are in some phase of clinical trials and some others have been forwarded for approval. Some of these products are listed in table 1.3 separately.

After considering all the factors it would be fair to state that biotechnology in the last few decades has become a part of pharmaceutical development. The recent decade in particular has seen some unprecedented developments in this field. Further, the developments will have an impact on the practice of medicine and on the pharmaceutical industry. Although this impact at present is unpredictable, exciting pathway are bound to open up carrying us into the twenty-first century.

Table 1.2 : Biotechnological drugs and vaccines tested and approved until 1992

S. No.	Generic Name	Product Name	Company	Approval Date
1.	Human insulin	Humulin	Eli Lilly	Oct., 1982
2.	Sometrem	Protropin	Genetech	Oct., 1985
3.	DigoxinImmune Fab	Digibind	Burroughs Wellcome	April, 1986
4.	Muromonab CD3	Orthoclone OKT3	Ortho Biotech	June 1986
5.	Interferon-α-2b	Intron A	Schering-Plough	June 1986
6.	Interferon-α-2a	Roferon-A	Hoffmann-La-Roche	June 1986
7.	Hepatitis-B-vaccine	Recombivax HB	Merk	July 1986
8.	Somatotropin	Humatrope	Eli Lilly	March 1987
9.	Alteplase	Activase	Genetech	Nov., 1987
10.	Heamophilus-B-conjugate vaccine	Hib Titer	Praxis Biologics	Dec., 1988
11.	Epoietin-α	Epogen	Amgen	June 1989
12.	Hepatitis-B-vaccine	Engerix-B	SmithKline Beecham	Sept., 1989
13.	Interferon-α-n3	Alferon N	Interferon Sciences	Oct., 1989
14.	Interferon-γ-Ib	Actimmune	Genetech	Dec., 1990
15.	Filgrastim	Neupogen	Amgen	Feb., 1991
16.	Epoitin-α	Procrit	Ortho Biotech	Feb., 1991
17.	Sargramostim	Prokin	Hoechst-Roussel	March 1991
18.	Sargramostim	Leukin	Immunex	March 1991
19.	Aldesleukin	Proleukin	Cetus	June, 1992

1.4. GMP COMPLIANCE AND BIO-PHARMACEUTICAL FACILITIES

Recent years have witnessed an explosive growth in the biotechnology industry in the area of development and manufacture of a variety of new products. Most of the interest has been focused on the development of biopharmaceutical products such as vaccines, therapeutic proteins and monoclonal antibodies.

The development activity has reached a point where companies are commercializing their products. Being the most developed country, in USA alone companies such as Genetech, Amgen, Centocor and others have brought products into the market. Some other companies have contracted with them around the globe for designing and construction of manufacturing facilities for these products in compliance with the FDA and cGMP. Most of the biopharmaceuticals being developed are derived from the application of recombinant DNA technology. For regulatory purpose, most of these products are classified as **biologicals**. It is important to recognize that United States FDA has two main regulatory groups with the responsibilities for different therapeutic products.

In most of the countries of the world and especially in Indian based concerns the regulatory considerations applicable are very similar to those in the FDA of United States. The Center for Drug Evaluation and Research (CDER) is concerned with the traditional products that are produced by fermentation or organic synthesis and which are readily characterized with well defined analytical methods. These include antibiotics, analgesics, anti-inflammatory agents, etc. Most of the recombinant therapeutic products with the exception of insulin are regulated by the Center for Biologics Evaluation and Research (CBER). Traditionally, CBER was responsible

