SOME ASPECTS OF DESIGN AND ANALYSIS OF BIOLOGICAL SEPARATION PROCESSES

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TO

MY PARENTS

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DECLARATION

Certified that the work incorporated in the thesis **"Some Aspects Of Design And Analysis Of Biological Separation Processes"** submitted by **Ujwal P. Shinde** was carried out by the candidate under my supervision. Such materials as has been obtained from other sources has been duly acknowledged in the thesis.

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(B.D. KULKARNI)
Research Guide

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SYNOPSIS OF THESIS

The breathtaking discoveries of today's biology not only revolutionize our views about living things but also make a profound impact on science and engineering. Genetic engineering, which came into existence about 10-15 years ago, has made it possible to change in a purposeful way the machinery of inheritance to *design the living matter*. This recent upsurge of interest in biotechnology has aroused renewed consideration of the range of products of commercial value that might be produced from biological systems. The techniques by which such products are produced and isolated from organisms often is the major bottleneck in their application to modern industry. A major task of today's science is to expand and deepen basic research and make it's findings work in practical fields. With extensive utilization of experimental methods, simulation studies and system analysis may promote biology to the rank of an exact science. In the present thesis, an emphasis on some of these points has been made.

Affinity separations utilizing immobilized antibodies is a technique that is now being used for the purification of proteins for use as pharmaceuticals or in other applications where the purity of the product is an important consideration. The technique involves the immobilization of antibodies to inert supports in order to form biospecific adsorbents that can be used in purification processes. The biospecific adsorbents that have a high affinity for a single compound are ideally suited for the purification of proteins from a variety of biological sources.

Chapter one introduces the subject matter of the thesis. The literature on the various topics covered is reviewed in order to justify the work presented. Briefly, the problems associated with the purification of proteins on a large scale have been presented and highlights of the potential of affinity separations as industrial unit operations for this purpose are discussed. The reasons for using immobilized antibodies, their advantages and disadvantages are also discussed.

In chapter two, details of procedures by which polyclonal antibodies against a protein (Galactosyltransferase) are raised, purified and characterized are presented. These antibodies are then immobilized and used to study the process of deactivation of the adsorbed antibodies.

Chapter three presents an analysis of antibody deactivation. The effect of deactivation over a large number of cycles for various types of reactors has been studied in order to give clues for designing reactors using immobilized antibodies for large scale separations.

Chapter four presents a mathematical model for checking the feasibility of continuous affinity separations using a magnetically fluidized bed.

Chapter five presents the use of novel matrix for immobilization of a hydrophobic antigen rice prolamin and using it to estimate easily but accurately the content of prolamin in rice.

The processes by which antibodies and other different secretory proteins are made to cross the membrane barrier in cells are discussed in chapter six. Details of the signal that are recognized by the translocation apparatus are discussed.

Chapter seven presents detailed analysis of structural aspects of signal peptides. Based on this analysis, a mechanism has been postulated for the initiation of protein export across membranes.

Chapter eight comprises aspects of specific degradation of signal peptide inside the lipid bilayer of the membrane. A detailed analysis of differences existing between transmembrane sequences and signal peptides has helped to identify regions which may be playing a vital role in specificity of signal peptide peptidases.

Application of the above analysis to *Escherichia coli* has been undertaken in chapter nine. Regions in Protease IV, a signal peptide peptidase, have been identified by topological studies on the probable membrane spanning regions of this protein. A model for protein degradation in the membrane has been postulated.

Membranes are known to be fractal in nature. Each component of the membrane, namely the different types of lipids and proteins, constitute a part of the fractal shape. These components are mobile in shape leading to a time based evolution of the fractal shape, however, maintaining the

over all fractal dimension. The effects of *mobile shapes of fractals* on biological processes and its relevance to processes often encountered in other disciplines of science and engineering have been evaluated in chapter ten.

Chapter eleven finally concludes the thesis giving a concise summary of the results obtained and brings out the importance of the work. In the end, a brief discussion on further research that should be carried out is given.

CHAPTER 1

INTRODUCTION

CHAPTER 1

1.1   Introduction

The range of compounds that can be produced from biological systems extends from molecules such as ethanol to large complex proteins with enzymatic activity. Recent advances in the techniques of genetic engineering have resulted in the creation of microorganisms capable of making proteins that are of considerable interest for use as pharmaceuticals and medical diagnostic reagents. Large scale purification of such macromolecular compounds to a very high degree of purity, which is essential for them to be used as pharmaceuticals, is a major difficulty often encountered by bio-chemical engineers (Horstmann and Chase, 1989). So, in the next section, we shall take a look at the problems in large scale protein purification.

1.2   Problems in Large Scale Protein Purification

Biological activity of a protein is critically dependent on its three-dimensional conformation which in turn is a function of the physical and chemical properties of its environment (Stryer, 1988). Many proteins irreversibly lose their activity after exposure to high pH and temperature. Most of the well established chemical engineering operations that have been successfully adapted for the isolation of low molecular weight compounds (Eg. Antibiotics) are not suitable for purification of proteins. Proteins cannot be separated or concentrated by distillation and the techniques involving solvent extraction or fractional crystallization have been usefully applied only in very few cases. Unless great care is taken through the entire procedure of protein purification, the conditions involved will result in an irreversible change in the structure of the protein causing a loss in biological

activity and corresponding commercial value. Hence purification methods should not involve extremes of pH, exposure to organic solvents, high shearing forces and high temperatures (Chase, 1984a).

Along with the problem of stability is the problem of yield of the desired protein. Many a times the desired product present in the starting crude is typically about 0.001% (w/v) and may constitute less than 1% of the total protein (Lowe and Dean, 1974).

While considerable advances have been made using molecular biology and genetic engineering for improving the yield of the desired product, there have not been equivalent advances in downstream processing techniques. There are therefore two approaches which can be followed:

(1)   The conventional methodologies of rupturing the cell wall and separating the desired one from the thousands of proteins present (Chase, 1984a).

(2)   Genetically engineer the cell to extrude the required protein into the growth medium without the lysis of the cell wall and then separate it from a less complex mixture of proteins (Pugsley, 1988).

In the first method, lysis of the cell does not allow for its reusability and therefore cells have to be produced in large quantities and the operations generally done in batch mode. The second method poses an advantage in that it keeps the cells viable. As a result of this, the cells can be cultured continuously and will extrude the desired protein into the medium which can then be isolated from the growth medium. This process holds promise for possible automation. But even so, easy as it may seem in theory, engineering the organism to extrude the protein would involve a thorough understanding of the process of translocation of proteins across biological membranes. Furthermore, any large scale purification of the protein for its utility as a pharmaceutical would involve highly selective and efficient purification methods.

The present work deals with some of these aspects and an attempt to analyze and design such processes has been made. Initially, immunoaffinity separation methods have been studied theo-

retically and experimentally. In the latter part of the thesis, analyses has been done of the way antibodies or any other secretory proteins cross biological membranes. Effects of membrane fluidity using fractal concepts have been extrapolated to other biological processes. The potential application of protein translocation for large scale separations has also been discussed.

Biochemists have successfully used techniques such as molecular sieving, precipitation, ion exchange etc. for obtaining pure proteins. However, most of these techniques are used in conjunction with each other resulting in multi-step purification procedures. The scale up of a suitable laboratory procedure for large scale isolation of a protein requires the successful scale up of each stage which inevitably results in a complicated plant almost exclusively dedicated to a particular product (Janson 1984). A technique now frequently used at the laboratory scale is affinity separation (Cuatrecasus *et al*, 1968).

1.3   Affinity Separations

Affinity chromatography is a technique that exploits specificity in the interaction between biological molecules. These biospecific interactions range from recognition by an enzyme of its substrate to the recognition by antibodies of the particular antigen that elicited its synthesis (Cuatrecasus 1984).

The principle of the technique is elucidated as follows: The crude starting material is contacted with an immobilized phase consisting of molecules with an affinity for the desired product, firmly attached (usually covalently) to an inert insoluble support. This immobilized phase is referred to as the adsorbent. The desired product (which is called the adsorbate) will bind to the molecules attached to the support and will therefore be adsorbed to the immobilized phase. Provided that the binding is sufficiently strong, the other compounds in the crude mixture can be washed away without loss of the adsorbate. By altering the physical or chemical conditions, the adsorbate can be sub-

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sequently removed from the adsorbent. This latter stage is known as elution and the solution used is called the eluent. Following elution, the adsorbent can be prepared for use in another cycle of operation (Chase 1984).

A variety of molecules have been immobilized for use in affinity separations and these range from triazinyl dyes, enzyme inhibitors and substrate analogues to antibodies. An ideal compound should have a high affinity for its target molecule and a very low affinity for all other compounds, so that only the target molecule would be bound to the adsorbent.

1.3.1   Immobilized Antibodies in Affinity Separations

Antibodies constitute a class of proteins known as immunoglobulins and are synthesized by animals in response to the presence of a *foreign* substance in their blood. The antibodies produced will bind tightly to the molecules (known as antigens) that elicited their synthesis. Antibodies can be raised against almost all proteins foreign to the host and also against compounds of low molecular weight provided they can be rendered antigenic by prior attachment to a suitable protein (Karush 1963).

The interaction between an antigen and an antibody is thought of as being very similar to the initial binding between an enzyme and its substrate. The dissociation constants for the antigen-antibody binding reaction range from 10^{-4} M to 10^{-10} M at 25°C. The forces involved in the formation of the non-covalent complex are a combination of electrostatic and hydrophobic (apolar) interactions, van der Waals and London dispersion forces, and hydrogen bonding (Capra and Edmundsun, 1977).

The strong binding and high specificity of interactions that can occur between antigens and antibodies makes the use of antibodies highly desirable in affinity separations. The immobilization of antibodies to an inert support creates an immunoadsorbent which can be used to purify the corresponding antigen. Both monoclonal and polyclonal antibodies have been successfully used

for purification of various biological compounds. Monoclonal antibodies have several advantages over polyclonal ones. However, the production costs in case of monoclonal antibodies may be exorbitant (Lambert, 1989; Chase, 1984a).

Immobilized polyclonal antibodies have been used as bioselective sorbents since 1951 and their applications have been extensively reviewed (Hill and Hirtenstein, 1983; Lowe and Dean, 1974; Desai, 1990). Immobilized antibodies have found particular application in affinity separation where it has not been possible to devise a more conventional affinity adsorbent, either because a molecule with a suitable specific affinity is not known (Eg. Interferon purification) or a suitable molecule may be difficult to obtain in large quantities (Eg. a hormone receptor for hormone puri-fication). As the synthesis of a biospecific adsorbent by immobilizing antibodies is an expensive technique, it is likely that this type of adsorbent will only be used on large scale for the purification of high value compounds that cannot be isolated by more conventional and cheaper techniques. Polyclonal antibody systems are not readily susceptible to analysis for scale up studies (Chase *et al*, 1989).

Chapter 2 presents the techniques by which polyclonal antibodies are raised against galac-tosyltransferase, purified, characterized and quantitated. Purity of the antibodies is extremely important for there use as ligands for effecting bioseparations. These antibodies should be specific towards the desired protein to bring about a high degree of purity which is a prerequisite for its use as a pharmaceutical chemical.

1.3.2   *Factors that Affect the Performance of Immunoadsorbents*

The characteristics of the equilibrium between antigen and immunosorbent are particularly important in determining adsorption performance. Studies of equilibrium adsorption isotherms yield values of the maximum capacity of the immunosorbent in terms of the amount of antigen that can

be adsorbed per unit volume of the packed adsorbent (q_m) (Horstmann and Chase, 1989). The strength of the interaction between antigen and immunosorbent is characterized by the dissociation constant (k_d) of the adsorbed complex (Chase *et al*, 1989).

1.3.3   *Influence of the Value of the Dissociation Constant k_d*

One of the fundamental choices in the design of an immunosorbent process is the appropriate value of the dissociation constant k_d. The properties of the antibody itself have the greatest influence in determining the magnitude of this parameter and, when feasible, the most appropriate antibodies should be selected for use in immunopurification. The molecular mass of the antigen plays a major role in determining the value of k_d required when the concentration of antigen is expressed in terms of mass/volume. Such considerations are particularly important in determining whether it is feasible to use an immunosorbent procedure to remove trace levels of high molecular mass contaminants. Although very low values of k_d promote efficient adsorption and washing, the interaction between antigen and immunosorbent is often so strong that elution without denaturation of antigen and/or antibody can be difficult. During elution, it is the dissociation constant of the antigen-immunosorbent complex in the presence of eluent that governs the efficiency of elution of adsorbed antigen rather than the strength of the complex during previous stages (Chase *et al*, 1989).

1.3.4   *Factors That Affect The Capacity of Immunosorbents*

The molecular mass of the antigen plays an important role in determining the capacity of the immunosorbent for antigen. It has been shown that the maximum capacities predicted for *ideal* immunosorbents fall rapidly as the size of the antigen decreases. Hence low process throughputs (in terms of mass of antigen purified per cycle per unit volume of the bed) will be obtained when using immunosorbents for purifying small antigens. A number of reasons can be proposed to explain the poor yield of antigen binding activity for large antigens including unfavorable orientation or

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location of the immobilized antibody and steric hindrance of vacant adsorption sites by adsorbed antigen. In addition, the rates of adsorption for the small antigen are much higher than those for systems involving large antigens (Horstmann *et al*, 1986).

1.3.5   Influence of Level of Antibody Loading

Experimental data has indicated that the dissociation constant and ligand utilization efficiency does not vary with ligand concentration. The maximum capacities of immunoadsorbents were directly proportional to the amount of immobilized antibodies. The mass transfer characteristics of the low-loaded preparations were much better than those of high-loaded preparations. The observation that kinetic properties are substantially improved at low loadings of immobilized antibody would suggest that use of such materials would enable separations to be run at faster flow rates with shorter cycle times. However, larger beds must be used to obtain the same total bed capacity. The use of larger beds would, in general, be undesirable for preparative purposes as it would result in increased use of wash and eluent buffers and the product would be eluted at lower concentrations. There would also be a greater risk due to nonspecific adsorption (Chase, 1984b).

Chapter 3 deals with the estimation of certain parameters that characterize the antibody-antigen interaction.

1.3.6   Methods of Antigen Desorption

Elution of the adsorbed antigens from immobilized antibodies can be a difficult stage in an immunoadsorbent separation process. Elution requires the complete dissociation of the adsorbent-adsorbate complex and, ideally, the adsorbate should be eluted in a high concentration, low volume of solution (Dunn and Chaiken, 1974).

Use of weak buffers for elution of the adsorbate is undesirable as the rate of elution in such cases is very slow and the product would be eluted in a very large volume.

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A method of elution that is generally used in affinity separations involves the exposure of the complex to a solution containing a high concentration of the ligands that were attached to the support in the synthesis of the adsorbent. Under such conditions there is competition between the soluble and immobilized ligands for the adsorbate. If the soluble ligands are in excess then the adsorbate will partition exclusively into the soluble phase which is then separated from the adsorbent. The soluble ligand is then separated from the adsorbate by exploiting the differences between the molecular size of the two species. This method is normally used when the affinity between the ligand and the adsorbate is small and the cost of the ligand is not exorbitant. In the case of immobilized antibodies it would be required to add excess of antibodies to elicit elution of the adsorbate. This would be economically unfeasible. Furthermore, removing the adsorbate from the antibodies present in the solution would be as difficult as desorbing the same from the adsorbent. Thus this method will serve no useful purpose.

In the last few years there has been considerable interest in the use of electrophoretic desorption methods for the separation of antibody-antigen complexes. The mechanism involves the migration of the adsorbate carrying an electric charge towards the appropriate electrode after dissociation from the adsorbent has occurred. The process of migration towards the electrode, however, involves repeated adsorption-desorption between the free ligands. The process of elution is therefore extremely slow. One advantage is that the elution is achieved in an extremely small volume. Application of this method to large scale systems may be unfeasible due to undesirable convections and heating effects (Morgan and Dean, 1978).

The method most frequently used to elute immunoadsorbents involves the alteration of physical and chemical properties of the solution which is in contact with the antibodies such that the avidity of the binding between the binding of the antibody and antigen is reduced. This type of elution is called as nonspecific elution.

Eluents used in non-specific elution fall into a number of categories:

(i) Those involving a change of pH. In this process, lowering the pH below 2.5, using solutions containing acetic acid, propionic acid or glycine\HCl buffer, often results in successful elution (Desai and Lyddiatt, 1990).

(ii) Protein denaturants such as urea (8M) or guanidine hydrochloride (6M) can denature some proteins in a reversible manner. They have been used as eluents at neutral pH particularly when the antigen involved is unstable at acidic pH (Desai, 1990).

(iii) Chaotropic agents or chaotropic ions reduce the strength of hydrophobic interactions which are important in determining the structure of proteins. These agents are non-specific desorbing agents and generally unacceptable as eluents for adsorbates which are of pharmacological value. The agents generally used for elution are thiocyanate ions or solutions containing iodide, chloride or magnesium ions (McLennan and Raney, 1985).

(iv) Polarity reducing agents. Hydrophilic interactions play an important role in the binding of an antigen to a antibody. Hence the use of a substance that reduces the polarity of the medium should facilitate desorption. The agents that have been successfully employed are dioxan (upto 10%) or ethylene glycol (upto 50%).

(v) Temperature effects have been exploited by workers to facilitate dissociation. It has been shown that the dissociation constant k_d for the reaction increases two-fold as the temperature increases from 4 to 43°C. This observation provides the basis for varying the strength of binding between the adsorbate and the adsorbent at various stages of the chromatographic procedure. Such methods are used when antibodies are stable at high temperatures (Chase, 1984a).

The effect of elution buffers on reusability of immunoaffinity matrices is also dealt with in chapter 3. Irreversible adsorption of antigen causes a loss in adsorption capacity of the matrix. This loss, which can affect the efficiency of the overall separation procedure, has been modelled over different types of reactors to give clues for optimizing reactors for affinity separations.

1.3.7   *Repetitive Operations*

The synthesis of adsorbents is an expensive process and the major cost involves the production of antibodies, purification and linkage to the chosen matrix. It is therefore desirable to the use the adsorbent in many cycles of operation in order that cost of the adsorbent itself becomes a less dominant feature in the overall cost of the purification procedure. Degeneration of adsorbent during an immunoadsorbent operation will limit its capacity for binding antigen and the number of cycles that it can be used for. There are a large number of factors that can limit the useful life of an immunoadsorbent (Janatova and Globel, 1985).

a)   Loss in biochemical activity of the immobilized antibodies will result in reduction of the capacity of the adsorbent and will affect the performance of the adsorption phase. Denaturation of the antibody could be caused by conditions chosen for desorption of the antigen, through poisoning or enzymatic degradation of the adsorbed antibody.

b)   Loss in capacity will also occur if there is incomplete desorption of the antigen during each cycle. Similarly, continual build up of non-specifically adsorbed species, that are neither removed by washing or elution, will eventually give rise to loss of capacity.

c)   Degeneration of the support by a change in the mechanical or chemical properties can affect the performance of the adsorbent to a large extent. Decrease in the mechanical stability of the material will result in compaction of compressible mechanical beads resulting in a decrease in the flow rates in a fixed bed reactor. Disintegration of the adsorbent due to shear will result in a drop in particle size and hence give difficulty in

separation of the solid phase in batch reactors.

Loss in activity during the operation process would affect the overall throughput and hence an analysis of the process of deactivation would help, not only in identifying factors that affect the life of the immunoadsorbent but also in design of reactors which would help in minimizing the extent of deactivation (Desai, 1990).

Chapter 4 deals with mathematical modelling of a novel technique which can be used as a continuous affinity separation procedure. The effects of dispersion gives clues to achieve efficient separations.

Development of a sensitive separation method using S-14 zeolite as a matrix is discussed in detail in chapter 5. The results indicate that zeolites hold potential as stable matrices for affinity separations.

In chapters 6-9, aspects of protein translocation across biological membranes are dealt with.

1.4   Export of Proteins Across Membranes

How do proteins pass through or integrate into membranes? This is one of the fundamental unsolved questions in biology. A great majority of the proteins are synthesized in the cytoplasm, although their final destination is varied. In a simple case of a gram negative cell like *Escherichia coli*, there can be six different compartments: the cytoplasm, the inner membrane, the outer membrane, the periplasmic space and the extracellular medium. In a more complex cell over twenty such destinations are possible. Although each of the various types of membranes pose the same problem-*how a hydrophilic protein can be transported across a hydrophobic lipid bilayer*-the cells have not evolved one solution. Many common features are however found among different systems.

An era of intensive study of the mechanistic details in this field began mainly with formulation of the *signal hypothesis* by Blobel and Dobberstein (1975). Since the initial research on protein export across the rough endoplasmic reticular membrane, a wide range of other membrane systems such as bacterial, mitochondrial, chloroplast etc. have been investigated and well documented.

