ENZYMES: THE BIOLOGICAL CATALYSTS OF LIFE

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Summary

The function of catalysts is to speed up reactions without becoming a part of the reaction products. Enzymes, the biological catalysts are highly specific, catalyzing a single chemical reaction or a very few closely related reactions. The exact structure of an enzyme and its active site determines the specificity of the enzyme. Substrate molecules bind themselves at the enzyme's active site. Substrates initially bind to the enzymes by non-covalent interactions, including hydrogen bonds, ionic bonds and hydrophobic interactions. Enzymes lower the activation energy and the reactions proceed toward equilibrium more rapidly than the uncatalyzed reactions. Both prokaryotic and eukaryotic cells commonly use allosteric regulation in responding to changes in conditions within the cells. Allosteric regulation can be positive or negative. Regulation by allosteric inhibitors is common in many biosynthetic pathways. A protective peptide in zymogens regulates by inactivating the protein. Zymogens are proteolytically activated. Catalytic activity of some enzymes may be regulated by cofactors. Metal ions or other small molecules serve as the cofactors. Some enzymes contain tightly bound cofactors termed prosthetic groups. Protein degradation is also

one central way to regulate the enzyme levels. Hundreds of enzymes are commercially available. Some of these have increasing importance in industry and in medical and clinical applications.

1. Introduction

Enzymes are proteins functioning as catalysts that speed up reactions by lowering the activation energy. A simple and succinct definition of an enzyme is that it is a biological catalyst that accelerates a chemical reaction without altering its equilibrium. During the reactions the enzymes themselves undergo transient changes. In the overall process, enzymes do not undergo any net change. The enzyme catalysts regulate the structure and function of cells and organisms. They catalyze the synthesis and breakdown of biochemical building blocks and macromolecules, the transmission of genetic information, the transport of compounds across the membranes, motility of organisms and conversion of chemical energy. Enzyme catalysis is essential for making biochemical reactions proceed at appropriate speed in physiological conditions. They speed up the reactions in the cells so that they may occur in fractions of seconds. In the absence of catalysts most cellular reactions would not occur even over time periods of years. Without rapid cellular reactions life in its present form would not be possible. One characteristic feature of enzymes is their specificity. Thus each reaction in the cell is catalyzed by its own, specific enzyme. The substances that are acted upon by enzymes are **substrates**. Substrate can be a small molecule or a macromolecule like an enzyme itself. For example, trypsin is the enzyme that uses polypeptides as the substrate and hydrolyses the peptide bonds.

The exact number of different enzymes in various cells is not known. However, because the number of different reactions in the cells of higher eukaryotes is in the tens of thousands, the number of different enzymes in the cells has to be in the same scale. Based on results of the genome sequencing projects, the estimated number of enzymes in the cells is now much more accurate than before these sequencing projects were initiated. For example, in an *Escherichia coli* cell, there are roughly 4300 proteins and almost 3000 of them are enzymes. Mammals have more than ten times the number of proteins and enzymes than there are in *E. coli*.

Enzymes have been utilized for thousands of years in microbial processes. Microbes and their enzymes have been applied for preparation of wines, beer, cheeses and other milk products. The role of enzymes in the fermentation process has been known for less than two hundred years. In the 1850s Louis Pasteur presented a theory that sugar is converted into ethanol in yeast by "ferments". He also concluded that these ferments are inseparable from the living yeast cells. At the end of the eighteenth century, more information on the nature of fermentation was obtained when Buchner was able to ferment using a yeast filtrate. Processes for the isolation of enzymes and studies of their properties were ready to start.

The ultracentrifugation technique was applied for enzyme studies in the 1920s. These studies revealed the physical nature of the enzymes. Solving the amino acid sequence of ribonuclease gave the enzyme structure in chemical terms. The 3-dimensional structure of lysozyme was solved in 1965 by using X-ray diffraction analysis. On the basis of the

3-dimensional structure, the significance of the active center in the action mechanism was postulated. Today thousands of amino acid sequences of proteins are known. Equally, the number of known 3-dimensional protein structures is thousands. Most proteins have tight globular structure, and they contain one or several **subunits.** However, enzymes show considerable flexibility. Some are regulated by small molecules (**effectors**). In the cells there are small molecules, often end products of the enzyme reaction, which regulate the level of enzyme activity. This is called **allosteric regulation**. Allosteric regulation is achieved by conformational changes in protein structure and subsequent changes in activity.