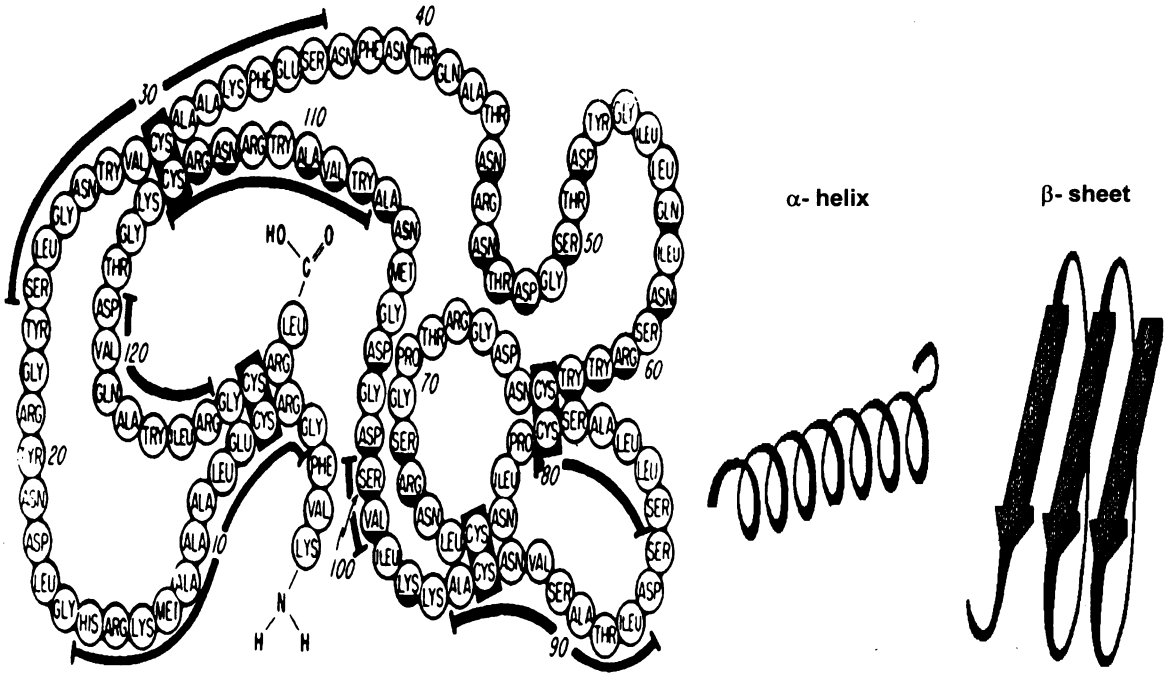


Fig. 2.2 : Primary structure of lysozyme

2.3.2. Tertiary structure

The overall folding of the polypeptide chain, which includes the organized secondary structure as well as random stretches, is attributed to the tertiary structure (Fig. 2.3). At present one of the most reliable and powerful methods to determine the three dimensional structure of a protein, and in combination with the primary sequence, for portraying the relative stereochemical positions of the atoms is X-ray crystallography. X-rays are utilized because their wavelength and molecular dimensions in the protein crystal are of the same order of size.

The enzymes listed in table 2.4 that catalyze dissimilar reactions have been found to possess unique tertiary structures, but several generalizations can be afforded due to the accumulated atomic details. In spite of the regional flexibility, all the compactly folded molecules have very little space inside to accommodate even small water molecules. This interior mainly consists of hydrophobic side chains (of leucine, phenylalanine, tryptophan, valine, etc.) and is surrounded by the polar amino acids (arginine, aspartic acid, etc.). The enzyme involved in catalyzing similar reaction types and possessing homology in their primary structures, especially in the sequence of their non-polar residues (the aggregation of which provides the driving force for protein folding) also possess three-dimensional homology. For instance, all the members of the serine proteases family have tertiary conformations with a common hydrophobic core.

A second type of localized three-dimensional homology, that is not evident from amino acid sequence data, has been observed with enzymes binding similar cofactors. For example, tertiary structures of dogfish-muscle lactate DH (dehydrogenase), horse-liver alcohol DH and lobster and *B. stearothermophilus* glyceraldehyde-3-phosphate DH, all of which require NADH as coenzymes, can be divided into separate domains, i.e., polypeptide regions associated with particular functions. The two domains being : a catalytic domain which is structurally unique to each individual enzyme and a coenzyme binding domain whose construction is remarkably similar to all. The coenzyme binding domain is comprised of six parallel strands of β-plated sheet and four α-helices, and these are found arranged in the sequence β-α-β along the primary structure. The continuous amino acid sequences constituting the domain are however not located identically in the overall primary structures.

substrate concentration with a gradual approach to saturation of enzyme with substrate. There are two cardinal points in such plots.

1. K_m - the substrate concentration giving half maximum velocity.
2. V_{max} - maximum velocity toward which the rate approaches at infinitely high substrate concentration.

Michaelis and Menten suggested that much additional informations can be derived from the hyperbolic saturation curves of enzymes when they are translated into a simple mathematical form. The Michaelis and Menten equation is basically an algebraic expression of the hyperbolic curves in which the important terms are substrate concentration[S], initial velocity (V_o), V_{max} and K_m. According to early views of Henri and of Michaelis and Menten, it may be assumed that the substrate [S], combines with the enzyme, to form the complex [ES], according to the reversible reaction.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad \text{---(2.1)}$$

k_1 the velocity constant characteristic for the reaction that leads to the formation of ES
k_2 the characteristics for the dissociation of ES

The next step involves the formation of product P and the generation of free enzyme

$$ES \xrightarrow{k_3} P + E \qquad \text{---(2.2)}$$

Then $[E_t]$ represents the total enzyme concentration (the sum of free and combined enzyme). [ES] is the concentration of the enzyme-substrate complex $\{[E_t]-[ES]\}$ represents the concentration of free or uncombined enzyme [S], the substrate concentration, is ordinarily far greater than [Et], so that the amount of [S] bound by [E] at any given time is negligible compared with the total concentration S

The rate of formation of [ES] in reaction (2.1) is assessed by following equation

Rate of formation = k_1 {[conc. of free enzyme]}[conc. of substrate]

$$= k_1 \{[E_t]-[Es]\} [S] \qquad \text{---(2.3)}$$

where k_1 is the rate constant of reactions (2.1). The rate of formation of ES from E+P by reversible reaction (2.2) is very small and may thus be neglected.