It is now a well established fact that signal peptides are almost universally responsible for initiation of protein translocation. Several lines of evidence argue that signal sequences from various organisms work much in the same way (Gierasch, 1989). Many features of the export pathway appeared to be shared by all species, since most exported proteins can be translocated and processed correctly by the export machinery from several organisms (Mueller *et al*, 1982). Yet, despite this striking conservation of critical cellular function, signal sequences display a remarkable lack of primary sequence homology, even among closely related species (Verner and Schatz, 1988). How such diverse signals are responsible for initiating protein translocation across different membranes has been critically analyzed in the latter part of the thesis.

In chapter 10, the concept of mobile fractal shapes has been applied to fluidity of membranes. This concept of *fractiles* (or mobile fractal shapes) has then been extrapolated to problems in other diverse areas. The fractile concept gives possible reasons for rate enhancements over the limit set by three-dimensional diffusion control in many physical, chemical and biological reactions.

CHAPTER 2

PURIFICATION AND CHARACTERIZATION
OF
GALACTOSYLTRANSFERASE ANTIBODIES

Polyclonal antibodies have been raized against galactosyltransferase, an enzyme with immense clinical importance. Purification, quantitation and characterization has been undertaken. Once assured of the purity of these antibodies, their subsequent attachment to Sepharose attempted in order to utilize this matrix as an affinity adsorbent in immunoadsorbent separations.

CHAPTER 2

2.1   Introduction

In immunoaffinity chromatography, before antibodies can be attached to a suitable matrix, it is of utmost importance to characterize and quantitate them. In the present chapter, immunological studies are described for galactosyltransferase, an enzyme that is turning out to be quite important in clinical biochemistry, especially for the detection of cancer (Mawal *et al, 1990 c)*. Hence development of rapid techniques to isolate this enzyme in a pure form and to quantitate it are gaining importance.

Galactosyltransferase (UDP-galactose:N-acetyl-D-glucosamine B-1, 4-galactosyl-transferase, EC 2.4.1.38) is present in soluble form as well as bound to the membrane of the golgi apparatus (Schahter *et al*, 1970). The enzyme catalyses transfer of galactose from UDP-galactose (UDP-gal) to N-acetylglucosamine (GlcNAc) synthesizing N-acetyl-lactosamine which forms a part of the carbohydrate side chain of glycoproteins. In addition to this reaction, galactosyltransferase can also synthesize lactose from UDP-galactose and glucose in the presence of α−lactalbumin which functions as modifier protein (Brew *et al*, 1968). The protein is well characterized from the milk of cows, rat, porcine, bovine and human sources (Sojar and Mawal, 1981). However, very little information is available on its immunological characterization. The antibodies raised against galactosyltransferase have then further been used for immunochromatographic studies.

2.2   Materials and Methods

2.2.1   *Chemicals:*

All chemicals were obtained from SD's (India), E. Merck (India) or Glaxo (India) and were always of *Analar* or *Guaranteed* Reagent specifications. Complete and Incomplete freunds adjuvant and agar were from Difco (USA). Sephadex G-25, Sepharose CL-6B, CL-4B and Sephadex G-200 were from Pharmacia (Uppsala, Sweden). Trizma base, Glycine, Iodogen (1,3,4,6-tetrachloro-3a, 6a-diphenyl glycoluril), Tyrosine, Bovine Serum Albumin, Goat anti-Rabbit IgG-Horseradish peroxidase conjugate, 5-Amino salicylic acid, β −Mercaptoe-thanol, Agarose, Coomassie brilliant blue R-250, Polyethylene glycol (M. W. 8000) and Dextran T-40 were from Sigma Chemical Co. (USA). $Na^{125}I$ (carrier free) was from Bhabha Atomic Research Centre, Bombay (India). Nitrocellulose paper was from Schleicher and Schuell (Germany). Microtitre plates were obtained locally from Laxbro Co., Pune (India). Collodion bag (10,000 cut off) was from Sartorius (W. Germany). Kodak X-O-Mat (AR5) X-ray film for autoradiography was from Kodak (USA). Divinyl Sulphone (DVS) was from Aldrich (USA).

2.2.2   *Raising Antiserum to Galactosyltransferase:*

A variety of animals like rabbit, guineapig, horse, goat and mouse can be employed as hosts for immunization. However, for most of the immunological work, rabbits are preferred on account of their moderate size, well known immune response and the ease with which enough serum can be obtained by small bleeding to carry out preliminary characterization of antibodies.

In the present studies one to three month old New Zealand White rabbits were injected and antibodies raised against galactosyltransferase. For immunization, galactosyltransferase

was dissolved in PBS (Phosphate Buffered Saline, ie. 0.1 M disodium hydrogen orthophosphate, 0.4 M monosodium dihydrogen orthophosphate, 0.14 M sodium chloride, pH 7.2) at 2 mg/ml concentration and was injected into the rabbits, 1 mg at each time in complete freund's adjuvant (Mishell and Shiggi, 1980). The route of immunization was at multiple subcutaneous sites. Six to eight booster doses of 1 mg protein incomplete freund's adjuvant were administered at fortnightly intervals and the animals were bled one week after the final booster dose. The blood was allowed to clot and serum was collected by centrifugation. The serum was divided into 1 ml aliquots and stored at -70°C. Generally greater than 1:16 titre of the antibodies in the serum was obtained.

2.2.3   *Preparation of Antigen and Antibody Affinity Matrices:*

Sepharose CL-4B, activated with divinyl sulphone (DVS) serves as a useful matrix to which a number of proteins can be attached as affinity ligands. Here, 10 g Sepharose CL-4B beads were activated with 2 ml DVS, for 70 minutes at room temperature (Ouchterlony, 1949). The beads were washed with water to remove unreacted DVS and resuspended in 0.3 M sodium bicarbonate, pH 9.0. These are the DVS activated beads to which suitable proteins can be coupled. To this activated Sepharose beads (9.5 ml volume), 5 mg galactosyltransferase (for antigen-affinity matrix) was added and the mixture incubated for six hours at room temperature with very gentle intermittent rotating of the beads. The unbound antigen was washed with 0.3 M sodium chloride by decanting the supernatant. Free activated sites (ie. the sites not bound by the antigen) on beads were blocked with 0.3 M glycine. As determined by absorbance at 280 nm, generally 85-95% of the protein was bound to the beads.

For antibody-affinity matrix, galactosyltransferase antibodies was bound to DVS-activated Sepharose CL-4B beads as described above for the antigen-affinity matrix. Both the affinity matrices were stored in PBS containing 0.02% sodium azide as preservative.

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2.2.4   Purification of Antibodies from Rabbit Serum by Antigen-affinity Chromatography:

Prior to purification of antibodies from the serum, the antibodies were precipitated with 40% ammonium sulphate at 4°C for 30 minutes. The precipitated antibodies were obtained by centrifugation at 8000 g for 10 minutes at 4°C. The pellet was resuspended in 2 ml PBS. Ammonium sulphate was removed by extensive dialysis against PBS.

Since the serum contains total antibodies, it is of utmost importance to purify specific antibodies, which was performed on antigen-affinity chromatography using specific antigen (galactosyltransferase).

Affinity beads were packed in a column upto 5 ml volume and washed thoroughly with PBS till no absorbance could be measured at 280 nm. One ml galactosyltransferase antiserum was passed repeatedly (3 to 5 times) through the above column and the unbound proteins were then washed off completely till no absorbance could be measured at 280 nm. Bound IgG's were then eluted with 0.3 M Glycine-HCl, pH 3.0, and immediately neutralized with solid Tris to pH 7.0 to 7.4. The purified IgG fractions were checked for specificity by Ouchterlony double-diffusion (Ouchterlony, 1949) as described later in section 2.2.6.1. The IgG fractions were pooled and dialysed against PBS. These purified antibodies were used for all further immunological work.

2.2.5   Radioactive (^{125}I) Labelling of Proteins:

Labelling of the antigen was essential since labelled antigens were used for quantitative and qualitative immunological estimation of proteins. Labelling of proteins *in vitro* is generally achieved by labelling tyrosine residues of proteins with ^{125}I. The reaction is quantitative and labelled proteins of high specific activity can be obtained. The labelling reaction was

carried out essentially according to Fraker and Speck (1978). Protein (50 to 100 μg) was usually labelled with 0.1 to 0.2 mCi $Na^{125}I$ (obtained carrier free, specific activity 13-17 mCi/μg) in the presence of Iodogen for 10 minutes on ice in a total volume of 100 μl. The reaction was terminated by addition of 20 μl tyrosine (0.4 mg/ml in 0.2 M Borate buffer, pH 8.4, containing 0.075 M sodium chloride), by binding to the excess ^{125}I. 1% BSA (0.4 ml) in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.14 M sodium chloride and 1 mg/ml potassium iodide were then added to the reaction mixture. Iodinated proteins were freed of unincorporated $Na^{125}I$ by extensive dialysis against PBS. Radioactivity due to ^{125}I was measured in a gamma counter (LKB, Clinigamma model number 1272) and stored at -70°C till further use.

2.2.6   Characterization of Galactosyltransferase Antibodies

To determine the antigenicity of galactosyltransferase approaches like Ouchterlony double-diffusion, Rocket and immunoelectrophoresis, Enzyme Linked Immunosorbent Assay, Radioimmunoassay were employed. After characterization of antibodies, matrices with varying concentrations of attached antibodies were prepared and their kinetic parameters evaluated.

2.2.6.1   Ouchterlony Double-diffusion :

This is the simplest technique employed to characterize the antigen-antibody reaction. It was first described by Ouchterlony (1949), and is most commonly used (i) to estimate titre of antibodies and (ii) to compare reactivities of different antigens and antibodies with each other. Double-diffusion was carried out on microscopic slides or small sized glass plates (4 cm X 6 cm), in 1% agarose gel made in PBS containing 0.1% sodium azide. The glass plates

were coated with a thin film of agar which was allowed to dry prior to pouring molten agarose on it. When the agarose had set completely, circular (~6 mm diameter) wells were punched out of the gel. Appropriate quantities of the antigen and the antibodies were loaded into the wells and these were allowed to diffuse through the gel over a period of 48-72 hours, at 4°C in a humid chamber. When diffusion was complete, antigen-antibody precipitin lines were observed. The gels were dried *in situ* and stained with 0.25% Coomassie brilliant blue R-250 in ethanol:acetic acid:water (4:1:5 v/v) and the slides were again dried. The stained bands were then photographed using a visible light source.

To check the antibody titre, various dilutions of antibodies, ie. 1:2, 1:4, 1:8, 1:16 and 1:32, were loaded in the peripheral wells. Galactosyltransferase was loaded in the central well. Diffusion was allowed to take place for 48-72 hours at 4°C, after which a precipitin arc was observed indicating antigen-antibody reaction (Figure 2.1).

2.2.6.2   *Rocket and Immunoelectrophoresis*

In order to determine the number of antigenic proteins, rocket and immunoelectro-phoresis were carried out as described in LKB application note number 249 (1978). Rocket electrophoresis (Figure 2.1) was carried out on a microscopic slide precoated with a thin film of agar on 1% agarose gel containing 0.2% (v/v) antiserum. After the gel was set completely, circular (~6 mm diameter) wells were punched about 1 cm from the base. Appropriate quantity of antigen was loaded into the well. Electrophoresis was carried out at 200V for 18-19 hours at 4°C. The gels were dried *in situ*, stained and destained as mentioned above in the Ouchterlony double-diffusion technique. Immunoelectrophoresis was carried out on a microscopic slide in 1% agarose in PBS. Circular (~6 mm diameter) wells were punched and an appropriate quantity of antigen was loaded into these wells. Proteins were electrophoresed for 200
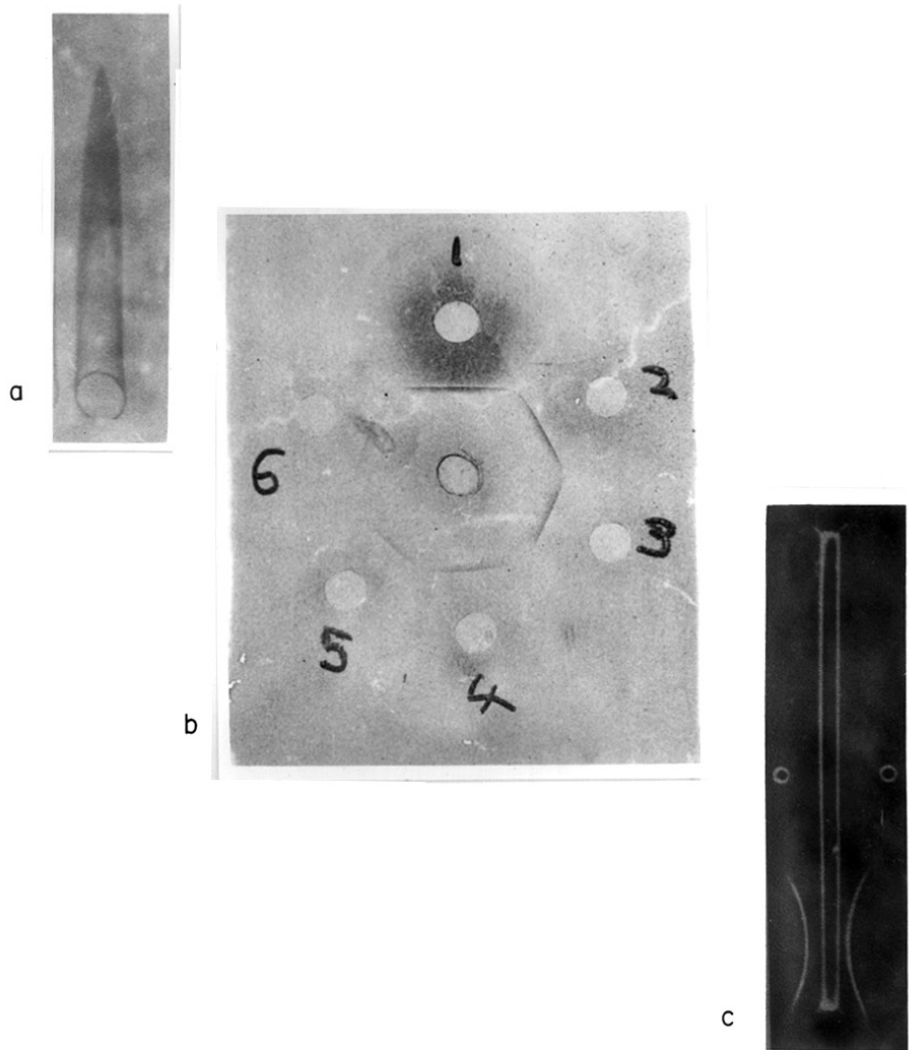
FIGURE 2.1   Characterization of galactosyltransferase antibodies.
a) Rocket electrophoresis
b) Double diffusion
c) Immunoelectrophoresis

Volt-hours. After electrophoresis a central slot was cut in the gel and approximately 100 μl antiserum was loaded into it, followed by incubation at 4°C in a humid chamber. The gels were dried *in situ*, stained and destained as mentioned in the previous section.

2.2.6.3   Radioimmunoassay (RIA)

When assaying antibodies or antigen, it is often convenient to have either the antigen or antibody immobilized by attachment to a solid surface. Polystyrene beads, red cells, agarose beads etc. can be used as a support but it is more convenient to use a plastic test tube or a microtitre plate as the carrier for the antigen or the antibody under test. After washing, antibody bound to the antigen is revealed by adding a labelled antibody directed against the first immunoglobulins. This label may be a radioisotope, such as ^{125}I (Radioimmunoassay), or an enzyme such as horseradish peroxidase (Enzyme Linked Immunosorbent Assay). For quantitation of galactosyltransferase, RIA was established according to Pillai and Mohimen (1982) with some modifications. Varying amounts of galactosyltransferase (0-1000 ng) was mixed with PBS to a final volume of 300 μl. To this solution 200 μl PBS, 20 μl anti-galactosyltransferase antibodies and 10 μl competing labelled galactosyltransferase (~ 10,000 cpm) was added. The reaction mixture was incubated at 25°C for 4 hours, for the antigen-antibody reaction to take place. Here varying amounts of cold or unlabeled antigen is used while the concentration of galactosyltransferase antibodies and competing antigen is kept constant. As the concentration of unlabeled galactosyltransferase increases there is a decrease in the binding of the labelled galactosyltransferase (Figure 2.2). 500 μl the aqueous solution of 35% polyethylene glycol (M.W. 8000) was added and the mixture was left at 4°C for 4 hours to precipitate the antigen-antibody complex. After thorough washing of the
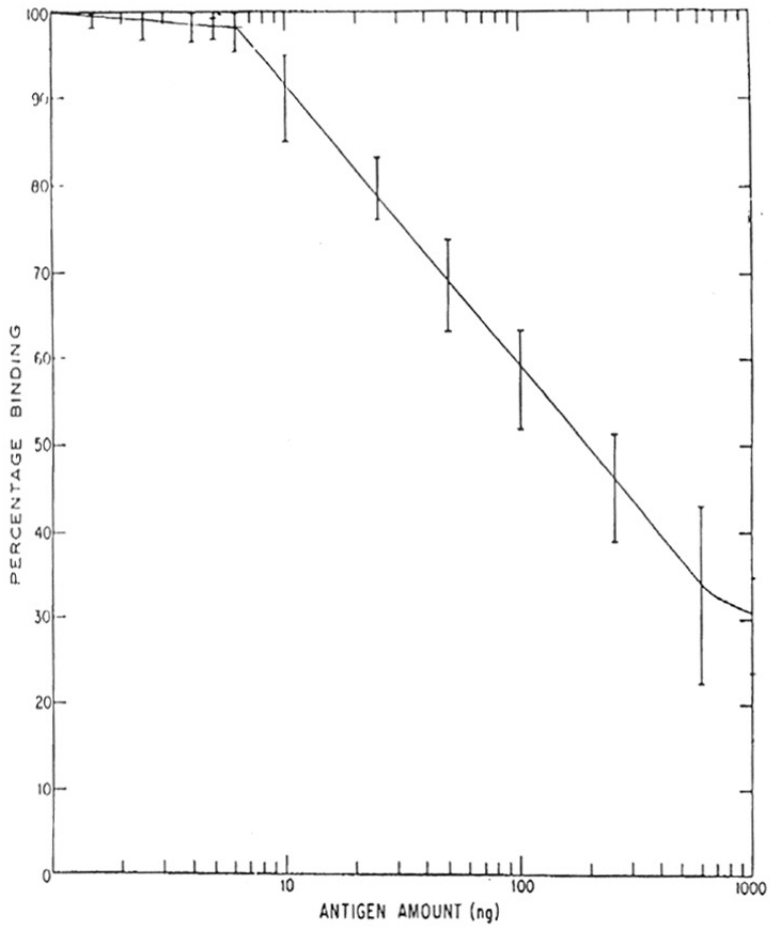
FIGURE 2.2 Fraction of labelled galactosyltransferase bound to solid phase galactosyltransferase antibodies in the presence of various amounts of competing galactosyltransferase.

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unbound antigen by PBS, the precipitated labelled antigen was removed by centrifugation at 2000 g for 10 minutes at 25°C. The radioactivity in the precipitate was measured in a gamma counter (LKB Clinigamma model number 1272).

2.2.6.4   *Enzyme-linked Immunosorbent Assay (ELISA)*

ELISA was carried out according to the method of Hudson and Hay (1980). In this experiment varying amounts of antigen and BSA (as a negative control) were used in the range of 0-10,000 ng in 100 μl volume. These were then allowed to adsorb overnight onto the wells of ELISA microtitre plate at 4°C. Unadsorbed proteins were then aspirated out. 2% BSA in 100 μl PBS was added to these wells for nonspecific binding and incubated at 37°C for 2 hours. BSA was then aspirated out and the adsorbed antigen was allowed to react with 1:300 dilution of anti-galactosyltransferase serum for one hour at 37°C. Unbound antiserum was aspirated out and the wells were washed once with PBS. To each well, 100 μl of 1:2000 dilution of goat anti-rabbit IgG-horseradish peroxidase conjugate was added and was incubated for one hour at 37°C. The peroxidase was then assayed with hydrogen peroxide and 5-amino salicylic acid in sodium acetate buffer, pH 5.0. The intensity of colour was allowed to develop in dark for 30 minutes, following which the reaction was stopped by addition of 50 μl 0.1 N HCl. The colour in each well was measured at 450 nm and a graph of absorbance at 450 nm was plotted against antigen concentration (Figure 2.3).