Development of recombinant DNA technology in the 1970s had enormous impact in understanding of protein structure/function relationship. By applying gene manipulation it was possible to study in a rational manner amino acid residues involved in protein stability, substrate binding, enzyme catalysis and subunit interaction. It also facilitated in developing methods for protein purification and even opened new views for designing proteins with desired structure and properties. One example of the power of the techniques is changing of lactate dehydrogenase to malate dehydrogenase. By site directed mutagenesis, certain amino acid residues were changed in the active center resulting in change in substrate specificity and catalysis.

Most biological catalysts are proteins, but not all. RNA can also act as a catalyst in RNA hydrolysis speeding up the reaction up to 10^{11} -fold. These catalysts are called **ribozymes.** Small ribozymes containing approximately 30 nucleotides are called hammerhead ribozymes. They catalyze hydrolysis of the phosphodiester bonds. Also antibodies which are not "classical enzymes" catalyze reactions via a high-energy transition state.

2. Enzymes as Biological Catalysts

In the enzyme catalyzed reaction, the substrate is changed to the product via a high energy transition state. The state has a very short life cycle and it is stabilized by the enzyme. For an enzyme catalyzing a simple reaction from substrate to product the overall reaction can be written in two steps:

$$E + S \Longleftrightarrow ES \qquad (1)$$
$$ES \longrightarrow E + P$$

An example of this type of reaction is decomposition of hydrogen peroxide into water and oxygen. Although the reaction is strongly favored thermodynamically, it is very slow unless catalyzed. Catalase increases the uncatalyzed rate of H_2O_2 decomposition. In the presence of catalase, the decomposition of H_2O_2 occurs 10^8 times faster than in the absence of catalase. Indeed, enzymes may increase the rate of a reaction as much as 10^{17} -fold. Reactions required to digest food, send nerve signals or contract muscle simply do not occur at a useful rate without enzymes. Enzymes provide a specific environment within which a given reaction is energetically more favorable.

2.1. Factors Affecting Activity

Generally, an increase in temperature increases the activity of enzymes. Because enzymes function in cells, the optimum conditions for most enzymes are moderate temperatures. At elevated temperatures, at a certain point activity decreases dramatically when enzymes are denatured. Purified enzymes in diluted solutions are denatured more rapidly than enzymes in crude extracts. Incubation of enzymes for long periods may also denature enzymes. It is more suitable to use short incubation time in order to measure the initial velocities of the enzyme reactions. The International Union of Biochemistry recommends 30 ^{o}C as the standard assay temperature. Most enzymes are very sensitive to changes in pH. Only a few enzymes function optimally below pH 5 and above pH 9. The majority of enzymes have their pH-optimum close to neutrality. The change in pH will change the ionic state of amino acid residues in the active site and in the whole protein. The change in the ionic state may change substrate binding and catalysis. The choice of substrate concentration is also crucial because at low concentrations the rate is dependent on the concentration, but at high concentrations the rate is independent of any further increase in substrate concentration.

2.2 Active Site

Enzymatic catalysis relies on the action of amino acid side chains arrayed in the active center. Enzymes bind the substrate into a region of the **active site** in an intermediate conformation (see Figure 1).