The rate of breakdown of ES in reactions (2.1) & (2.2)

$$\text{Rate of breakdown} = k_2 [ES] + k_3 [ES] \qquad \text{---(2.4)}$$

in which k_2 and k_3 are the rate constants for the reverse reaction (2.1) and the forward direction of reaction (2), respectively.

When the rate of formation of [ES] equals the rate of breakdown, and the [ES] concentration remains to be constant, such state of reaction is referred as steady state.

Rate of formation of [ES] = rate of breakdown of [ES]

$$k_1\{[E_t]-[ES]\} [S] = k_2 [ES] + k_3[ES] \qquad \text{---(2.5)}$$

$$k_1 [E_t] [S] - k_1 [S] [ES] = k_2 [ES] + k_3 [ES]$$

$$k_1 [E_t] [S] = [ES] \{k_1 [S] + k_2 + k_3\}$$

therefore,
$$[ES] = \frac{k_1 [E_t] [S]}{\{k_1[S] + k_2 + k_3\}}$$

$$[ES] = \frac{[E_t] [S]}{\frac{[S] + k_2 + k_3}{k_1}} \qquad \text{---(2.6)}$$

The initial velocity, according to the Michaelis and Menten theory, is determined by the rate of breakdown of [ES] in reaction (2.2), where rate constant is k_2. Thus we have

$$V_o = k_3 [ES]$$

$$[ES] = \frac{V_o}{k_3}$$

This concept may explain why enzymes are proteins and thus much larger than most substrate molecules. The conformational changes align amino acid residues or other groups on the enzyme in the correct spatial orientation for substrate binding, catalysis, or both.

2.6.2. Proximity and orientation

Mutual orientation of reactants is a very specific property of enzymes enabling them to accelerate the conversion (to increase the reactivity of substrates) by a few orders of magnitude. The contact site of the enzyme active center binds specifically the substrate, providing thereby for their mutual orientation and approach so as to facilitate the intervention of catalytic groups. The formation of enzyme substrate complex may take place in such a way that the susceptible bond is in a close proximity to the catalytic group and also is precisely oriented, thus greatly increases the probability that the ES complex will enter the transition state (Fig. 2.12).

Orientation of two or more molecules, incapable of realization via chaotic collisions in an aqueous medium or on the surface of an inorganic catalyst, favors a drastic increase in the reaction rate. An ordered arrangement of substrates leads to a drop in entropy and, consequently, to diminution of the activation energy.

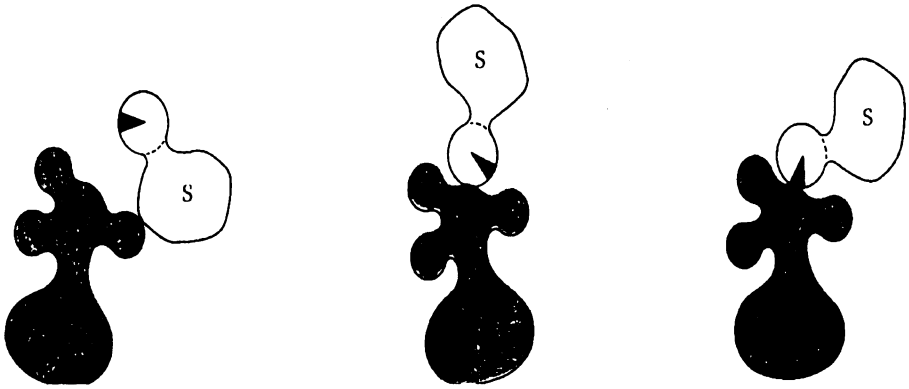
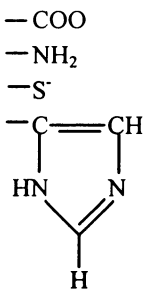


Fig. 2.12 : Diagrammatic representation of proximity and orientation

2.6.3. General acid-base catalysis

The active site of an enzyme possesses functional groups of specific amino acid residues capable of acting both as an acid (good proton donors) and as a base (good proton acceptors). When the substrate is anchored at the active site, its molecule becomes liable to the influence of electrophilic and nucleophilic groups of the catalytic site, which results in an electron density redistribution in the substrate molecule regions accessible to attack by acid base groups. Such general-acid or general-base groups are powerful catalysts for many organic reactions in aqueous systems. Histidine exhibits clearly defined acid-base properties. Blocking of the histidine residue entails the enzyme inactivation. The acid-base catalysis is typical of hydrolases, lyases and isomerases.

a. Some proton-donating groups



b. Some proton-accepting groups