2.2.6.5   *Determination of Affinity Constant*

The antibody-affinity matrices were prepared as described in section 2.2.3. The only difference here was the use of varying concentrations of antibodies to be attached.
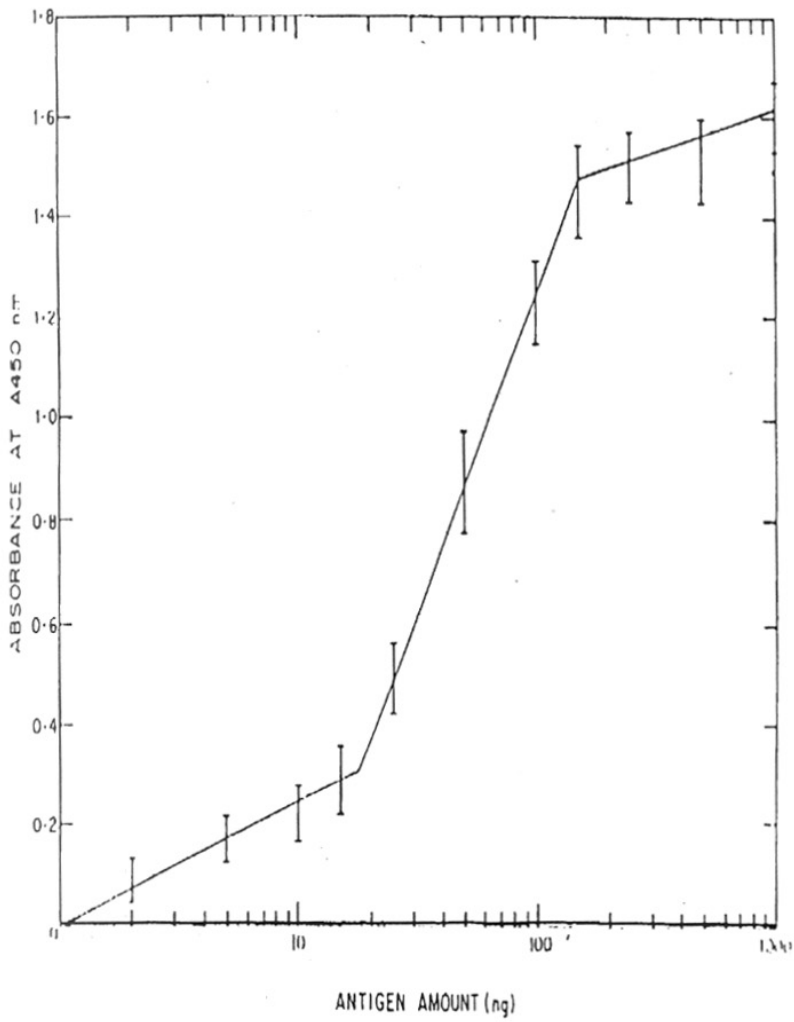
FIGURE 2.3   ELISA of galactosyltransferase

The affinity adsorbent was added to flasks containing different amounts of galacto-syltransferase in 50 mM Tris-HCl, pH 7.2, in a final volume of 10 ml. The flasks were shaken in a water bath for 4 hours and then samples of 1 ml each were removed and centrifuged (5000 g, 3 min) and the amount of protein in the supernatant was determined from optical measurements at 280 nm. The amount of galactosyltransferase bound to the adsorbent (q^*) was then calculated as the total amount of enzyme present at the beginning of the experiment less the amount still present in the supernatant (c^*) at equilibrium. Controls were carried out to ensure that there was no loss of enzyme activity under these conditions.

Using the equation (Chase, 1984b)

$$\frac{c^*}{q^*} = \frac{c^*}{q_m} + \frac{K_d}{q_m} \tag{2.1}$$

values of K_d and q_m were determined from the straight-line plots of c^*/q^* against c^*. The intercept of such plots on the c^* axis is at $-K_d$ and the gradient of the line is $1/q_m$ (Figure 2.4).

The adsorption isotherm measured for the binding of galactosyltransferase to immo-bilized galactosyltransferase antibodies is depicted in figure 2.5.

2.2.6.6 Determination of Adsorption Rate Constants k1 and k2

The estimation of the rate constants k1 and k2 was done essentially by the method of Chase (1984a). In this procedure, in order to determine the value of adsorption rate constant k1 that is applicable to the kinetic profile of batch adsorption, the experimental data was compared with a series of predicted results that were constructed by computer methods. The predicted results were calculated using the equation
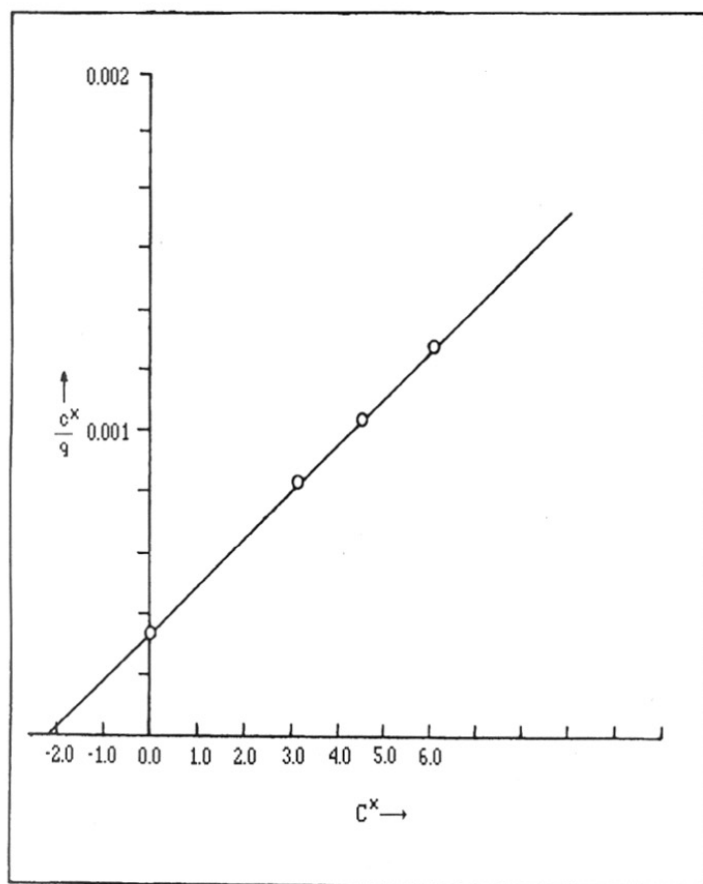
FIGURE 2.4   Profile of ratio of $\frac{c^{\cdot}}{q}$ versus $c^{\cdot}$. The intercept on the $c^{\cdot}$ axis is K_d and the slope of the line is $\frac{1}{q_m}$.
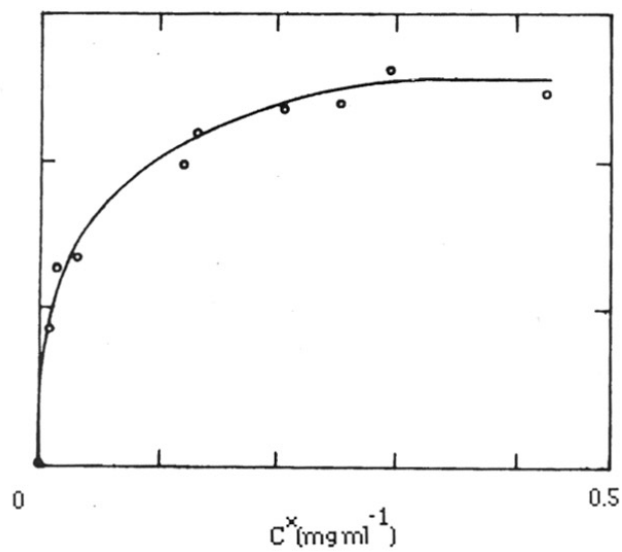
FIGURE 2.5 Isotherm for binding of galactosyltransferase to anti-galactosyltransferase adsorbed to Sepharose CL-6B.

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$$\frac{dq}{dt} = k1c(q_m - q) - k2q \qquad (2.2)$$

where c is the concentration of the adsorbate in the solution, q is the solid-phase concentration of the adsorbed material per unit volume of settled adsorbent, and q_m is the maximum adsorption capacity of the adsorbent.

Values of Kd and q_m were calculated as described in section 2.2.6.5. The experimental kinetic results were compared with the predicted results for a range of k1. The value of k1 applicable to the system investigated was taken to be that value which resulted in a predicted time course that fitted the experimental results (Figure 2.6).

2.3   Results and Discussions

The concentration of the antibodies was determined by Ouchterlony double-diffusion using various dilutions of the antiserum. Antibodies having titre of 1:32 were used for all immunological work. Figure 2.1a shows formation of the precipitin arc for galactosyltransferase at a dilution of 1:32 indicating a high antibody titre. Reactivity of the antiserum for galactosyltransferase was checked against BSA and Ovalbumin by Ouchterlony double-diffusion which indicates that the antiserum reacts with the galactosyltransferase alone.

The purity of the antibodies for galactosyltransferase was further confirmed by immunoelectrophoresis (where a single precipitin arc is observed figure 2.1b) and rocket electrophoresis which confirms the presence of a single antigenic protein.
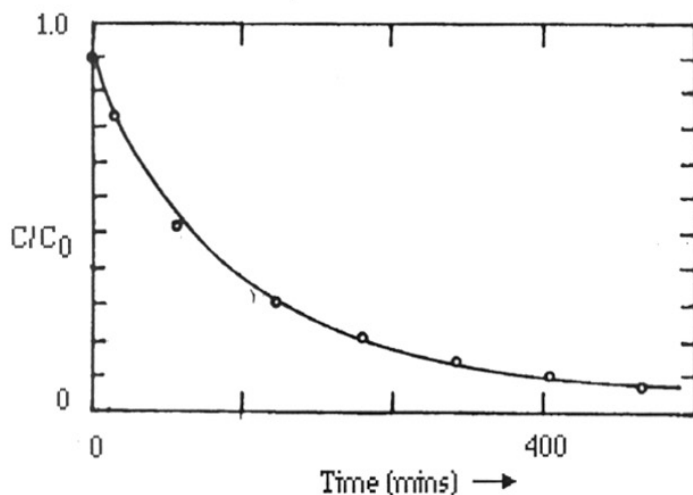
FIGURE 2.6   Profile depicting the adsorption of galactosyltransferase as a function of time. The figure shows experimental points (O) and the solid line is the predicted performance using equation 2.2. The values used in the prediction were K_d = 2.3 X 10^{-4} mg ml^{-1}, qm = 4.5 X 10^{-9} mol ml^{-1}, and k1 = 2.8 X10^3 M^{-1} sec^{-1}.

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The standard RIA was first established for galactosyltransferase using the antibodies raised in rabbits. The antigen concentration are used in the range of 0-1000 ng, the lower limit of detection being 1 ng. From figure 2.2 it is clear that approximately 180 ng of unlabelled galactosyltransferase are required to dislpace 50% of labelled galactosyltransferase in this comparative assay.

The results of peroxidase assay using goat anti-rabbit IgG-peroxidase conjugate and dextran-peroxidase conjugate are depicted in figure 2.3. It can be seen from this figure that the goat anti-rabbit IgG-horseradish peroxidase conjugate can detect galactosyltransferase upto 1 ng when the dilution of 1:1000 is used.

From the adsorption isotherm (Figure 2.5) and the plot of the straight-line plots of c^*/q^* against c^* (Figure 2.4), the values of effective dissociation constant (Kd) and maximum capacity of adsorbent (qm) were determined as 2.3×10^{-4} mg ml^{-1} and 2.2 mg ml^{-1}, respectively.

Values for the rate constants k1 and k2 for the forward and reverse steps in the adsorption reaction were measured in batch experiments using the method described in section 2.2.6.6 are depicted in figure 2.6. It can clearly be seen that the experimental and the predicted results match. The values of the parameters used in the prediction were Kd = 2.3×10^{-4} mg ml^{-1}, qm = 4.5×10^{-9} mol ml^{-1}, and k1 = 2.8×10^{3} M^{-1} sec^{-1}. Similar experiments were carried out using different values of initial concentrations of adsorbate and different amounts of adsorbents to ensure the same value of k1. In this way a unique value of k1 is determined.

The k_d of the antigen-antibody interaction is high and ideal for immunoseparations. The affinity is optimal for increasing the efficiency of separation reducing nonspecific adsorption. This is because higher binding constants would mean relatively easier desorption and hence washing of the column would result in loss of desired antigen. A lower dissociation constant

would mean a stronger binding and a minimization of interference by contaminating proteins. A low dissociation constant however requires harsh or drastic elution techniques which result in deactivation of antibodies.

In conclusion, the polyclonal antibodies raised are pure and possess antigenicity specifically towards galactosyltransferase and therefore can be safely used in the preparation of a suitable adsorbent for immunoaffinity chromatographic separations. Aspects of deactivation of adsorbed antibodies are evaluated in chapter 3.

CHAPTER 3

STUDIES
ON DEACTIVATION OF
ADSORBED ANTIBODIES

Deactivation of adsorbed antibodies is an undesired step that is often encountered in immunochromatographic separation procedures. An analysis to this effect has been attempted in order to understand factors that affect deactivation in order to increase the life of the immunoadsorbent and efficiency of the process.

CHAPTER 3

3.1   Introduction

The previous chapter dealt with the purification, characterization and quantitation of galactosyltransferase antibodies. Once certain about the purity of the antibodies, the next step concerns their attachment to a suitable matrix and separation of the antigen (galactosyl-transferase in this case) using the adsorbed antibodies. The present chapter deals with the aspect of deactivation of such adsorbed antibodies. Deactivation of bound ligands is an undesired step in affinity separation techniques. This phenomenon is even more dominant in immunoaffinity chromatographic methods as very often, when the binding constants are low, drastic methods are used for desorption. This results in loss in activity of adsorbed antibodies, affecting the subsequent efficiency and predictability of a reactor (Chase, 1984a,b).

A majority of uses of immobilized antibodies in immunoadsorption chromatography have involved polyclonal antibodies. However such systems are not readily susceptible to analysis for scale-up studies, mainly due to the *first cycle* effect (Eveleigh and Levy, 1977) and subsequent deactivation of antibodies. This may be due to irreversible binding and denaturation due to the buffer systems used for binding and elution. For commercial applications it is of utmost importance to minimize the deactivation losses because the catalyst life significantly influences the economic feasibility of the process. Selection of eluents for immunoadsorbents has been investigated and optimized for elution process by Kennedy and Branes (1981).

In this chapter results are presented of some experiments on deactivation of immobilized polyclonal antibodies in a batch type reactor. A computer simulation of the performance of different reactors, namely

1) Batch type reactor

2) Semi-batch type reactor

3) Plug flow reactor

has been conducted with the intention of obtaining clues for maintaining and improving the efficiency of separation over a large number of cycles.

3.2   Materials and Methods

The antibody-affinity matrices were prepared as described in section 2.2.3. The only difference here was the use of varying concentrations of antibodies to be attached. Galacto-syltransferase was labeled using $Na^{125}I$ as described in section 2.2.5.

3.2.1   Deactivation studies

The immunoadsorbent matrix was first treated with *Bovine Serum Albumin* (BSA) in order to block possible sites of non-specific adsorption. To this BSA treated matrix, excess purified labeled galactosyltransferase was added and the system allowed to equilibrate for one hour. The beads were thoroughly washed and the adsorbed galactosyltransferase then eluted using 0.3 M Glycine-HCl, pH 3.0, and immediately neutralized with solid Tris to pH 7.0 to 7.2. The amount of protein eluted after the first cycle was measured in a gamma counter

(LKB Clinigamma model number 1272) and used as the maximum protein that can bind to the matrix. This matrix, called here as the *first cycle matrix,* was then used for subsequent studies in a batch type reactor.

3.2.1.1   Deactivation due to adsorption and elution buffer

The BSA treated matrix (30 ml) was taken in a 100 ml beaker. 50 ml of Tris-HCl buffer, pH 7.2, was added to it and incubated at 4°C for one hour with constant shaking. The beads were then centrifuged and treated with 0.3 M Glycine-HCl, pH 3.0, for one min and then immediately neutralized with solid Tris to pH 7.0 to 7.4. The beads were then centrifuged and washed thoroughly with Tris-HCl, pH 7.2. 500 µl of this matrix was taken and labeled galactosyltransferase added to it. The amount of galactosyltransferase bound was calculated by the difference between galactosyltransferase added and the amount in the supernatant. The unused matrix was then again subjected to treatment by adsorption and elution buffer. The amount of protein that binds to 500 µl of matrix with repeated number of cycles of exposure was plotted. The experiments were repeated for different exposure time varying from 1 min to 10 min.

3.2.1.2   Deactivation due to irreversible binding

The *first cycle matrix* (1 ml) was taken in a test tube. To it was added 5 ml of Tris-HCl buffer, pH 7.2, and 0.5 ml labeled galactosyltransferase (13-15 mCi/µg) and incubated at 4°C for one hour with constant shaking. The tube was then centrifuged (5000 g, 2 min) and the galactosyltransferase in the supernatant was estimated on a gamma counter. Amount of protein adsorbed to the matrix was estimated by the usual method. The centrifuged beads were treated with 0.3 M Glycine-HCl, pH 3.0, for two min and immediately neutralized with solid Tris to

pH 7.0 to 7.4. The beads were once again centrifuged and washed thoroughly with Tris-HCl, pH 7.2. Eluted protein was once again estimated and the amount of protein lost due to irreversible binding was calculated. The experiments were repeated using the same matrix and the loss in capacity of the adsorbent with number of cycles was calculated. The experiments were again repeated using matrices with different values of q_m.

3.3   Theoretical Analysis

As an initial approximation for a simple theoretical analysis, it has been assumed that the immobilized binding sites (Ab) on the affinity adsorbent have an affinity for only one antigenic determinant. The overall binding interaction of the polyclonal antibodies for different antigenic determinants can be described by a single overall rate constant Kf, while the deactivations are assumed to follow first order kinetics. The overall mechanism is depicted as:
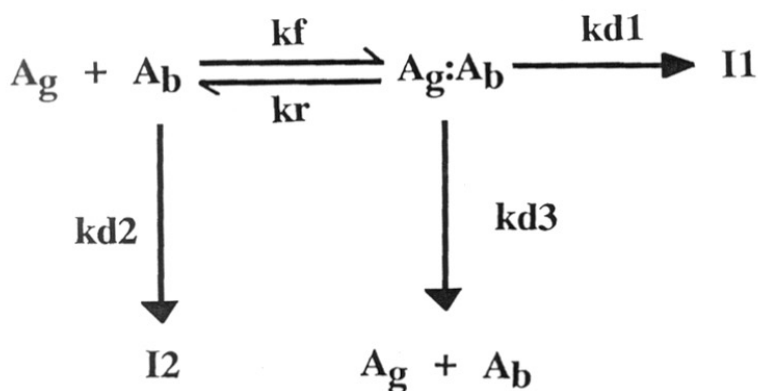
The types of deactivation considered here are:

i) The A_g:A_b complex formed undergoes deactivation due to a strong irreversible binding interaction.

ii) The antibodies (A_b) can themselves undergo irreversible denaturation mainly due to the incubation with buffers used for adsorption and elution.

Here, in the model, it is assumed that the affinity matrix used has been treated for the first cycle effect.