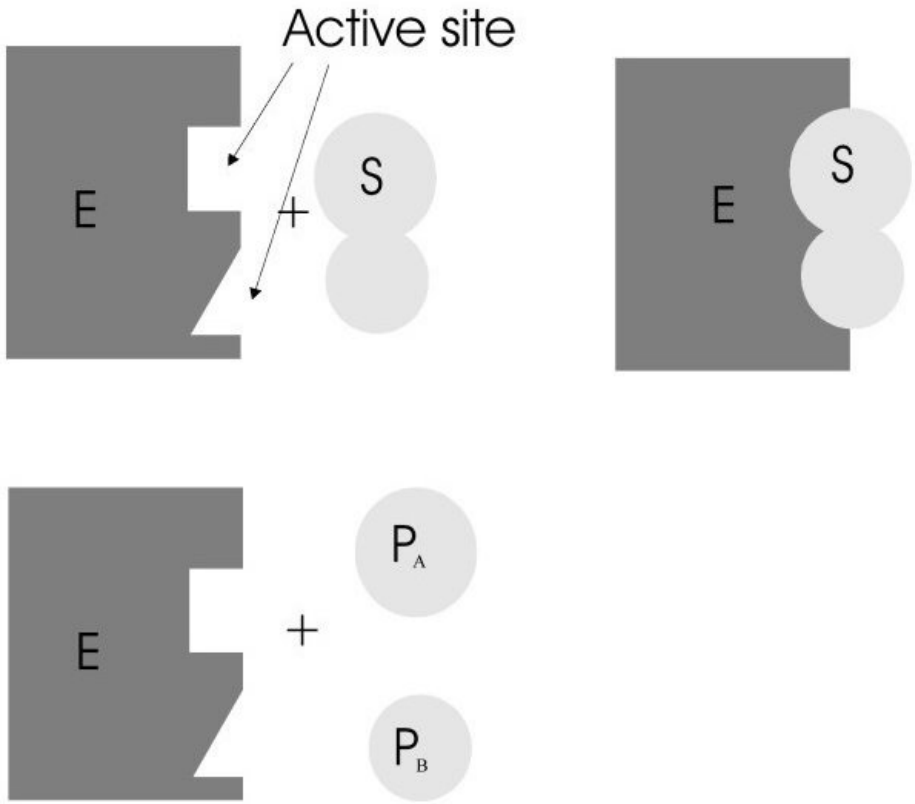

Figure 1. The induced-fit model of interaction between enzyme and substrate

The active site is often a pocket or a cleft formed by the amino acids that participate in substrate binding and catalysis. The amino acids that make up the active site of an enzyme are not contiguous to one another along the primary amino acid sequence. The active site amino acids are brought to the cluster in the right conformation by the 3-dimensional folding of the primary amino acid sequence. Of the 20 different amino acids that make up protein, the polar amino acids, aspartate, glutamate, cysteine, serine, histidine and lysine have been shown most frequently to be active site amino acid residues. Usually only two to three essential amino acid residues are directly involved in the bond leading to product formation. Aspartate, glutamate and histidine are the amino acid residues that also serve as the proton donors or acceptors.

The active site is surrounded by the amino acid residues that help substrate binding. The activation energies for formation of the transition state and for conversion of the intermediate to the product are lower than the activation energy for the uncatalyzed reaction. Most enzymes are composed of several hundreds of amino acids. The folding of the long amino acid sequence gives the protein some crucial properties. The active site pocket is very specific, protected by the surrounding polypeptide chain (α-helix or β-sheet structure). The enzyme and its catalytic cleft is very flexible allowing its high catalytic power.

Some enzymes contain a non-protein component called a **prosthetic group**. The prosthetic group is similar to coenzymes in function, but they are more tightly bound to the enzyme. Heme-iron is a prosthetic group e.g. in catalase and functions as the electron acceptor. The heme-iron prosthetic group is located at the active site of catalase and is indispensable for the catalytic activity of the enzyme.

2.3 Enzyme Kinetics

Kinetic analysis can be applied to characterize enzyme-catalyzed reactions by using unpurified or purified enzyme preparations. The usual way to measure the rate of an enzymatic reaction is mix the enzyme with the substrate and record the formation of the product or disappearance of the substrate at close to neutral pH and at temperatures from 25 to 37 ^{o}C. The formation of a product or disappearance of a substrate is generally measured continuously as a function of time. Very rapid reactions can be measured by using special "stopped flow" devices. They make it possible to follow kinetic measurements within 1 ms after mixing the substrate and the enzyme.

The enzyme catalyzed reaction rates usually increase linearly with the substrate concentration at low concentrations. At higher concentrations the reaction rate becomes independent of the substrate concentration (see Figure 2).

