved in catalysis. For example, flavin and heme cofactors are often involved in redox reactions.

5. Coenzymes

Coenzymes are small organic molecules that transport chemical groups from one enzyme to another. Some of these chemicals such as riboflavin, thiamine and folic acid are vitamins. Such compounds cannot be made in the body and must be acquired from the diet. The chemical groups carried include the hydride ion (H^{-}) carried by NAD or $NADP^{+}$, the acetyl group carried by coenzyme A, formyl, methenyl or methyl groups carried by folic acid and the methyl group carried by *S*-adenosylmethionine.

Since coenzymes are chemically changed as a consequence of enzyme action, it is useful to consider coenzymes to be a special class of substrates, or second substrates, which are common to many different enzymes. For example, about 700 enzymes are known to use coenzyme NADH.

Coenzymes are usually regenerated and their concentrations maintained at a steady level inside the cell.

6. Inhibitors

Enzyme inhibitors are substances which alter the catalytic action of the enzyme and consequently slow down, or in some cases, stop catalysis.

Most theories concerning inhibition mechanisms are based on the existence of the ES complex. Substrate inhibition will sometime occur when excessive amounts of substrate are present.

There are three common types of enzyme inhibition – competitive, non-competitive and substrate inhibition. Besides these inhibitor types, a mixed inhibition exists as well.

6.1. Competitive inhibitors

Competitive inhibition occurs when the substrate and a substance resembling the substrate are both added to the enzyme. The 'lock–key theory' of enzyme catalysts is used to explain why inhibition occurs.

The concept holds that one particular portion of the enzyme surface has a strong affinity for the substrate. The substrate is held in such a way that its conversion to the reaction products is more favourable. However, when an inhibitor which resembles the substrate is present, it will compete with the substrate for the position in the active site. When the inhibitor is bound to the active site of the enzyme, it blocks this position in the active site, but is not converted by the enzyme. In this way, the active site remains blocked. Hence, the observed reaction is slowed down because a part of the available enzyme sites is occupied by the inhibitor. If a dissimilar substance which does not fit the site is present, the enzyme rejects it, accepts the substrate and the reaction proceeds normally.

In competitive inhibition, the maximal velocity of the reaction is not changed, but higher substrate concentrations are required to reach a given velocity, increasing the apparent K_m.

6.2. Uncompetitive inhibition

In uncompetitive inhibition, the inhibitor cannot bind to the free enzyme, but only to the ES complex. The EIS complex thus formed is enzymatically inactive. This type of inhibition is rare, but may occur in multimeric enzymes.

6.3. Non-competitive inhibition

Non-competitive inhibitors are considered to be substances which, when added to the enzyme, alter the enzyme in a way that it cannot accept the substrate.

Non-competitive inhibitors can bind to the enzyme at the same time as the substrate; that is, they never bind to the active site. In this way a complex is formed of enzyme, inhibitor and substrate (EIS). Both the EI and EIS complexes are enzymatically inactive. Because the inhibitor cannot be driven from the enzyme by higher substrate concentration (in contrast to competitive inhibition), the apparent V_{max} changes. But because the substrate can still bind to the enzyme, the K_m stays the same.

6.4. Mixed inhibition

This type of inhibition resembles the non-competitive, except that the EIS complex has residual enzymatic activity.

In many organisms, inhibitors may act as part of a feedback mechanism. If an enzyme produces too much of one substance in the organism, that substance may act as an inhibitor for the enzyme at the beginning of the pathway that produces it, causing production of the substance to slow down or stop when there is sufficient amount. This is a form of negative feedback. Enzymes which are subject to this form of regulation are often multimeric and have allosteric binding sites for regulatory substances. Irreversible inhibitors react with the enzyme and form a covalent adduct with the protein.