3.3.1 The Model

For a batch type reactor the mass balance equations can be given as follows:

$$\frac{dN_{A_g}}{dt} = -VkfC_{A_g}C_{A_b} + VkrC_{A_gA_b} \tag{3.2a}$$

$$\frac{dN_{A_b}}{dt} = -VkfC_{A_g}C_{A_b} + VkrC_{A_gA_b} - Vkd_2C_{A_b} \tag{3.2b}$$

$$\frac{dN_{A_gA_b}}{dt} = VkfC_{A_g}C_{A_b} - VkrC_{A_gA_b} - Vkd_1C_{A_gA_b} \tag{3.2c}$$

$$\frac{dN_{I1}}{dt} = Vkd_1C_{A_gA_b} \tag{3.2d}$$

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$$\frac{dN_{I2}}{dt} = Vkd_2C_{A_b}$$

(3.2e)

For the semi-batch type reactor the mass balance equations are:

$$\frac{dN_{A_g}}{dt} = qC_{A_g0} - VkfC_{A_g}C_{A_b} + VkrC_{A_gA_b}$$

(3.3a)

$$\frac{dN_{A_b}}{dt} = qC_{A_b0} - VkfC_{A_g}C_{A_b} + VkrC_{A_gA_b} - Vkd_2C_{A_b}$$

(3.3b)

$$\frac{dN_{A_gA_b}}{dt} = VkfC_{A_g}C_{A_b} - VkrC_{A_gA_b} - Vkd_1C_{A_gA_b}$$

(3.3c)

$$\frac{dN_{I1}}{dt} = Vkd_1C_{A_gA_b} + qC_{I1_0}$$

(3.3d)

$$\frac{dN_{I2}}{dt} = Vkd_2C_{A_b} + qC_{I2_0}$$

(3.3e)

For a packed bed reactor:

$$\frac{dC_{A_g}}{dt} = u\frac{dC_{A_g}}{dl} - kfC_{A_g}C_{A_b} + krC_{A_gA_b}$$

(3.4a)

$$\frac{dC_{A_b}}{dt} = -kfC_{A_g}C_{A_b} + krC_{A_gA_b} - kd_2C_{A_b} \tag{3.4b}$$

$$\frac{dC_{A_gA_b}}{dt} = kfC_{A_g}C_{A_b} - krC_{A_gA_b} - kd_1C_{A_gA_b} \tag{3.4c}$$

$$\frac{dC_{I1}}{dt} = kd_1C_{A_gA_b} \tag{3.4d}$$

$$\frac{dC_{I2}}{dt} = kd_2C_{A_b} \tag{3.4e}$$

$$\text{where } V = V_0 + qt \tag{3.5a}$$

$$\text{and } \frac{dV}{dt} = q \tag{3.5b}$$

$$N_{A_g} = VC_{A_g}; N_{A_gA_b} = VC_{A_gA_b}; N_{A_b} = VC_{A_b}; N_{I1} = VC_{I1}; N_{I2} = VC_{I2} \tag{3.5c}$$

Non-dimensionalize using

$$L = \frac{lkR}{u} \tag{3.6a}$$

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$$X = \frac{C_{A_g}}{C_{A_{g0}}}; \quad Y = \frac{C_{A_b}}{C_{A_{g0}}}; \quad Z = \frac{CA_{gA_b}}{C_{A_{g0}}} \tag{3.6b}$$

$$I1 = \frac{C_{I1}}{C_{A_{g0}}}; \quad I2 = \frac{C_{I2}}{C_{A_{g0}}}; \quad I1_0 = \frac{C_{I10}}{C_{A_{g0}}} \tag{3.6c}$$

$$Y_0 = \frac{CA_{b0}}{CA_{g0}} \tag{3.6d}$$

to get

in case of a batch reactor :

$$\frac{dX}{dT} = -\alpha XY + Z \tag{3.7a}$$

$$\frac{dY}{dT} = -\alpha XY + Z - KD_2 Y \tag{3.7b}$$

$$\frac{dZ}{dT} = \alpha XY - Z(KD_1 + 1) \tag{3.7c}$$

$$\frac{dI1}{dT} = KD_1 Z \tag{3.7d}$$

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$$\frac{dI2}{dT} = KD_2Y \tag{3.7e}$$

in case of semi-batch reactor :

$$\frac{dX}{dT} = \left\{ \frac{1-X}{T} \right\} - \alpha XY + Z \tag{3.8a}$$

$$\frac{dY}{dT} = \left\{ \frac{Y_0 - Y}{T} \right\} - \alpha XY + Z - KD_2Y \tag{3.8b}$$

$$\frac{dZ}{dT} = \alpha XY - Z\left(\frac{1}{T} + KD_1 + 1 \right) \tag{3.8c}$$

$$\frac{dI1}{dT} = KD_1Z + \left\{ \frac{I1_0 - I1}{T} \right\} \tag{3.8d}$$

$$\frac{dI2}{dT} = KD_2Y + \left\{ \frac{I2_0 - I2}{T} \right\} \tag{3.8e}$$

in case of a packed bed reactor :

$$\frac{dX}{dT} \ = \ -\frac{dX}{dL} - \alpha XY + Z \tag{3.9a}$$

$$\frac{dY}{dT} \ = \ -\alpha XY + Z - KD_2Y \tag{3.9b}$$

$$\frac{dZ}{dT} \ = \ \alpha XY - Z(KD_1 + 1) \tag{3.9c}$$

$$\frac{dI1}{dT} \ = \ KD_1Z \tag{3.9d}$$

$$\frac{dI2}{dT} \ = \ KD_2Y \tag{3.9e}$$

˙Where

$$T \ = \ Kr\left\{\frac{V_0}{q} + t\right\} \tag{3.10a}$$

$$\alpha \ = \ \frac{KfCA_{g0}}{Kr} \tag{3.10b}$$

$$KD_1 = \frac{kd_1}{Kr} \tag{3.10c}$$

$$KD_2 = \frac{kd_2}{Kr} \tag{3.10d}$$

The nondimensional equations were solved using the variable step-size Merson method.

3.4 Results and Discussions

3.4.1 Deactivation due to Adsorption and Elution Buffer

Many times the affinity of the antibody for antigen is high. This criteria, though desirable for increasing the efficiency of separation, forces one to use harsh methods to elicit elution of the adsorbed antigen. This often results in partial inactivation of the adsorbed antibodies, which ultimately leads to a drop in the efficiency and predictability of the separation procedure. The aim, therefore, should be to minimize the deactivation in order to make the separation procedure cost effective. Figure 3.1 depicts the effect of adsorption and elution buffer on the matrix. As expected, the deactivation of adsorbed antibodies is greater when the time of exposure to the eluting buffer is more. This, therefore, requires that we find the minimum time that elicits complete elution of antigen so as to maintain the activity of the matrix over a large number of cycles. Figure 3.2 indicates that the minimum exposure time of the matrix to the eluting buffer (in this case) is 1.5 min. During this time nearly 95% of adsorbed antigen is eluted. Longer exposure of matrix to extreme conditions may also result in loss in specific activity of the enzyme. The profile of ratio of protein eluted at a particular exposure time (over 5 cycles) and the extent of deactivation due to the buffer at that time indicates an optimum
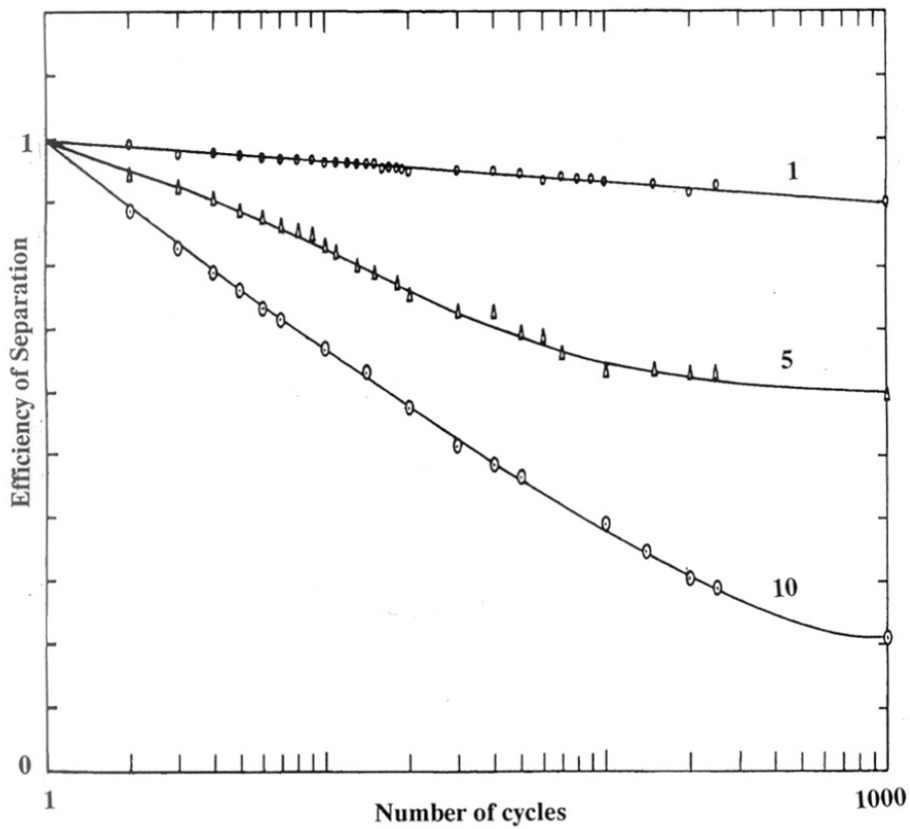
FIGURE 3.1   Profiles for efficiency of separation as a function of number of cycles, depicting the effect of adsorption and elution buffer on adsorbed antibodies, for various exposure times. Efficiency is defined as the ratio of antibodies adsorbed in first cycle and antibodies adsorbed in the cycle under consideration.
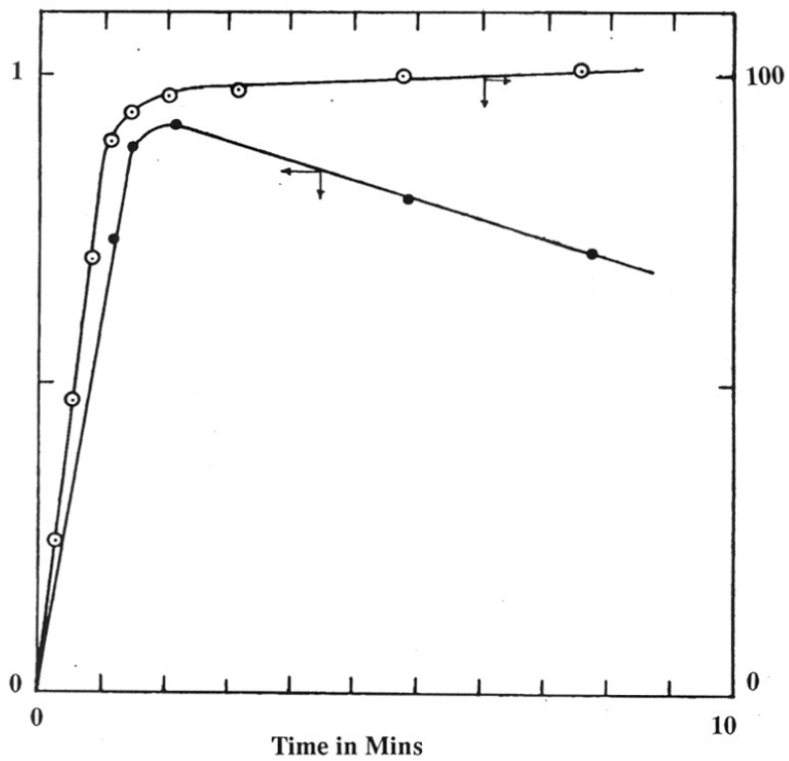
FIGURE 3.2   Profiles depicting extent of elution ( ) and ratio of eluted protein and extent of deactivation as a function of exposure time (For details see text).

exposure of 1.5 to 2 min. As the exposure time increases, though the extent of elution increases, the loss of adsorbent due to deactivation is far greater (as depicted in figure) than that at lower exposure times. Therefore, though the efficiency of elution is about 95%, the life of the matrix is greatly increased.

3.4.2 Deactivation due to Irreversible Binding

Irreversible binding of proteins to the matrix also reduces the efficiency of separation. Sources for irreversible binding may be incomplete masking of reactive ligands to which the antibodies are attached or attachment to the adsorbent matrix via multiple interactions (a cumulative effect of matrix-antigen and antibody-antigen interactions). Whatever the mechanism, such a deactivation results in unpredictability in the extent of separation and a drastic drop in the efficiency of separation. Figure 3.3 shows the effect of ligand concentration on deactivation. As the concentration of attached antibodies increases, irreversible attachment also increases. One of the reasons for this may be attachment of antigen to the matrix via interaction between various antigenic determinants and antibodies. A profile depicting the ratio of protein % eluted over five cycles to the % deactivated due to irreversible binding during these cycles is shown in figure 3.3. Loss in ligand concentration due to irreversible binding is calculated as the difference between the total deactivation and deactivation due to the buffer system. The optimum ligand concentration for maximum efficiency is 2.3 mg ml^{-1}.

3.4.3 Theoretical studies

The primary aim of this study is to arrive at a simple model for deactivation in a way so as to be able to predict the reactor performance over a large number of cycles and to optimize the separation procedure by designing appropriate reactors.
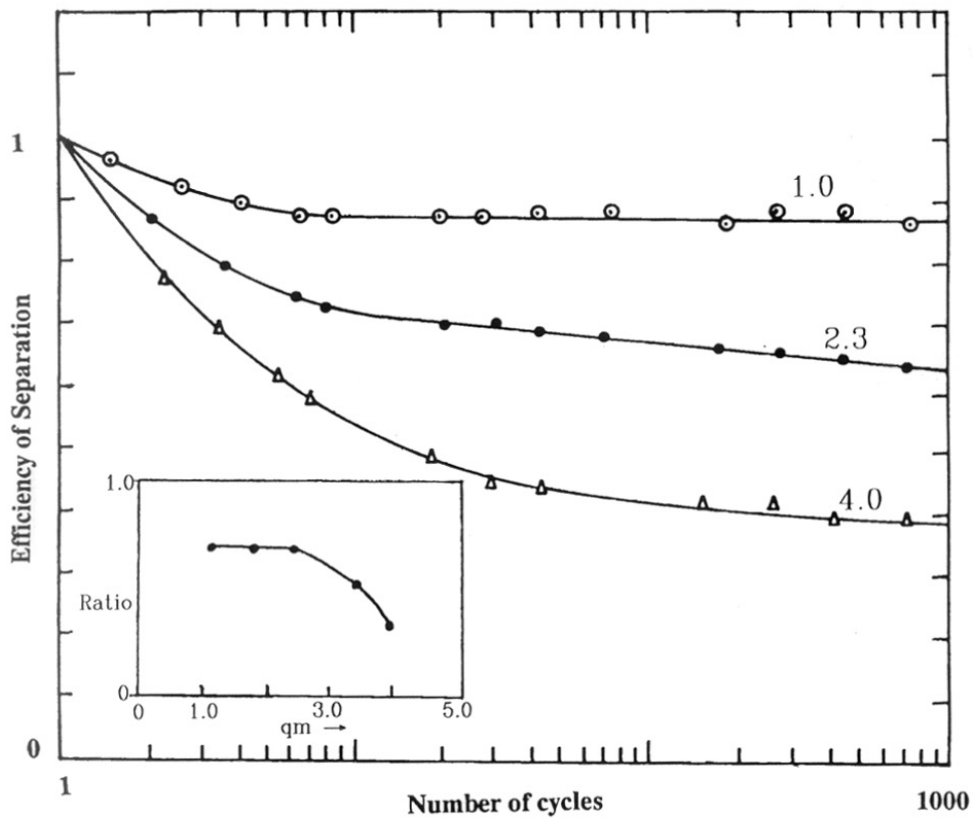
FIGURE 3.3   Effect of ligand concentration on efficiency separation as a function of number of cycles.

Inset in figure 3.3 presents ratio of protein eluted and amount of protein deactivated as a function of ligand concentration.

A simple deactivation mechanism, as described in equation 3.1, seems adequate as an initial approximation. Using this mechanism, simulations for a batch type reactor were performed and compared with the experimental data. From figure 3.4 it can be seen that experimental and theoretical results match reasonably well for the mechanism described by equation 3.1. Using this mechanism the performance of a semi-batch type, batch type and a column reactor was evaluated. Two cases are considered in the present study:

(i) the free ligands undergo deactivation at a rate lower than the Ag-Ab complex.

(ii) the free ligands undergo deactivation at a rate higher than the Ag-Ab complex.

Case 1: The free ligands undergo deactivation at a rate lower than the Ab-Ag complex.

Initially the batch type reactor gives higher binding of protein to the matrix over a small period of cycles, when compared to the semi-batch type reactor (A1 and A2 in figure 3.5). In this case, therefore, the batch type reactor is superior to the semi-batch type. However, as the number of cycles increases, the semi-batch type reactor shows higher conversion as compared to the batch type reactor. The ligand concentration and the forward binding constant, however, do play an important role. For a higher ligand concentration, the protein binding in a semi-batch type is nearly equal to that in the batch type. However, for lower ligand concentrations, the batch type reactor proves to be superior. However, as the number of cycles increase, the performance of the semi-batch type is better than that of the batch type reactor.

When comparing performances of column and batch type reactors, it can be seen (B1 and B2) in figure 3.5 that the ratio of adsorbed protein in a column to that in a batch type, when the ligand concentration is high, is found to be much smaller in magnitude than that for a lower ligand concentration. This implies that for lower ligand concentrations, a column type of reactor is advantageous, whereas, for higher ligand concentrations the batch type of reactor may be preferred to a column.
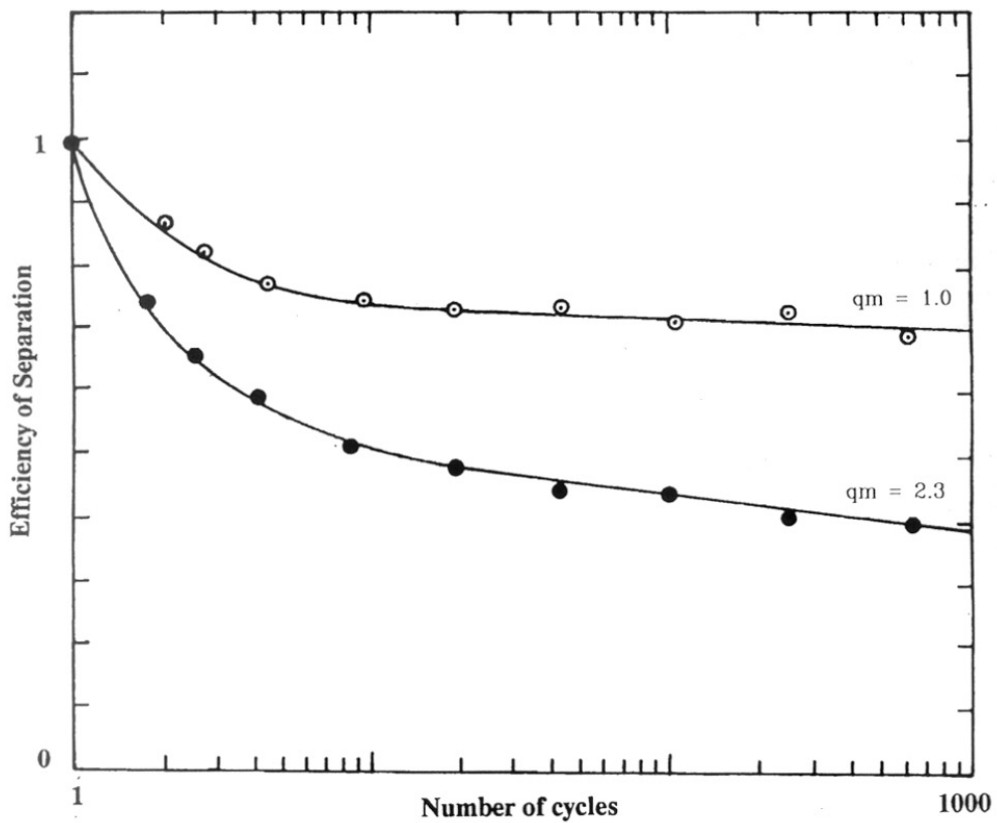
FIGURE 3.4 A comparison between simulated results and experimental data for different ligand concentrations. Circles depict experimental data while solid line depicts simulated results.
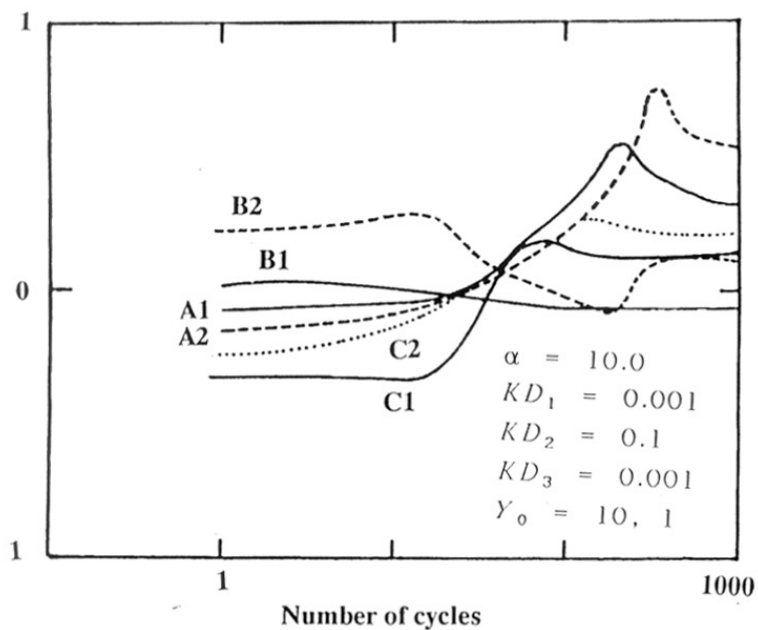
FIGURE 3.5   Ratio of efficiency of separation in different reactors as a function of number of cycles.
A1 and A2 ---- Semi-batch to batch
B1 and B2 ---- Column to batch
C1 and C2 ---- Semi-batch to column
1 and 2 ---- depict ligand concentrations of 10 and 1 respectively.

The performance of the semi-batch reactor is inferior to that of the column type during the initial cycles (C1 and C2 in figure 3.5). As the number of cycles increase, the performance of the semi-batch type supersedes that of the column. This is because in a column, even though no protein is binding to the lower parts, the buffer which is flowing over these ligands undergoes deactivation. In the semi-batch type, the matrix which is added towards the end does not undergo extensive deactivation. Hence the above mentioned behavior. The ratio of protein binding to the matrix in semi-batch to that in the column reduces with the increase in ligand concentration. In such cases, it would therefore be ideal to use a high ligand concentration in a broad diameter and narrow length columns, preferably in the form of a membrane to reduce the extent of deactivation and, at the same time, achieve higher efficiency.

Case 2: The free ligands undergo deactivation at a rate higher than the Ab-Ag complex.