At the saturation level, enzyme molecules have their active sites occupied. All the enzyme is in the form of the ES complex. The product is made at the maximal rate (**V_{max}**) depending only on the rate at which the substrates are converted to products. The rate of product formation can be written as:

$$v = \frac{V_{max}\,[S]}{\qquad\qquad} \qquad (2)$$

$K_m + [S]$

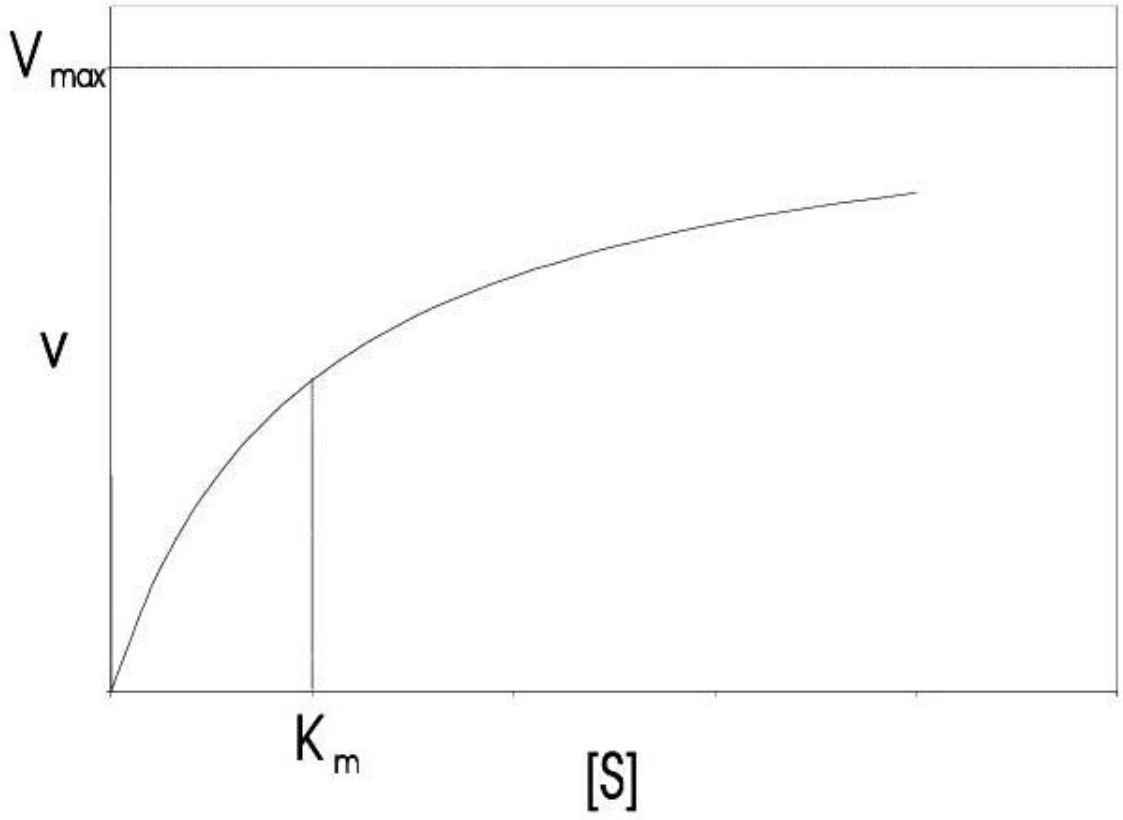

Figure 2. The reaction velocity **v** as a function of the substrate concentration **[S]** for an enzyme catalyzed reaction. At saturating substrate concentration the reaction velocity reaches $\mathbf{V_{max}}$. $\mathbf{K_m}$ is the substrate concentration where the reaction velocity is half maximal.

This equation is known as the Michaelis-Menten equation. The value of V_{max} for any enzymatic reaction varies with the enzyme concentration. The more enzyme molecules are present the faster the reaction will proceed at saturation. In other words, V_{max} provides information about the enzyme's catalytic efficiency. Each enzyme has a typical **turnover number**. This number refers to the number of substrate molecules that the enzyme molecule saturated with the substrate can convert to product per unit time. The turnover numbers of the most efficient enzymes are several hundred thousands and the less efficient enzymes have turnover numbers less than one molecule per second. For example, catalase has a turnover number $4x10^7$ and lysozyme 0.5 molecules per second. The constant $\mathbf{K_m}$ is called the Michaelis constant. It determines the substrate concentration at which the velocity is half maximal. The Michaelis constant is a measure of the affinity of an enzyme for a substrate and the value of the constant varies over a wide range but generally falling in the range of 10^{-6} to 10^{-1}. Enzymes with large K_m values show a reluctance to dissociate from the substrate. These enzymes are generally less active than the enzymes with low K_m values. Enzymes that catalyze reactions involving more than one substrate have K_m values for each substrate.