When the Ab-Ag complex undergoes denaturation, the batch type reactor has a distinct advantage over the semi-batch type for large number of cycles. The performance of batch type with that of the semi-batch then rapidly deteriorates over a very small period of cycles (A3 and A4 in figure 3.6). The number of cycles for which the batch type is advantageous over the semi-batch type is controlled by the ligand concentration. Higher ligand concentrations will prove to be distinctly advantageous for a batch type reactor. The ratio of deactivation will remain constant over a large number of cycles.

When the column and batch type reactors are compared (B3 and B4 in figure 3.6), it is observed that the ratio of protein binding in a column to that in a batch type is less than one indicating that the performance of the column is inferior to that of the batch type reactor throughout all cycles. Thus, it would be advantageous to use a batch type reactor. Lower is the ligand concentration, better is the performance of the batch type reactor to that of the column type.

The figure shows a plot with the y-axis ranging from 1 (top) through 0 (middle) to 1 (bottom), and the x-axis labeled "Number of cycles" ranging from 1 to 1000.

The plot contains the following parameters:

$$\alpha = 10.0$$
$$KD_1 = 0.1$$
$$KD_2 = 0.001$$
$$KD_3 = 0.001$$
$$Y_0 = 10, 1$$

Curves are labeled: A3, A4, B3, B4, C3, C3.
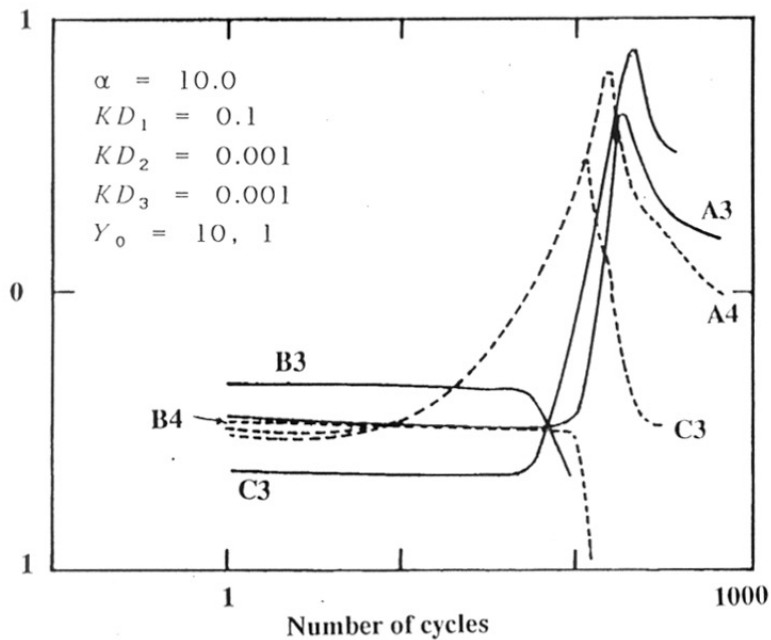
FIGURE 3.6 Ratio of efficiency of separation in different reactors as a function of number of cycles
A3 and A4 ---- Semi-batch to batch
B3 and B4 ---- Column to batch
C3 and C4 ---- Semi-batch to column
3 and 4 ---- depict ligand concentrations of 10 and 1 respectively.

The performance of the semi-batch reactor is found to be inferior to that of the column reactor during the initial cycles (C3 and C4 in figure 3.6). As the number of cycles increase, the performance of the semi-batch reactor proves to be superior to that of the column reactor.

Thus, from the above analysis, it can be seen that depending on the affinity between the antibody-antigen and concentration of antigens and antibodies the extent of deactivation can vary in different types of reactors. Therefore, to minimize deactivation, the type of reactor chosen should depend on the factors discussed above. A particular system with different parameters may behave differently. Therefore, before undertaking ways to minimize deactivation losses, the parameters of that system should be estimated.

MODELLING
CONTINUOUS
AFFINITY SEPARATION REACTORS

Continuous affinity separation using a fluid bed reactor is a novel technique that can be utilized for large scale production of high purity biologically active materials. Model equations describing the physical process have been formulated and analyzed to study the effects of dispersion on the concentration profiles. The theoretical analysis gives important clues for better design of these reactors.

CHAPTER 4

4.1   Introduction

Chapter 3 dealt with aspects of deactivation of adsorbed antibodies as a function of number of cycles and time. In the present chapter, we deal with theoretical simulations of a technique which holds potential as a continuous affinity chromatography separation procedure. The technique involves the use of a matrix which is magnetic in nature and is fluidized in a magnetically stabilized bed.

Rapid advances in genetic engineering and molecular biology have paved the way for large scale production of specific proteins and enzymes (Ulmer, 1983). The range of compounds produced can vary vastly in their molecular weights and properties (Demain, 1981) and are of considerable interest in the manufacture of pharmaceutical and medical diagnostic reagents (Gilbert and Villa-Komaroff, 1980). The downstream processing methods have however lagged behind considerably (Hawtin, 1982). One of the most difficult and challenging tasks facing large scale biotechnology has been the need to find and develop appropriate separation and purification methods. Most of the present conventional biochemical methods of separation are laborious and time consuming, one of the major reasons being the batchwise method of operation. Furthermore, the purification folds obtained by the above methods have been only about two to ten times, often resulting in about three to ten steps to produce a product of acceptable purity (Lilly and Dunill, 1969). However, the advent of affinity methods of separations have led to some heartening progress.

Affinity purification techniques employ the unique property of biospecific recognition between two molecules (Cuatrecasus et al, 1968; Lerman, 1953; Cuatrecasus, 1971). The interactions involved are highly specific in nature and vary from system to system (Porath et al, 1967). Very high purification, of the order of about a hundred to a thousand fold have been reported (Cuatrecasus, 1972; Allen and Majerus, 1972). Even so these systems have remained in the state of infancy (for large scale purification) (Chase, 1984a), predominantly due to the exorbitant cost of affinity matrices, which make large scale batch mode of operation rather unfeasible. Furthermore, since peak emergence from the column is time dependent, it becomes difficult for automation and

control of the process. Such problems have been encountered by earlier workers who have used various columns as preparative tools (Berg, 1946). All these factors have led to the use of affinity separation techniques mainly as laboratory scale methods of purification and characterization of proteins.

Continuous separation processes have made a fair amount of progress in conventional methods of purification (Sussman, 1976; Fox et al, 1969; Janson and Hedman, 1983). They have also been extensively modeled (Gobie et al, 1985; Noble, 1985). However their application to affinity pose enormous difficulties. This is mainly due to the two discrete steps involved:

1) Adsorption

2) Elution

both of which require different specific but diverse conditions. To make affinity a continuous method will therefore involve the movement of the matrix. Such systems have been used for large scale separation but have met with limited success for industrial applications (Robinson et al, 1972). Hence it is necessary to study such methods theoretically and experimentally in an attempt to increase the feasibility of their industrial application to continuous separation methods.

Burns and Graves (1985) have illustrated the use of a magnetically stabilized fluidized bed as a viable and attractive method for continuous affinity separation. The advantages in the use of such a system are many and hence could prove to be ideal for scaling up of affinity chromatography.

Rosensweig (Rosensweig, 1979a; 1979b) has shown that unlike ordinary fluid bed reactors, dispersion and backmixing is very low in a magnetically stabilized fluidized reactor. Burns and Graves (1985) have assumed the system to follow ideal plug flow characteristics. However we believe that this could lead to a fair amount of over prediction of concentrations. The main reasons for the deviation from ideality being:

(1)   The use of a fluidized bed in which the countercurrent movement of the solids and the liquid occurs

(2)   A continuous adsorption desorption phenomena occurring throughout the length of the column.

This will lead to considerable back mixing in the liquid phase warranting the incorporation of axial dispersion in the balance equations. The model proposed below is also valid for normal fluid bed reactors where magnetic stabilization may be uneconomical. Thus an attempt is made in the present work to study the effects of dispersion in continuous affinity columns. This would facilitate designing a system for high efficiency large scale protein separation in a rational manner.

4.2   Mathematical Modelling

The present mathematical model aims at the development of a set of simple equations to estimate the appropriate parameters required for a high efficiency continuous affinity chromatographic system. In our model (figure 4.1) we have considered the feed of the protein and the buffer to be at the bottom of the column unlike the middle feed as considered by Burns and Graves (1985), and hence it became necessary for us to remodel the ideal plug flow case with a modified set of boundary conditions.

Several simplifying assumptions have been made to obtain an analytical solution:

(i)   The rates of adsorption and desorption are assumed to depend on the concentration of protein in the liquid and solid phase, respectively.

(ii)   The forward and reverse rate constants are lumped constants which take care of the kinetic effects of protein/ligand binding.

(iii)   The effect of saturation of the ligand on the matrix is assumed to be negligible.

(iv)   The internal diffusion and mass transfer rates are assumed to be negligible for small bead size.

The modeling part has been divided into two parts: one without the effect of dispersion and the other with dispersion.

$V \leftarrow$ — W
$C_S = 0$

1

Z

0

V — $\rightarrow$ W
$C_l = 0$

A) IDEAL PLUG FLOW MODEL

$V \leftarrow$ — W
$C_S = 0$

$$\frac{dC_l}{dZ} = 0$$

V — $\rightarrow$ W

$$\frac{dC_l}{dZ} = P_e(1 - C_l)$$

B) DISPERSED PLUG FLOW MODEL
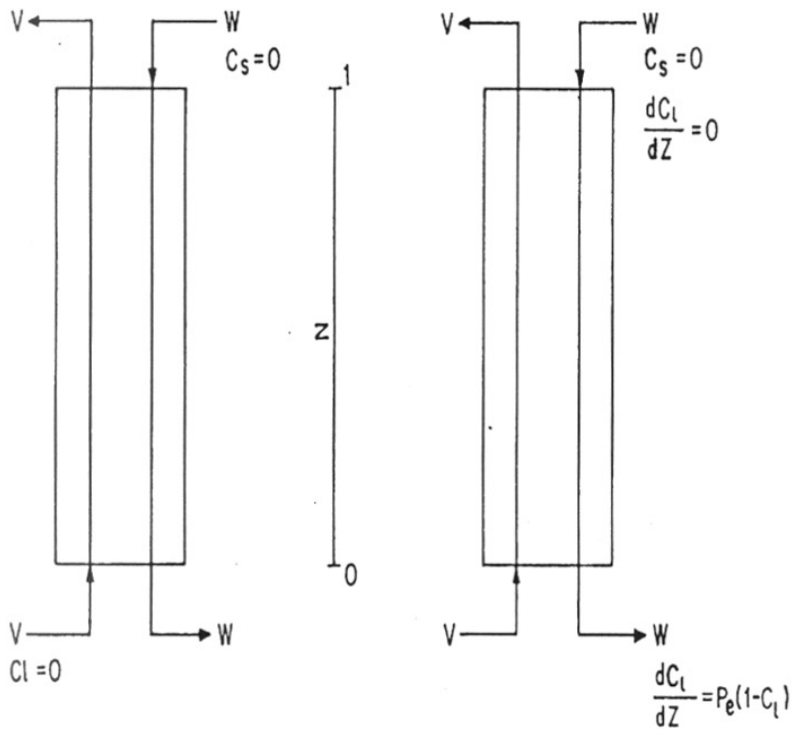
FIGURE 4.1 Column division for mathematical modelling of a fluidized bed chromatographic system. Pure solids enter the column at $Z = 0$ and solvents (with proteins) enters at $Z = 1$.
(A) Ideal plug flow model
(B) Dispersed plug flow model

4.2.1 The Ideal Plug Flow Model

The non-dimensional equations that are of a type similar to those considered by Burns and Graves (1985), are given below

$$\frac{dCl}{dZ} \;=\; \alpha \;\; (Cs - Cl) \tag{4.1a}$$

$$\frac{dCs}{dZ} \;=\; \beta \;\; (Cs - Cl) \tag{4.1b}$$

with the dimensionless parameters:

$$\alpha \;=\; \left\{ \frac{K_f A L \varepsilon}{V} \right\} \tag{4.2a}$$

$$\beta \;=\; \left\{ \frac{K_r A L (1 - \varepsilon)}{W} \right\} \tag{4.2b}$$

the boundary conditions used are:

$$Cs \;=\; 0 \quad at \quad Z \;=\; 1 \tag{4.3a}$$

This boundary condition states that as the solids enter the column the concentration of the solute on the matrix is equal to zero.

$$Cl \ = \ 1 \quad at \quad Z \ = \ 0 \tag{4.3b}$$

This condition states that at the inlet of the reactor, the concentration in the liquid phase is equal to the feed stream concentration.

Solving equations 4.1a and 4.1b analytically, using the above defined boundary conditions, we get:

$$Cl \ = \ F_1 \ + \ F_2 e^{rz} \tag{4.4a}$$

$$Cs \ = \ F_1 \ + \ K \, rF_2 e^{rz} \tag{4.4b}$$

where:

$$r \ = \ \beta - \alpha \tag{4.5a}$$

$$F_1 \ = \ \frac{-\beta e^{r}}{\alpha - \beta e^{r}} \tag{4.5b}$$

$$F_2 = \frac{\alpha}{\alpha - \beta e^r} \tag{4.5c}$$

$$K = \frac{\beta}{\alpha} \tag{4.5d}$$

4.2.2   The Dispersed Plug Flow Model

Taking into consideration dispersion, which will occur only in the liquid phase, we arrive at a new set of equations which can be given at steady state as

$$V\frac{dC}{dz} - ADe\frac{d^2c}{dz^2} = K_r nA(1-\varepsilon) - K_f cA\varepsilon \tag{4.6a}$$

$$W\frac{dn}{dz} = -K_r nA(1-\varepsilon) + K_f cA\varepsilon \tag{4.6b}$$

W and V denote the flow rates of the matrix and the buffer, K_r and K_f the reverse and the forward kinetic rate constants, A is the cross sectional area of the reactor, ε is the void fraction of the solid matrix and n and c depict the concentration of the solute on the solid and in the liquid phase, respectively. Any position along the length of the reactor is denoted by z.

Defining the following variables:

$$Z = \frac{z}{L} \tag{4.7a}$$

$$Cl \quad = \quad \frac{c}{cf} \tag{4.7b}$$

$$Cs \quad = \quad \frac{k_r n (1 - \varepsilon)}{k_f cf \varepsilon} \tag{4.7c}$$

the equations 4.6a and 4.6b can be rendered dimensionless as follows:

$$\frac{d^2 Cl}{dZ^2} \quad = \quad Pe \frac{dCl}{dZ} - Pe\alpha(Cs - Cl) \tag{4.8a}$$

$$\frac{dCs}{dZ} \quad = \quad \beta(Cs - Cl) \tag{4.8b}$$

where

$$Pe \quad = \quad \frac{VL}{AD\varepsilon} \tag{4.9}$$

Here α and β are the dimensionless forward and reverse rate constants respectively and have the same meaning as that illustrated in equation 4.2a and 4.2b, while K is the dimensionless equilibrium constant.

Using Danckwerts boundary conditions

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1) $Cs \ = \ 0 \ \ at \ \ Z \ = \ 1$

which implies that the concentration of the solute on the matrix is zero as it enters the top of the reactor.

2) $\frac{dCl}{dZ} \ = \ 0 \ \ at \ \ Z \ = \ 1$

which implies that as soon as the liquid phase leaves the reactor the dispersion is zero.

3) $\frac{dCl}{dZ} \ = \ Pe(1-Cl) \ \ at \ \ Z \ = \ 0$

Solving equations 4.8a and 4.8b analytically using the above boundary conditions, we get the equations for Cl and Cs as,

$$Cl \ = \ R_1 + R_2 e^{m1} + R_3^{m2} \tag{4.10a}$$

$$Cs \ = \ R_1 + J_1 R_2 e^{m1} + J_2 R_3 e^{m2} \tag{4.10b}$$

where:

$$m1 \ = \ \left\{ \frac{(Pe+\alpha) - \sqrt{(Pe+\alpha)^2 + 4Pe(\alpha-\beta)}}{2} \right\} \tag{4.11a}$$

$$m2 \ = \ \left\{ \frac{(Pe+\alpha) + \sqrt{(Pe+\alpha)^2 + 4Pe(\alpha-\beta)}}{2} \right\} \tag{4.11b}$$

$$J_1 \ = \ \frac{\alpha Pe + (Pem1) - m1^2}{Pe\alpha} \tag{4.12a}$$

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$$J_2 = \frac{\alpha Pe + (Pem2) - m?}{Pe\alpha}$$

(4.12b)

Further defining:

$$c1 = m1e^{m1}$$

(4.13a)

$$c2 = m2e^{m2}$$

(4.13b)

$$c3 = J_1e^{m1}$$

(4.13c)

$$c4 = J_2e^{m2}$$

(4.13d)

$$c5 = \frac{(m1 - Pe)}{Pe}$$

(4.13e)

$$c6 = \frac{(m2 - Pe)}{Pe}$$

(4.13f)

$$D = (c2c6) + (c2c3) - (c1c6) - (c1c4)$$

(4.13g)

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we get the equations for R_1, R_2 and R_3 as:

$$R_1 = \frac{(c3c2) - (c1c4)}{D} \qquad (4.14a)$$

$$R_2 = -\frac{c2}{D} \qquad (4.14b)$$

$$R_3 = -\frac{c1}{D} \qquad (4.14c)$$

Using equations 10a and 10b the values for Cl and Cs can be calculated for different parameters along the length of the column.

4.3 Results and Discussions

From the equations derived analytically (4.10a and 4.10b), it is possible to obtain the liquid and the solid phase concentration of protein (Cl and Cs) along the length of the reactor. Figure 4.2a and 4.2b depicts these profiles for the solid and the liquid phases, with the values of α and K as 6.00 and 0.06 respectively, for both the ideal and the dispersed plug flow models. It can be clearly seen that binding to the solid phase in the reactor with dispersion is always less than the ideal plug flow model. Further, we can also conclude that if the dimensionless binding constant α is large and the dimensionless equilibrium constant K is less than one, the overall conversion in the liquid phase is larger in the lower half of the reactor and the separation will occur within a very short distance from the point of entry of the feed into the reactor.
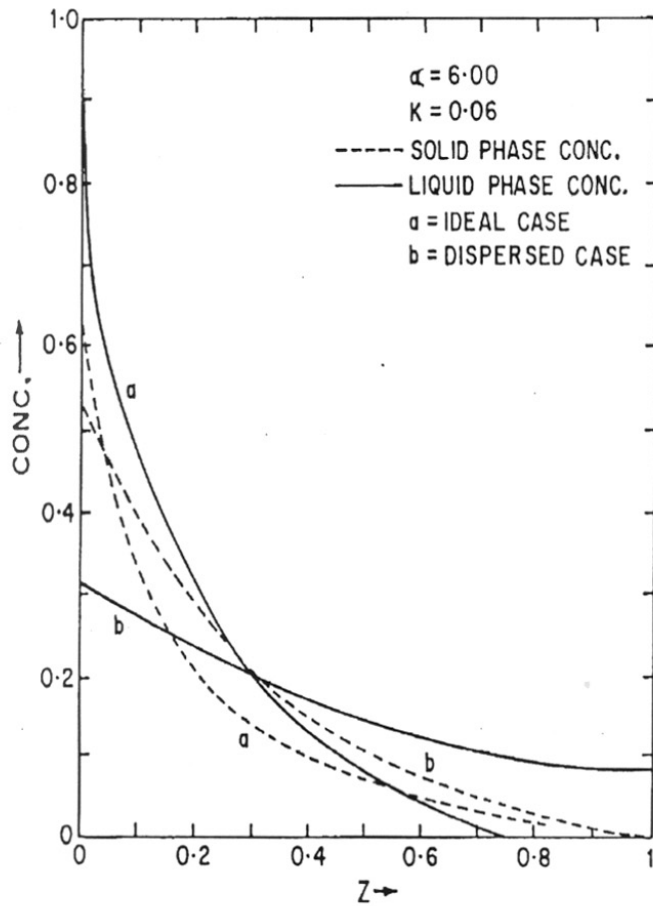
FIGURE 4.2 Concentration profiles for the solid and liquid phases in the reactor.

(a) depicts the ideal case
(b) depicts the dispersed case

For constant values of K less than one, an increase in the value of α will increase the efficiency of separation by increasing the binding of the solute to the solid phase. Similarly, for constant values of α, any decrease in the value of K will force more solute to bind on the matrix, thus increasing the value of concentration bound to the matrix at the exit point for the solid matrix (ie. Cs at $Z = 0$) in the reactor. Conversely, if the value of K is increased, keeping the value of α constant, the solute bound to the matrix at the exit for the solid phase in the reactor (Cs at $Z = 0$) is less.

The effects of α and K on the profiles are the same as observed in the ideal plug flow model. From equations 4.2a and 4.2b, it is seen that for a given column, for α to be large, the flow rate of the protein feed (V) should be small, while for the equilibrium constant (K) to be small the dimensionless constant β should be small. However a small α would mean a higher flow rate of the matrix (W). Therefore ideally suited for an efficient separation is a high W/V ratio. A high W/V ratio will further increase the dispersion. This dispersion in the liquid phase along the length of the reactor will affect the overall efficiency of the separation. Thus an optimum W/V ratio must be chosen to maximize the binding of the protein to the solid matrix.