In most cases, more than one intermediate is involved. In the hydrolysis reaction of ester catalyzed by chymotrypsin an acyl-enzyme intermediate is formed. The overall reaction can be written:

$$E + S \Longleftrightarrow ES \Longleftrightarrow ES' \longrightarrow E + P \qquad (3)$$

In some cases there is a decrease in reaction rate at high substrate concentrations. The decrease is known as the substrate inhibition. The decrease may be due to the presence of multiple substrate binding sites. The sites have different affinity to substrate.

Enzyme inhibitors may give information on the active site. **Competitive inhibitors** are reversible inhibitors that alter the K_m but not V_{max}. Structurally similar inhibitor and substrate bind to the same site (the active site) in the enzyme and the inhibitor inhibits competitively the enzyme. The inhibitor is prevented from binding if the active site is already occupied by the substrate. A practical example of competitive inhibition is the sulfa drug. The effective substance of the sulfa drug, sulfanilamide, resembles structurally p-aminobenzoic acid and inhibits metabolism of p-aminobenzoic acid. The inhibition prevents the production of folic acid. The shortage of folic acid causes death of bacterial cells. A general kinetic equation for competitive inhibition is as follows:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_{ES}}{V_{max}}\left[1 + \frac{[I]}{K_{EI}}\right]\frac{1}{[S]} \qquad (4)$$

The effect of a competitive inhibitor can be seen on a Lineweaver-Burk plot of kinetic data (see Figure 3).

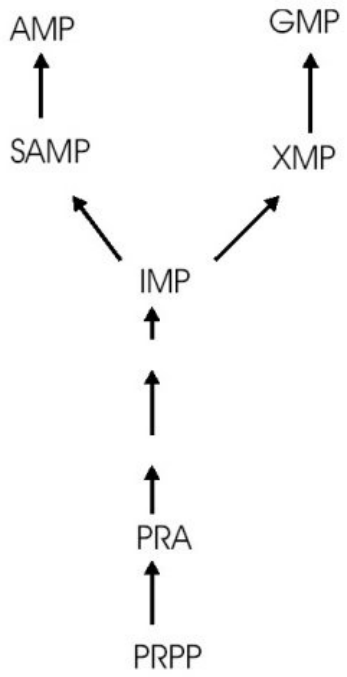

Figure 3. Double-reciprocal plot of reaction velocity vs. substrate concentration. The Lineweaver-Burk plot demonstrates the effect of a competitive inhibitor

Some inhibitors bind non-covalently and reversibly at the sites other than the active site. These inhibitors are called **non-competitive inhibitors**. They reduce the enzyme's turnover number and thus decrease V_{max} while K_m remains unaffected. Because non-competitive inhibitors do not resemble substrates and have their own binding sites, they usually act on broad spectrum of enzymes. For example, magnesium ion containing enzymes are **non-competitively** inhibited by a chelator, EDTA.

There are numerous inhibitors that react essentially irreversibly with enzymes. They are toxic substances that bind covalently to enzymes. The **irreversible inhibitors** generally form a covalent bond with a functional amino acid residue in the enzyme. These inhibitors may change either V_{max} or K_m or both. The irreversible inhibitors often provide clues to the active site residues of the enzyme. An example of an inhibitor of this group is diisopropyl fluorophosphate (DFP). DFP reacts irreversibly with serine hydroxyl groups of proteins. DFP very efficiently inhibits acetylcholinesterase and serine proteases.

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Biographical Sketches

Pekka I. Mäntsälä was born in February 1937.

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He is currently Professor in biochemistry at the University of Turku, and his fields of research are combinatorial biosynthesis, hybrid antibiotics, secondary metabolism, protein structure-function relationship, and purine nucleotide biosynthesis.

Dr. Jarmo Niemi was born 1958 in Pori, Finland. He graduated from the University of Turku in 1983 in biochemistry and has worked as a researcher and research manager at Leiras Pharmaceuticals 1983-1991, and as an instructor at the Department of Biochemistry and Food Chemistry at the University of Turku, where he completed his Ph.D. in 1998. He also worked as a researcher at the Turku Centre of Biotechnology in 1995-1999. His current research project involves enzymes participating in anthracycline antibiotic biosynthesis.