Figure 4.3 shows the concentration profiles in the reactor for various Peclet numbers. While the exit conversion (i.e. solute binding to the matrix at $Z = 0$) decreases with increasing dispersion, an interesting feature is observed in the profiles inside the reactor. There exists a point (crl) at which the concentration of the protein in the liquid phase remains totally unaffected by the extent of dispersion. Such a crossover point (crs) is also observed for the concentration profiles plotted for the solid phase, although it occurs at a different position. Any variation in the dimensionless parameters causes a movement of crl and crs in the same direction along the length.

A detailed study of this movement of the crossover point as a function of system parameters was then made. It is evident from figures 4.4a and 4.4b that any increase in α, with a constant value of K, shifts the values of crs and crl to a higher position along the length of the reactor. Similarly, any increase in K, with constant α, shifts the magnitude of crs and crl to a lower position along the length of the reactor.

Each crossover point, for both the solid and the liquid profiles, for a given set of parameters, has some fixed position (Z) along the length and some finite concentration (Ccrs or Ccrl). A plot
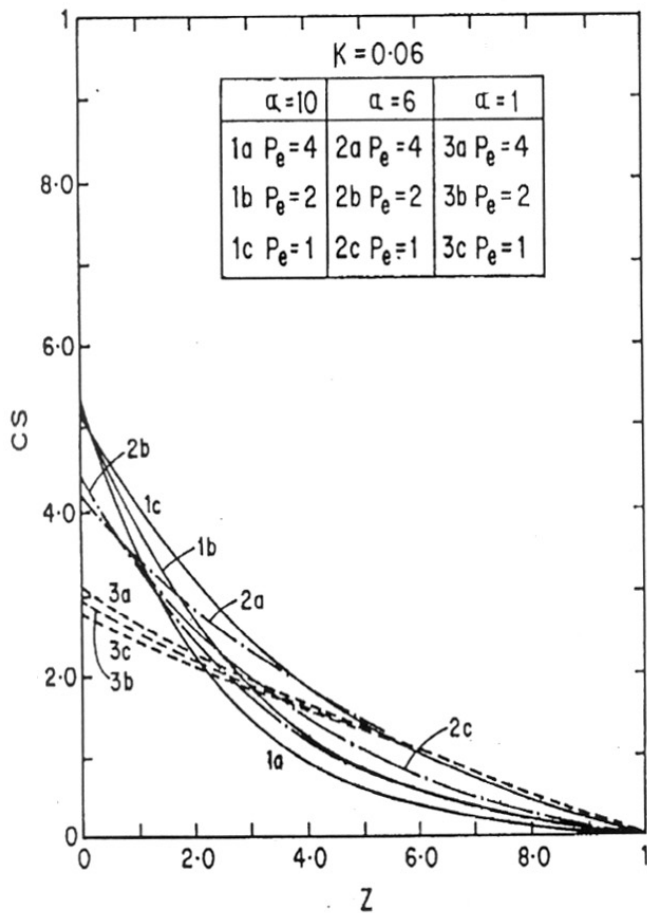
FIGURE 4.3A   Variation in the solid phase concentration profiles in the reactor with α as the parameter.
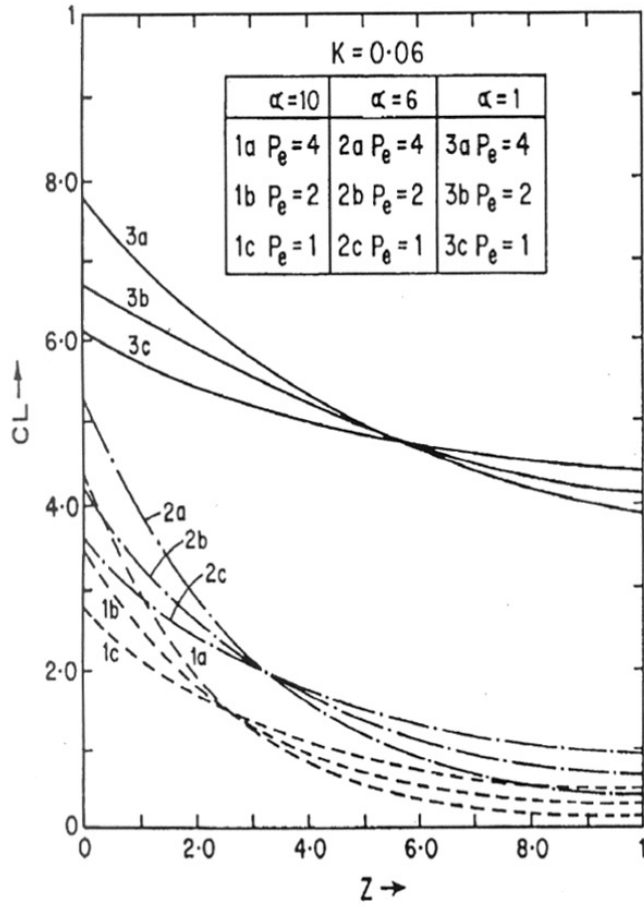
FIGURE 4.3B Variation in the liquid phase concentration profiles in the reactor with α as the parameter.
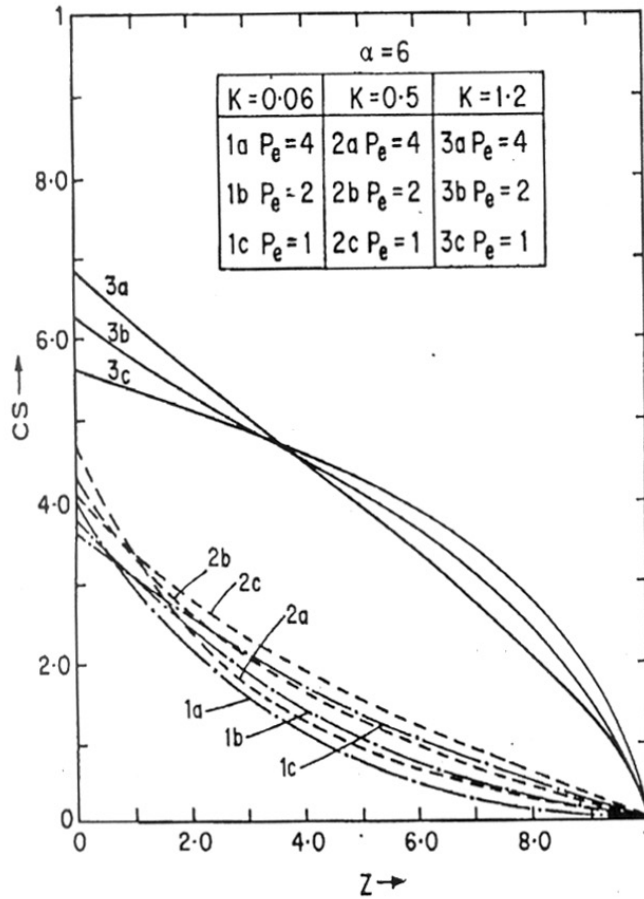
FIGURE 4.3C Variation in the solid phase concentration profiles in the reactor with **K** as the parameter.
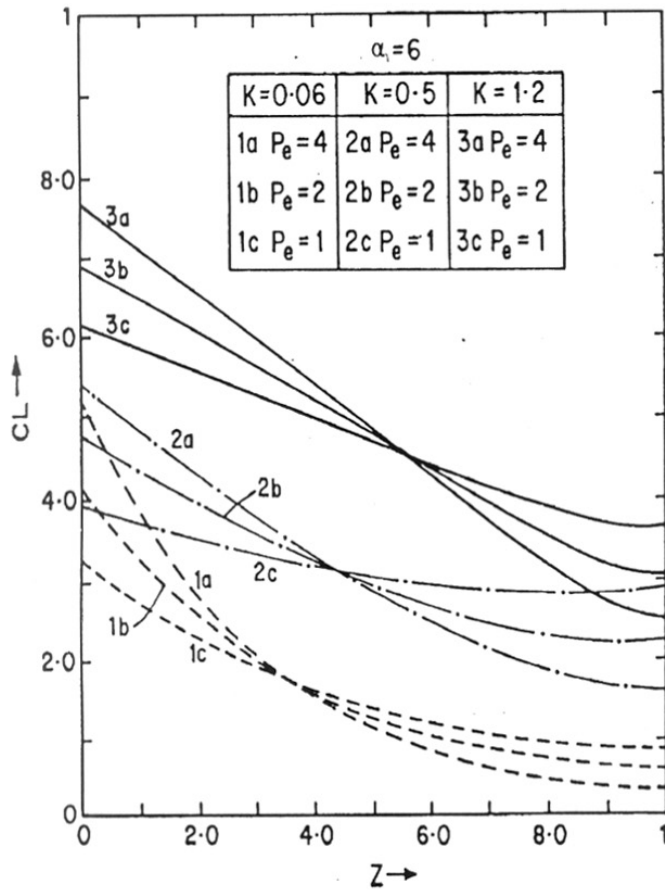
FIGURE 4.3D Variation in the liquid phase concentration profiles in the reactor with **K** as the parameter.
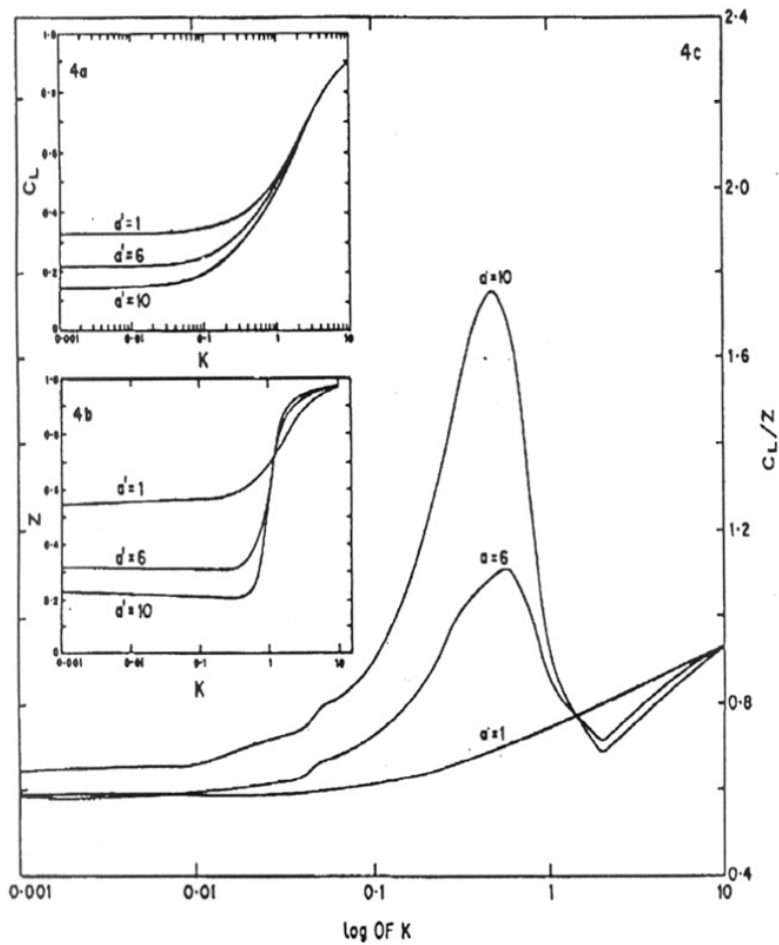
FIGURE 4.4 Liquids phase profiles for the variation of

    a)   the concentration at crossover point with the equilibrium constant

    b)   the position of the crossover point with the equilibrium constant

    c)   the crossover point and the ratio concentration/Z of the crossover point with the equilibrium constant.

of the position Z (at Ccrl) with α for various values of K is shown in figure 4.4a. A typical S shaped curve is observed for any particular value of α. A noteworthy feature of this profile is that for a range of about 0.0001 to 0.6 and about 2 to 10 the position of the crossover point remains nearly the same. However, in the range of about 0.6 to 1.5 there is a very sharp change in the position of the crossover point. As K increases, the crossover point moves to a higher position along the length of the column. The rate of increase is proportional to the value of α considered. Another important feature of the graph is the presence of another crossover point which indicates that at that value of K the dispersion in the column does not affect the separation. This value of K is found to be close to 1.2. If such a situation is considered a substantial amount of protein binding to the solid matrix is observed. This binding to the matrix, even when the reverse rate constant is greater than the forward rate constant, can be attributed to the simultaneous countercurrent movement of the solids and liquids and the bottom feed into the reactor.

If the concentration at the crossover point (Ccrs or Ccrl) for any constant value of α was plotted against the K (figure 4.4b), it was observed that the conversion at the crossover point is not greatly affected below a certain K. However, as K increases the conversion at the crossover point decreases. Figure 4.4c depicts the profiles of the ratio of (Ccrl/ Z) for any fixed value of α for various K. It is seen that a crossover point is present at K corresponding to about 1.2 This indicates that at this value of K, dispersion has no effect on the efficiency of the separation. Such profiles can also be plotted for the solid phase concentration and it is found that the trend is similar to that observed for the liquid phase profiles. The crossover points obtained above can be moved to any particular position desired in the reactor, depending on the system used, and therefore may be advantageously used for the optimum design of continuous affinity separation reactors.

The crossover point in the liquid and solid phase can be determined analytically by the equation:

$$\frac{d}{dPe}(R_1 + R_2 e^{m1} + R_3 e^{m2}) = 0 \qquad (4.15a)$$

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$$\frac{d}{dPe}(R_1 + J_1 R_2 e^{m1} + J_2 R_3 e^{m2}) = 0 \qquad (4.15b)$$

The parameters evaluated in the present study can fit in realistic cases, where the values of α can be made as high as 20 and K can take values of 10^{-3} to 10^{-10}, depending on the liquid and solid flow rates considered. Further the values of α, β and K can be maneuvered to suit the requirements of the system under consideration.

Thus, in conclusion, a parametric analysis has been conducted to gauge the effects of dispersion on the column performance. It is found that for a wide range of operating conditions of the system, dispersion can considerably reduce the binding of the protein to the matrix. Thus accurate estimation of dispersion and its incorporation in the mass balance equations is necessary for the realistic prediction of protein binding in continuous affinity separation columns. For the axially dispersed columns existence of crossover points in both the solid and the liquid profiles are detected. Depending upon the particular situations the crossover point can be advantageously manipulated such that it occurs at any required position in the column.

4.4   Nomenclature

A = the cross sectional area of the column

α = Dimensionless forward rate constant

β = Dimensionless reverse rate constant

c = concentration of the solute in the liquid phase

cf = concentration of the solute in the liquid phase feed entry

Cl = Dimensionless concentration of solute in the liquid phase

Cs = Dimensionless concentration of solute in the solid phase

$c1, c2, \ldots c6$ are the combined constants defined by equations 13 a,b,...f

crs = Crossover point for the solid profile

crl = Crossover point for the liquid profile

$Ccrs$ = Concentration at crossover point for the solid profile

$Ccrl$ = Concentration at crossover point for the liquid profile

D = Combined constant given by equation 13g

De = Dispersion coefficient

F_1 and F_2 = Combined constant given by equation 5b and 5c

J_1 and J_2 = Combined constant given by equations 12a and 12b

K = Dimensionless equilibrium constant

K_f = the forward reaction rate constant

K_r = the reverse reaction rate constant

L = Length of the matrix bed in the column

$m1$ and $m2$ = Combined constants given by equations 11a and 11b

n = Concentration of solute in the solid phase

Pe = Peclet number

R_1, R_2 and R_3 Combined constants given by equations

r = Combined constant given by equation 5a

t = Time

V = Liquid flow rate

W = Solid flow rate

Z = Dimensionless vertical position in the column

z = Vertical position in the column

Greek Symbols:

ε = Void fraction

CHAPTER 5

SILICALITE ZEOLITE
AS A MATRIX
FOR IMMOBILIZATION OF
HYDROPHOBIC PROTEINS

A simple and easy affinity purification of antibodies raised against a highly hydrophobic protein is described. This method employs silicalite zeolite, a solid inert matrix, to which prolamin is adsorbed. A rapid and sensitive immunoradiometric assay was developed to quantitate prolamin. The results also indicate that zeolites hold potential as ideal chromatographic separation matrices.

CHAPTER 5

5.1   Introduction

Chapter 4 dealt with a magnetite impregnated matrix which was fluidized in a magnetically stabilized bed reactor. This process could be utilized as an efficient and continuous affinity separation procedure. The present chapter deals with the use of zeolites as matrix for immobilization and estimation of a hydrophobic protein, prolamin.

Zeolites are crystalline microporous *tectosilicates* with framework based on infinitely extending three-dimensional networks of SiO_4 and AlO_4 units joined by sharing every corner oxygen atom. These framework structures contain channels and/or cages of definite dimension (which are comparable to the size of molecules) and are responsible for the sorption and molecular sieving property of zeolites. A unit negative charge is associated with every AlO_4 tetrahedron and is balanced by some cation in the channel of zeolites. These charges can often be freely interchanged giving rise to the ion exchange properties of zeolites. Furthermore, presence of strong intercrystalline electric field gradients can induce sorption of polar molecules. This, therefore, is responsible for the ability of some zeolites to act as dehydrating agents (Akolekar, 1987).

Silicalite is an aluminum free zeolite and a member of the isostructural ZSM-5 series (Lecluze and Sand 1980). This zeolite possesses a very high thermal stability and is stable in air upto 1373 K. It is stable to most mineral acids but reacts with HF in a way similar to quartz (Flanigen *et al*, 1978).

The development and wide usage of affinity chromatography depends upon the ready availability of support materials and facile methods to attach ligands and proteins on them. Many a times, standard methods of ligand or protein attachment fail on certain systems (Groman and Wilchek, 1987) and the reasons for this may vary from inability to attach the ligand/protein to a

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suitable matrix to denaturation of the ligand upon attachment to the matrix. An example of this is rice prolamin, which is an extremely hydrophobic protein and is difficult to separate and quantitate on matrices like sepharose, acrylamide, dextran etc.

The adsorption of proteins on solid surfaces has been demonstrated to be a necessary first step in the process of cellular biological adhesion and thus important in such areas of interest as immunoassays, tissue culture, biomaterial design and marine fouling.

Immunoassays have become an important tool in biomedical research and continuous efforts are being made to increase convenience, specificity and sensitivity of such assays. In recent years, seed proteins have also been analyzed by immunochemical methods which are extremely sensitive and specific. Prolamin is a major cereal storage protein and is hydrophobic in nature. In the present chapter, affinity purification of rice prolamin antibodies using zeolite as solid matrix is depicted. Silicalite zeolite is highly hydrophobic and organophilic and hence selectively adsorbs organic molecules over water (Flanigen *et al*, 1978). Also, an immunoradiometric assay is described for rapid quantification of seed prolamin from a single influorescence using silicalite zeolite as a solid matrix. It is expected that such a method will have an application in selecting and propagating plants with high prolamin content.

5.2 Materials and Methods

5.2.1 Seed Material

Seeds of rice (cv. Basmati 370) were obtained locally from Parekh Traders. Basmati 370 is a variety of superior quality based on its elongation on presoaking and cooking, its tenderness and its aroma. It has also been shown to have lower amylose content and gelatinization temperature (Mawal, 1989).

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5.2.2   Chemicals

All chemicals were obtained from SD's (India), E. Merck (India) or Glaxo (India), and were always of *Analar* or *Guaranteed Reagent* specifications. Sephadex G-50, Octyl-Sepharose, DEAE-Cellulose A-50, Sepharose CL-4B, CL-6B and standard proteins were obtained from Pharmacia (Sweden). N,N' methylene-bisacrylamide, TEMED (N,N,N',N' -tetramethyl ethylene diamine), ammonium persulphate, Trizma base, Glycine, Coomassie brilliant blue R-250, trypsin, ethyleneimine, ninhydrin, Phenyl isothiocynate and Guanidine hydrochloride were obtained from Sigma Chemical Co., (USA). Standard amino acid mixtures were obtained from Beckman (England).

5.2.3   Preparation of Seed Meal

A seed is a storage organ of plants accumulating considerable amounts of reserve food materials including lipids and fats. The fatty material must be eliminated before any of the seed storage proteins can be isolated on solubility basis. The seeds were finely powdered in a Remi make blender at maximum speed for 10-15 minutes and the fine powder was then packed into a column over a bed of anhydrous sodium sulphate. All fats and lipids were extracted by percolating n-hexane (approximately 5 volumes of the packed bed volume) (Mawal, 1989). After passing n-hexane, acetone was allowed to percolate through the column for removing all traces of n-hexane and moisture. The defatted seed was then allowed to dry at 60 °C to remove traces of acetone.

5.2.4   Purification of Rice Prolamin

Rice prolamin was isolated and purified as described in figure 5.1. After the removal of albumin and globulin, the residue was suspended in 70% ethanol for the extraction of prolamin. The supernatant obtained after centrifugation with 70% ethanol was mixed with 3 volumes of 50 mM Tris-HCl buffer, pH 8.0, containing 1 M NaCl and was centrifuged at 12,000 g for 15 min at

Flow sheet for purification of rice prolamin from defatted seed meal

(a)   **Partial purification on DEAE cellulose A-50 column.**

Defatted seed meal (200 mesh sieve)

|
| +   50mM Tris-HCl buffer (pH 8.00)
↓   containing 0.5 M NaCl and 1mM PMSF

Stir for three hours
Centrifuge, 1200 rpm, 10 min 4°C

Residue   Supernatant
(Prolamin and Glutelin) .   (Albumin and Globulin)

Extraction with 70 % ethanol, 37°C

Suspend in 3 volumes of 50mM Tris-HCl buffer (pH
8.00) containing 1 M NaCl

Centrifuge at 12,000 rpm for 15 min at 37°C

Supernatant   Residue (Prolamin)

                                                              (repeat cycle)

                                    Freeze and lyophilize ——

Dissolve (10 mg) in loading buffer (LB)
(50 mM Tris-HCl (pH 8.00) containing 8 M urea and
0.1 % β –mercaptoethanol)

Load on DEAE cellulose A-50 column (1.5 cm X 7 cm)
preequilibriated with LB

Allow to adsorb for 3 h and collect the unbound protein and dialyse extensively to
precipitate prolamin

(b)   **Purification on Octyl Sepharose CL-4B column.**

Dissolve DEAE Cellulose purified protein
(10 mg) in 70 % ethanol

Load on Octyl Sepharose CL-4B column (2.5 X 10 cm)
Wash unbound fraction with 70 % ethanol

The prolamin is eluted with 70 % ethanol containing 0.1 M HCl
at a flow rate of 25 ml/h. Pool peak fraction. Dialyse and Lyophilize.
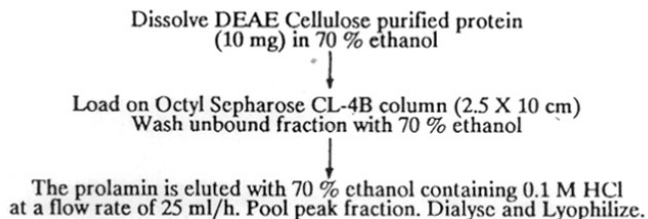
FIGURE 5.1

37°C. The pellet was suspended and then lyophilized. The pellet showed low solubility in 70% ethanol when redissolved. Hence, the pellet was resuspended in 3 volumes of 50 mM Tris-HCl, pH 8.0, containing 1 M NaCl and this slurry was centrifuged. The recycling improved the solubility to a very high extent. The recycled prolamin pellet was partially purified by DEAE cellulose A-50 column chromatography. In this method, the protein (10 mg) was dissolved in the loading buffer (50 mM Tris-HCl buffer, pH 8.0 containing 8 M urea and 0.1% β – mercaptoethanol and dialyzed for 3 hours against the same buffer at room temperature. The protein was then loaded on a DEAE cellulose A-50 column (1.5 cm x 7.0 cm) which was pre-equilibrated with the loading buffer. The protein was allowed to bind for 3 hours and the unbound protein fraction was washed out using the loading buffer. This fraction was then dialyzed extensively against water. Under these conditions the prolamin precipitated (since it is water insoluble).

The DEAE cellulose A-50 purified protein (10 mg) was dissolved in 70 % ethanol and then loaded on Octyl-Sepharose column (2.5 cm x 10.0 cm) pre-equilibrated with 70% ethanol. The unbound fraction was washed with 70% ethanol and prolamin was eluted with 70% ethanol containing 0.1 M HCl at a flow rate of 25 ml/hour. The column eluent was monitored continuously at 280 nm and the peak fractions were then pooled, dialyzed and lyophilized.

5.2.5   Synthesis of Zeolite S-14

Zeolite S-14 (TEBAOH-Silicate) was synthesized according to Choudhary and Akolekar (1988). The morphology of the zeolite crystal was determined by using a Cambridge Stereoscan Model 150 scanning electron microscope. The silicate was analyzed for its crystalline nature by X-ray powder diffraction methods using a Phillips X-ray generator with a Ni-filtered CuK radiation source and a scintillation counter.

5.2.6   Iodination of Prolamin

During conventional iodination, the protein is dissolved in PBS and is iodinated using iodogen. Rice prolamin, a highly hydrophobic protein, is soluble in alcohol (70% ethanol) while with the decrease in ethanol concentration the protein precipitates. With this in view, prolamin (50 μg) was labelled with 200 μCi of Na^{125}I (specific activity 13-17 m Ci/μg) in the presence of iodogen for 10 min on ice in a total volume of 100 μL. Iodination under these conditions was optimum and overall incorporation did not increase beyond 10 min. The addition of BSA (400 μL, 2% BSA in PBS) resulted in precipitation of prolamin which later redissolved by itself during dialysis against 70% ethanol. The iodinated purified prolamin gave a single spot at 8.3 kDa on SDS electrophoresis (Mawal 1989).

5.2.7   Affinity Purification of Rice Prolamin Antibodies

Antibodies against rice prolamin were raised in New Zealand white rabbits as described in section 2.2.2.

Zeolite S-14 was packed in a column (1.5 cm X 5 cm) and washed thoroughly with 70 % ethanol. Rice prolamin (10 mg in 1 ml of 70 % ethanol) was loaded on the column and allowed to equilibrate for one hour. Unbound prolamin was washed with 70 % ethanol. One ml of serum was passed over the column and allowed to equilibrate for 30 mins. Unbound antibodies were washed out with phosphate buffered saline (PBS, 0.1 M Na$_2$HPO$_4$, 0.4 M NaH$_2$PO$_4$, 0.8 % NaCl, pH 7.2). The antibodies were eluted with 0.3 M glycine, pH 3.5, and the eluate immediately neutralized by adding solid Tris.

5.2.8   Immunoradiometric Assay

Antibodies were iodinated as described in section 5.2.6. Rice prolamin of varying concentrations were incubated with S-14 zeolite for 1 hour at 37°C. Unbound antigen was removed after

spinning the zeolite matrix in an eppendorf at 10,000 g for 2 mins. To this matrix, radiolabelled antibodies (approximately 10,000 cpm) are added and incubated for one hour at 37°C. Any traces of unbound antibody were removed by washing with PBS. The antibodies bound were measured on Clinigamma counter 1272.

5.3   Results and Discussions

5.3.1   *Affinity purification of Prolamin antibodies*

In an RIA which is intended for the examination of crude extract of plant materials, it is of utmost importance to use purified antibodies. Prolamin is a highly hydrophobic protein (Mawal *et al*, 1989) and thus the affinity purification is hampered. A simple solid matrix, S-14 zeolite is of great importance in helping the purification of antibodies as it itself is a highly hydrophobic matrix and binds very strongly to rice prolamin. The optimal conditions for attaining high binding was an incubation period of 30 mins and no difference was observed with an increase in time. As the antibodies were very specific for prolamin, it can now be used in immunoradiometric assay. The possible mechanism of attachment of prolamin to zeolite may, in addition to hydrophobic interactions, be the penetration of certain unbranched *chain-like* or phenyl R-groups of amino acids (Akolekar, 1987).

5.3.2   *Application of Immunoradiometric assay*

Using iodinated rice prolamin antibodies a standard assay was established for varying concentrations (1 to 1000 ng) of unlabelled antigen (Figure 5.2). From this figure it is clear that the lower limit of detection of prolamin is 10 ng.

Since prolamin constitutes a major class of proteins in most cereals, this standard immuno-radiometric assay was used for quantitation of prolamin during seed germination. The results which are obtained are depicted in figure 5.3 indicate that rice prolamin was synthesized optimally around 16 days post anthesis and this results were similar to those obtained by radioimmunoassay of rice prolamin as reported earlier (Mawal *et al*, 1989). In this method, the optimal concentration as suggested by immunoradiometric assay is between 2 and 400 ng (Mawal *et al*, 1990). Furthermore, a high concentration of protein during coating leads to increased desorption during incubation and immunoreaction. This sometimes gives rise to undesirable prozone phenomena. In conclusion, it is suggested that if affinity purified antibodies are used the sensitivity of the assay will increase and such assays will open a new area of technology for radioimmunoassay.

Affinity purification and immunoradiometric assay of a hydrophobic protein, rice prolamin, are reported for the first time. This method can be used as an assay for quantitation of rice prolamin during development. Furthermore, zeolites can also be used as matrices for adsorption of other hydrophobic proteins.
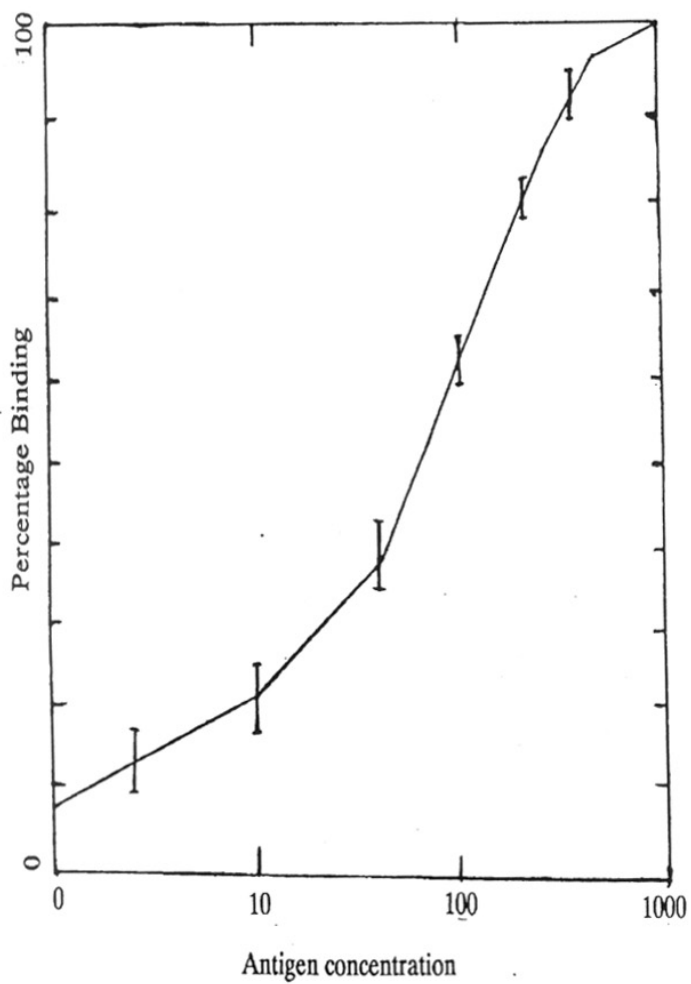
FIGURE 5.2 Profile depicting the variation in the percentage of iodinated rice prolamin antibodies adsorbed as a function of antigen concentration.

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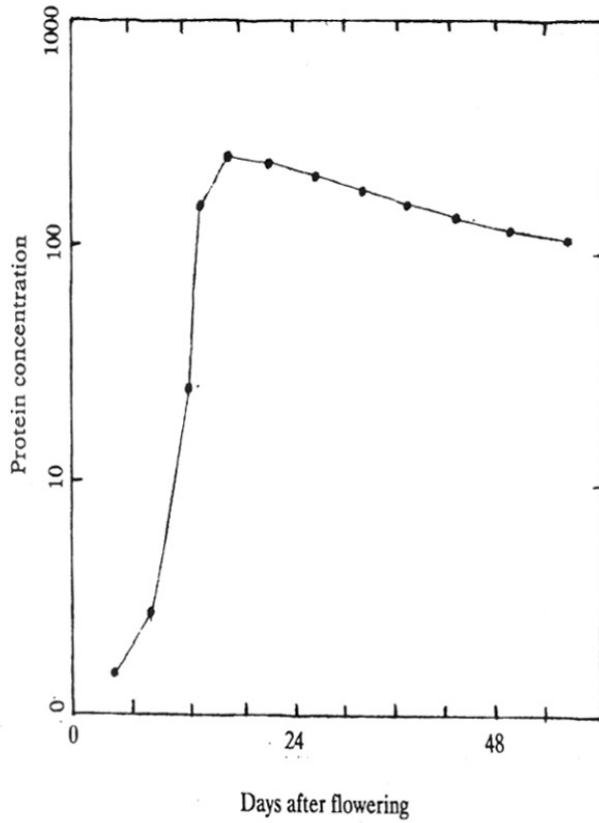
FIGURE 5.3 Application of the radiometric assay in quantitating the extent of prolamin produced in rice seeds at specific days post anthesis. Each value represents an average of three experiments at each stage of development.

ASPECTS OF
PROTEIN TRANSLOCATION
ACROSS BIOLOGICAL MEMBRANES

Protein synthesis takes place in the cytoplasm. Some of these proteins, either after their synthesis or some time during their synthesis may have to cross certain biological membranes. An important constituent of the apparatus responsible in process of protein translocation is a small peptide called either the signal peptide or leader peptide. The present chapter reviews the structural organization of this peptide and its interactions with other components of the translocation apparatus mainly from the structure-function relationship. Various models of protein translocation are also discussed critically.

CHAPTER 6

6.1　　Introduction

An average cell synthesizes 10^3 to 10^4 different types of polypeptides, each of which must reach a specific intracellular location to perform its function. Some proteins are inserted into a specific membrane. Others pass through one or even several membranes to reach their destination. Which signal tells a protein where to go? How are these signals decoded? And how can a protein with many of its hydrophilic groups penetrate a membrane? The present chapter introduces aspects of protein translocation across membranes. More specifically, membranes which are translocation competent, the signals responsible for translocation, components of the translocation apparatus, the conformations acquired by the signal peptide and the role of specific proteases in the entire process are discussed. Furthermore, different models for protein translocation and the way the signal peptides interact with different components of the translocation apparatus have been discussed.

6.2　　Membranes Capable of Protein Translocation

Of the many known biological membranes, only a subset of these can translocate proteins. These are the endoplasmic reticulum (ER), the peroxisomal membrane, the bacterial plasma membrane, the inner membrane of the mitochondria, and the inner and thylakoid membrane of the chloroplasts (Gierasch, 1989; Verner and Schatz, 1988; de Vrije, 1990). Of these, the mitochondrial membrane is unique in that it can transfer proteins in both directions.

The smooth endoplasmic reticulum, the Golgi complex, secretory vesicles, endosomes, lysozomes, the plasma membrane, and perhaps also the nuclear inner membrane are translocation incompetent and arise by differentiation of the rough endoplasmic reticulum. It is

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still unclear whether the outer membrane of mitochondria, chloroplasts, and gram negative bacteria are translocation-competent by themselves, since they are attached to the corresponding inner membrane through characteristics *contact sites* (Hackenbrock, 1968). Outer membrane proteins might first be inserted into these sites and subsequently be sorted into the outer membrane.

6.3   Signals Responsible for Protein Targeting

The signal directing a protein to its target membrane is usually a short stretch of amino acids at or near the NH_2-terminus of the protein itself (Blobel and Sabatini, 1971; Milstein *et al*, 1972; Blobel and Dobberstein, 1975). Many peroxisomal and a few mitochondrial targeting signals, however, are at the COOH-terminal (Gierasch, 1989). Most of the amino terminal sequences, often called as signal sequences or leader sequences, are proteolytically removed by specific proteases called as signal peptidases. These proteases are located on the trans side of the target membrane. The targeting signal need not always be proteolytically removed from the precursor and when removal is blocked, in many cases, the precursor is still translocated (von Heijne, 1979; Beckwith and Silhavy, 1983; Schatz, 1987).

When targeting signals are deleted or suitably altered, the protein remains in the compartment where it was synthesized (Bassford *et al*, 1979; Emr *et al*, 1980; Ferenci and Silhavy, 1987). Conversely, when a signal peptide is fused to the NH_2-terminal of a cytoplasmic protein, the fusion protein is usually translocated across the same target membrane as the precursor that served as the source of the targeting signal (Ellis, 1985). However, not all fusion proteins translocate efficiently. This may be due to the fact that the *passenger protein* may interfere with a heterologous targeting signal (Talmadge *et al*, 1980; Lingappa *et al*, 1984). Generally, a specific interaction between two proteins involves complementary

steric fit of the surfaces at the interface and thus requires particular amino acids at precise positions in the structure of both proteins. Since signal peptides for a specific target membrane lack significant primary sequence homology, the information must be encrypted as a common secondary or tertiary structure that should differ between precursors targeted to different membranes (Randall *et al*, 1987).

Although, given the evidence available, we must conclude that the primary structure is not directly involved in the role of signal peptides, it does not imply that the signal peptides do not mediate the interaction of precursors with some protein. However, this interaction may be mediated in an unconventional manner. Consider the possibility that a factor might bind precursors via the backbone irrespective of the side chains and the role of the leader might be to retard folding so that the backbone would remain exposed. Although such an interaction might seem improbable, a precedent for this proposal is the binding by the protease La (product of the *lon* gene) of its denatured substrate (Waxman and Goldberg, 1986).

Most of the signal peptides belong to one of two groups. The first *(hydrophobic)* group has a tripartite structure: a relatively hydrophilic NH_2-terminus with one or two basic residues; a hydrophobic core of seven to eight amino acids residues; and a relatively hydrophilic COOH terminus often ending with an amino acid carrying a small side chain. (von Heijne, 1981; Watson, 1984; von Heijne, 1985). These hydrophobic signals target proteins across the endoplasmic reticulum and bacterial membranes. Although bacterial and endoplasmic reticulum signal sequences differ slightly from each other, they are functionally interchangeable (Watts *et al*, 1983).

The second *(hydrophilic)* group lacks uninterrupted stretches of hydrophobic residues, is rich in basic and hydroxylated residues, and contains few, if any, acidic residues. These hydrophilic signals target proteins into mitochondria and chloroplasts (Schatz, 1987; Roise and Schatz, 1988) and possibly into the peroxisomes (Gould, *et al*, 1987; Small *et al*, 1988;

Swinkels *et al*, 1988). The targeting signals for chloroplasts and mitochondria appear to differ slightly in their COOH-terminal regions, but are partly interchangeable at least under certain conditions. The two thirds of a NH_2-terminal of a chloroplast presequence can direct an attached passenger protein into yeast mitochondria *in vivo* although the efficiency of targeting is low (Hurt *et al*, 1986). There, however, seems to be very little missorting if any of proteins among mitochondria, chloroplasts and the peroxisomes (Boutry *et al*, 1987).

The question as to how targeting signals with different primary sequences direct proteins into the same membrane has always been an intriguing one. One common feature noted with some of the chemically synthesized bacterial and mitochondrial signal peptides is their amphiphilicity (Roise and Schatz, 1988; Briggs *et al*, 1985). The bacterial signal peptides have a hydrophobic moment in the COOH --> NH_2 direction, with their NH_2-terminus as the polar domain. The mitochondrial signal peptides are amphiphilic only when they are folded into a secondary structure with the hydrophobic moment in a direction perpendicular to their long axis. Several observations have indicated the importance of this amphiphilicity in proper functioning of protein translocation (von Heijne, 1988).

That targeting signals are amphiphilic does not preclude the participation of protein catalysis in translocation. These sequences, due to their amphiphilicity, can mediate protein-protein interactions (Popot and Engelman, 1990). Indeed, other proteins are involved in the process of translocation. How the amphiphilicity of the presequences contribute to their targeting function is still a mystery.

The degeneracy of these signal sequences suggests that they arose during evolution by point mutations or DNA transpositions. This is particularly relevant for mitochondrial signal sequences since most of the proteins imported into present day mitochondria may be descendants of proteins originally synthesized within endosymbiotic mitochondrial ancestors (Gray and Doolittle, 1982). Experiments in yeast mitochondria have indicated that a

plasmid-encoded protein lacking a mitochondrial targeting sequence can acquire one by point mutations in the mature sequence or by local DNA arrangements or by mutations that add a new NH_2-terminal domain to the protein (Vassarotti *et al*, 1987; Bibus *et al*, 1988). Although most of these newly created signals are only weakly active, they could presumably be improved by further mutation and selection.

6.4   Components of Protein Translocation Apparatus

Protein translocation *in vitro* is stimulated by cytosolic proteins and inhibited by treating the cytosolic face of the target membrane with proteases (Wickner and Lodish, 1985; Imanaka *et al*, 1987). In microbial systems, protein translocation can also be blocked by mutations of genes encoding soluble or membrane proteins (Beckwith and Ferro-Novick, 1986; Deshaies and Schekman, 1987; Yaffe and Schatz, 1984). In humans, Zellweger's disease appears to be caused by a single recessive mutation that abolishes the import of proteins into the peroxisomal matrix (Santos *et al*, 1988). This suggests that protein translocation across all translocation competent membranes is mediated by receptor-like systems that involve proteins in the cytosol and on the target membrane. Studies on mammalian endoplasmic reticulum have identified four distinct components of a receptor like system (Walter *et al*, 1984; Gilmore and Blobel, 1985). The first one is a ribonucleoprotein particle called as the signal recognition particle, or SRP, which binds to the signal peptide as it emerges from the ribosome, resulting in a ternary complex called as the 70mer translation arrested complex. The second is a 72-kD integral endoplasmic reticular protein known as the SRP-receptor or the docking protein (Meyer *et al*, 1982; Gilmore *et al*, 1982). This protein is responsible for the release in translation arrest thereby allowing the signal sequence to interact with the third component, a 35 kD integral membrane glycoprotein protein called the signal sequence receptor (Wiedmann *et al*, 1987).

We know almost nothing about the subsequent translocation steps except that most proteins are simultaneously elongated and translocated through a membrane site that is accessible to hydrophilic protein denaturants such as urea (Walter *et al*, 1984; Gilmore and Blobel 1985). In the yeast *Saccharomyces cerevisiae*, however, some proteins (such as the pheromone precursor, prepro-α-factor) can be translocated as completed polypeptide chains (Hansen *et al*, 1986; Rothblatt and Meyer 1986; Waters and Blobel, 1986). It is therefore interesting that an SRP-like component has not been detected in yeast, although *Schizosac- charomyces pombe* contains an essential 7S RNA that is homologous to the 7S RNA in mammalian SRP (Brennwald *et al*, 1988). Instead, 70-kD heat-shock proteins (and probably additional proteins) are components of the endoplasmic reticulum translocation machinery in yeast. Translocation of prepro-α-factor is stimulated by purified 70 kD heat-shock proteins in a cell-free yeast system, and arrested by genetic elimination of at least three 70 kD heat-shock-like proteins *in vivo* (Deshaies *et al*, 1988; Chirico *et al*, 1988). Elegant studies on yeast mutants, which are temperature sensitive for translocation, into the lumen of the endoplasmic reticulum have identified novel genes that appear to encode components of the yeast endoplasmic reticulum translocation system (Deshaies and Schekman, 1987). Some of these may be the yeast equivalents of known components of the mammalian endoplasmic reticulum translocation system, but others may be new, and thus enhance our understanding of the endoplasmic reticulum translocation system.

In *Escherichia coli*, elegant genetic experiments have identified many genes whose mutations can interfere with protein export from the bacterial cell (Beckwith and Ferro-Novick, 1986). However, many of these mutations have been difficult to interpret because

    (i) they affect export of only a subset of proteins;

    (ii) they are in genes encoding proteins (such as periplasmic ribose binding proteins) whose involvement in protein export is difficult to rationalize;

(iii) null mutations in some of these genes do not alter the cells'
phenotype;

(iv) until recently, biochemical analysis of these mutants was difficult, because
efficient *in vitro* systems for studying protein translocation across bacterial
membranes were not available.

However, at least two *E. coli* genes encode bonafide components of the bacterial protein
export machinery: *sec A*, encoding a 92 kD cytosolic protein, and *sec Y*, encoding a 42 kD
integral membrane protein (Oliver and Beckwith, 1981). No bacterial SRP equivalent has
been found. Instead, several other cytosolic protein factors promote protein translocation
across bacterial membranes *in vitro* (Mueller and Blobel, 1984; Fecycz and Blobel, 1987;
Weng *et al*, 1988). One of these (termed *trigger factor*) is associated with ribosomes and
retards refolding of a urea denatured bacterial precursor protein *in vitro* (Crooke and Wickner,
1987). Some of these factors may thus be functionally similar to the heat shock like proteins
in the eukaryotic system. Indeed, cytosolic fractions from yeast and *E. coli* can substitute for
each other in reconstituted systems (Fecycz and Blobel, 1987; Weng *et al*, 1988).

Less is known about protein translocation into eukaryotic organelles. Translocation of
many (but not all) protein precursors into mitochondria is stimulated by cytosolic factors
(Attardi and Schatz, 1989), which appear to include the same 70 kD heat-shock proteins that
mediate protein translocation across the endoplasmic reticulum (Deshaies *et al*, 1988; Chirico
et al, 1988)). Import also requires proteins on the mitochondrial surface (Attardi and Schatz,
1989), which may be concentrated at sites of close contact between the two mitochondrial
membranes (Kellems *et al*, 1975; Schwaiger *et al*, 1987). In chloroplasts, anti-idiotypic
antibodies against antibodies recognizing the presequence of an imported chloroplast protein
inhibited protein import into chloroplasts and identified a 31 kD protein at contact sites
between the two envelope membranes (Pain *et al*, 1988).

No unifying picture of these receptor systems can yet be drawn. For example, some precursor proteins insert into the mammalian endoplasmic reticulum without the aid of SRP/docking protein, or the bacterial plasma membrane without *sec A* or *sec Y*, respectively (Oliver and Beckwith, 1981; Wickner, 1988; Schlenstedt and Zimmermann, 1987; Mueller and Zimmermann, 1987); docking protein inserts into the endoplasmic reticulum by yet another route which is as yet poorly characterized (Hortsch and Meyer, 1988); Some mutations in the signal sequence of bacterial precursor proteins block not only translocation, but also synthesis of these proteins even though bacteria appear to lack an SRP like component. Many of these puzzles may reflect the existence of several parallel pathways which need not be mutually exclusive.

Despite these uncertainties, it appears that proteins destined to be translocated across membranes are generally recognized by receptor like components in the cytosol and on the target membrane. In the mammalian endoplasmic reticulum system, some of these components act sequentially, which may greatly enhance the specificity of the system. Similar multistep *filters* may operate with all translocation-competent membrane systems. As described below, some of these receptors may not only select proteins destined for translocation, but may also maintain, or generate, a translocation-competent conformation.

6.5   Signal Peptide Conformation and Translocation Competence

Proteins cannot be translocated through a membrane in a tightly folded state (Randall and Hardy, 1986; Schleyer and Neupert, 1985; Eilers and Schatz, 1986; Eilers *et al* 1988; Muller and Zimmermann, 1988).

(i) Maltose binding protein is exported to the *E. coli* periplasm only while it exists in a protease sensitive (presumably loosely folded) conformation in the cytosol.

(ii) Precursor proteins trapped during their import into isolated mitochondria by low temperature or antibodies against the *mature* moiety appear to be partly extended, with their NH_2-termini exposed to the matrix and at least part of their mature moiety exposed on the mitochondrial surface.

(iii) Import of precursor proteins into isolated mitochondria is blocked by ligands that stabilize the native conformation of the mature moiety.

(iv) Denaturation of some precursor proteins with urea accelerates their translocation across membranes.

(v) Progressive destabilization of a precursor protein by point mutations in the mature moiety progressively accelerates its import into isolated mitochondria.

(vi) Precursors whose mature moiety is extensively cross-linked by internal disulphide bridges are not translocated across membranes.

A translocation competent loose conformation may be achieved by several mechanisms. In the simplest case, the presequence itself may interfere with tight folding. Indeed, the precursor forms of two exported *E. coli* proteins (maltose-binding protein and ribose-binding protein) refold more slowly after denaturation than the corresponding mature proteins (Park *et al*, 1988). However, some fusion proteins that are imported by isolated mitochondria are folded as tightly, and refold with similar kinetics, as the corresponding presequence-free proteins (Endo and Schatz, 1988). Also, refolding of maltose binding protein in the *E. coli* cytosol is retarded by *prl A* suppressor mutations, suggesting that the presequence is not the only factor in slowing down refolding *in vivo*. Interactions of a nascent precursor with the translocation machinery may thus represent a second mechanism for ensuring a translocation competent conformation: tight folding would be prevented from the outset. A third mechanism may be energy dependent partial unfolding of precursors in the cytosol or on the target

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membrane. Adenosine-5'-triphosphate (ATP) is necessary for posttranslational translocation of proteins across all known membrane systems. Its possible role in changing the conformation of precursor proteins was first suggested by the observation that COOH-terminally truncated, incompletely folded nascent chains of an artificial mitochondrial precursor protein were imported by isolated mitochondria in the absence of ATP, whereas import of the completed, tightly folded precursor was ATP dependent (Verner and Schatz, 1988). Denatured or internally deleted precursor proteins, or isolated signal peptides also require little or no ATP for import into isolated mitochondria (Chen and Douglas, 1988).

An ATP dependent *unfoldase* (Rothman and Kornberg, 1986) that participates in protein translocation across the membrane has not yet been identified. Unfolding may be mediated by heat-shock-like proteins, which selectively bind partly denatured proteins and release them again in the presence of ATP (Pelham, 1986). In some instances, unfolding may also be mediated by acidic phospholipids on the target membrane (Endo and Schatz, 1988).

Thus, more than one system may be involved, depending on the type of membrane system and the type of precursor studied.

6.6   Role of Proteases in Protein Translocation

At least two distinct peptidases are involved in protein translocation. They are

(i) signal peptidases which are responsible for cleavage of the signal peptide from the precursor protein and

(ii) Signal peptide peptidases which are responsible for the degradation of the signal peptide in the membrane

6.6.1 *Signal Peptidase*

Signal peptidases are responsible for the specific removal of signal peptides from the amino terminus of most secretory proteins. The removal of the signal peptide by the membrane bound endopeptidase serves to release the secretory protein from the membrane and make the transfer an irreversible event (Ray *et al*, 1986). The signal peptide cleavage site has been well characterized and identified as a tripeptide. Signal peptidases of bacterial origin (*E. coli*) have been more extensively characterized than eukaryotic enzymes.

6.6.2 *Bacterial Signal Peptidase I*

E. coli signal peptidase I was first described by Mandel and Wickner (1979), who observed specific processing of the precursor of M13 phage major coat protein (precoat) by an enzyme located in the cell envelope. The enzyme was overproduced, purified and was found to be an inner membrane protein with a molecular weight of about 36,000 (Pugsley, 1989). The precoat was found to be processed by the signal peptidase either in a detergent soluble form or in proteoliposome (Geller and Wickner, 1985), and the enzyme also correctly processed other proteins of *E. coli in vitro* (Pugsley, 1989). A further indication that most *E. coli* secretory protein precursors are processed by signal peptidase I is that this enzyme is inhibited *in vitro* by purified signal peptides.

Signal peptidase has two transmembrane domains. One of these is the C-terminus which includes the catalytic domain and is exposed to the periplasm (Dalbey and Wickner, 1985; Moore and Miura, 1987). This corresponds with the observation that cleavage takes place only when precursors have penetrated inside the cytoplasmic membrane (Pugsley, 1989).

6.6.3 Bacterial Lipoprotein Signal Peptidase

A limited number of secretory proteins produced by both gram-positive and gram-negative bacteria are unique in that they have a Cys residue at a position +1 which must be acylated before processing by signal peptidase can occur. The enzyme which recognizes these sequences has been called as signal peptidase II or bacterial lipoprotein signal peptidase (Ichihara *et al*, 1981) Antibody studies on signal peptidase I and II have confirmed that the two signal peptidases are distinctly different and do not have overlapping substrate specificities.

E. coli lipoprotein signal peptidase is a 18-kD cytoplasmic membrane protein (Dev and Ray, 1984). Sequence analysis has indicated that it has four probable transmembrane domains and the catalytic domain is located in the periplasm. Lipoprotein signal peptidase is unique to bacteria (Tokunagu *et al*, 1983).

6.6.4 Other Bacterial Signal Peptidases

Although the combined activities of the above described peptidases account for all signal peptidase requirements in *E. coli*, a third enzyme with the same specificity as signal peptidase I has been purified and tentatively named as signal peptidase III (Ray *et al*, 1986). The specific activity of this enzyme is about 500 times higher and its molecular weight is almost two times that of signal peptidase I. The role of this peptidase is still unknown.

6.6.5 Eukaryotic Signal Peptidase

Eukaryotic signal peptidases have been characterized in relatively crude detergent extracts of microsomal membranes (Fujimoto et al, 1984). Many of the secretory proteins are not processed by the extracted enzyme, probably because once released they fold into conformations which cannot be recognized by the enzyme.

The catalytic site of the eukaryotic signal peptidase is located on the trans, noncytoplasmic face of the membrane (Yadeau and Blobel, 1989). Although the signal peptidase is located exclusively in the rough endoplasmic reticulum membrane, smooth microsomes can also process secretory protein precursors. It is not known how these two peptidases are different from one another (Tartakoff and Vassalli, 1983).

6.6.6 Signal Peptide Peptidases

Once the signal peptide is cleaved from the precursor protein, it must be degraded rapidly (Zwizinski and Wickner, 1981; Habener et al 1979). The reasons for this are

(i) Free signal peptides might prevent productive interaction between signal peptides on other secretory proteins and the bacterial cytoplasmic membranes and rough endoplasmic reticulum membranes (Briggs et al, 1986);

(ii) Free signal peptides have been shown to have deleterious effects on lipid monolayers and bilayers in vitro and therefore may exhibit similar effects in vivo if they were to accumulate in large amounts (Austen et al, 1984; Chen et al, 1987; Koren et al, 1983; Wickner et al, 1987).

Protease IV and Oligopeptidase A are the most well studied hydrolyases. These proteases do not degrade the signal peptide while it is still attached to the mature protein (Ichihara et al, 1984; Novak et al, 1986, Wickner et al, 1987). To explain this observation, it was suggested

that these enzymes are carboxypeptidases and require a free carboxy-terminal end of the signal peptide to initiate degradation (Ichihara *et al*, 1984; Ichihara *et al*, 1985; Novak *et al*, 1986, Wickner *et al*, 1987). However, it was elegantly shown by Novak and Dey (1988) that both these enzymes are endopeptidases and their results support the hypothesis that Protease IV is the major signal peptide peptidase that initiates degradation and that subsequent degradation is carried out by other cytoplasmic or membrane associated proteases.

6.7 Existing Models For Protein Translocation

6.7.1 The Signal Hypothesis

This hypothesis was proposed by Blobel and Dobberstein (1975) to explain secretion and integration of proteins inside membranes. In this process, the m-RNA binds to the ribosome and protein synthesis is initiated in the cytoplasm. After about 80 amino acids of the protein have been synthesized, the signal peptide protrudes from the base of the ribosome. At this point, the signal recognition particle binds to the signal sequence leading to a translation arrested complex called as the *~70-mer* complex. This arrested complex then moves towards the membrane and interacts with a membrane associated protein, called as the docking protein or the signal recognition particle receptor, which induces a release in the translation arrested complex allowing for protein translation to proceed. Protein synthesis continues and the protein traverses through a pore formed by certain other membrane proteins. During or after translation, the signal peptide is cut by the signal peptidase. Release of the completed protein causes detachment of the ribosome from the endoplasmic reticulum and m-RNA closing of the membrane pore.

The signal hypothesis can describe the process of secretion as well as insertion of proteins inside the membrane. Evidence for this hypothesis was derived from extensive experimentation in eukaryotic systems.

6.7.2   The Membrane Trigger Hypothesis

Wickner (1980) proposed a mechanism for protein translocation called the *membrane trigger hypothesis*. It was proposed by him that the signal peptide influences the precursor to fold into a conformation that can spontaneously insert inside the hydrophobic core of the membrane. In prokaryotes the membrane potential causes the protein to traverse the membrane bilayer. The protein then regains its water soluble form and is expelled into the medium. Signal peptidase removes the signal peptide from the precursor after this process. Thus secretory proteins or domains are transported across the membrane posttranslationally without the aid of a proteinaceous secretory apparatus. An energy source such as the membrane potential is required for such a mechanism of protein translocation.

6.7.3   The Loop Model

Based on the analysis of amino acid sequences of signal peptides, Inouye and Halegoua (1980) proposed the loop model of protein translocation. In this mechanism, positively charged residues at the amino terminus of the signal peptide bind to the negatively charged phospholipid head groups at the membrane surface. The proline and glycine residues induce a β-turn in the signal sequence such that it can enter the membrane as a loop. As the peptide elongates, the loop protrudes further into the membrane. The cleavage site is eventually located at the outer face of the cytoplasmic membrane, while the charged amino terminus remains anchored at the inner face. This hypothesis is supported by work done on β-galactosidase and

a lipoprotein signal sequence (Hayashi *et al*, 1985; Perreria and Lingappa, 1986).

6.7.4   *The Helical Hairpin Hypothesis*

This hypothesis was proposed by Engelman and Steitz (1981), which along with the direct transfer model of von Heijne and Blomberg (1979), shares an emphasis on the thermodynamic basis of secretion. This mechanism involves two helical arms which are long enough to span the membrane in either an α-helical or a 3_{10} helix. One arm is composed of the signal sequence and the other arm of 15-20 amino acid residues of the mature protein. The two arms form a side by side *hairpin-like* helical structure. The rest of the protein is then cotranslationally translocated across the membrane as part of the helical structure. This model predicts that export is initially driven by the favorable free energy of transfer of the hydrophobic signal peptide from the cytoplasm to the membrane.

6.7.5   *The Amphiphilic Tunnel Hypothesis*

This hypothesis suggested by Rapoport (1985) is based on thermodynamic considerations. The model is similar to the signal hypothesis proposed by Blobel and Dobberstein (1975). It is assumed here that the export apparatus of the membrane consists of an amphiphilic membrane that can bind to both hydrophilic and hydrophobic regions of the precursor protein. The tunnel might be formed of a protein with several types of binding sites, or of lipids arranged to provide both a polar and a nonpolar environment for protein export. The translocation process begins when the signal peptide of the protein binds to the hydrophobic region of the tunnel. The signal peptide enters the tunnel and folds into a low energy conformation. Hydrophilic regions of the protein are generally not retained in the tunnel and are exported into the aqueous phase while hydrophobic and amphiphilic domains remain in the membrane,

either until they assemble into a polar domain or until translocation is completed. When translation terminates, the amphiphatic tunnel disassembles, and portions of the nascent chain remaining in the membrane are either transferred to the outer face or retained in the bilayer, depending on the compatibility of the peptide with a hydrophobic environment. This hypothesis models for both protein secretion and insertion of the membrane proteins.

6.7.6   Singer's 'Unitary Mechanism' for Protein Translocation

In the mechanism proposed by Singer *et al* (1987 a, b), the presence of a translocator protein is hypothesized. This protein has certain structural features which enables it to carry out

(i) the binding of the signal peptide on polypeptide chain;

(ii) the seeding of the formation of successive subdomains of the chain within the translocator protein;

(iii) the energy dependent translocation of these subdomains one after another until the entire chain is translocated.

Throughout the process, the ionic residues remain in contact with water while the hydrophobic residues are shielded from the aqueous environment by the translocator protein. This mechanism allows for the translocation of a wide range of polypeptides. Furthermore, the entire process could occur either cotranslationally or posttranslationally.

EXPORT OF PROTEINS ACROSS MEMBRANES :
THE HELIX REVERSION HYPOTHESIS

A model is presented which explains the biological role of the leader peptide in protein export. Along the lines of this model, the conformational changes of the signal peptide with environment serves as a general mechanism for translocation. The leader peptide in the cytoplasm takes a hairpin like conformation which reverts to an extended helix upon integration into the membrane. The essential features of this model are in accord with recent results of protein export.

CHAPTER 7

7.1 Introduction

The previous chapter reviews the status of studies in protein translocation. The present chapter analyses signal peptides both from prokaryotic as well as eukaryotic sources in order to decode the structure-function relationships of signal peptides. Furthermore, this chapter also considers an alternative model for the process of protein translocation. The model can account for the stability of signal peptides, both in hydrophobic as well as hydrophilic environments. Interaction with components of the translocation apparatus are also well accounted for by this model. Biochemical evidence which strengthens this model have been provided and critically discussed.

It is now a well established fact that signal peptides are almost universally responsible for initiation of protein translocation. Several lines of evidence argue that signal sequences from various organisms work much in the same way (Gierasch, 1989). Many of the features of the export pathway appear to be shared by all species, since most exported proteins can be translocated and processed correctly by the export machinery from several organisms (Mueller et al, 1982). Yet, despite this striking conservation of critical cellular function, signal sequences display a remarkable lack of primary sequence homology, even among closely related species (Verner and Schatz, 1988).

Secondary structure prediction methods for signal peptides have not been very successful and have often given contradictory results (Austen, 1979; Gierasch, 1989). Comparing signal peptides which vary drastically in their length, but have the same structural organization

and perform the same function, is difficult. Todate all the analysis of signal peptides align either the N-terminus or the C-terminus and attempt to identify conserved regions. These have helped to identify regions which define the cleavage site (Von Heijne, 1988).

In the present work, based on the analysis of normalized sequences, it has been proposed that in the cytoplasm the leader peptide alone takes a hairpin like conformation which is stabilized in the vicinity of the ribosome. This conformation, as it passes from the aqueous cytoplasm into the hydrophobic membrane, undergoes a change. This *conformational reversion* could play a very vital role in the initiation of export of the precursor across the membrane.

7.2   Materials and Methods

All signal peptides were obtained from the current version of the SIGPEP data bank (Von Heijne, 1987) which holds a total of about 200 prokaryotic and 900 eukaryotic sequences. Only those sequences whose amino acid sequences were completely known were utilized in this analysis.

The theoretical conformational assessments, comparison or attempts to arrive at consensus patterns of signal peptides should take into account the differences in length. To facilitate the comparison between various signal peptides, the procedure developed for normalization of signal peptide length may be useful.

7.2.1   *Normalization of length of signal peptides*

An algorithm is developed for normalization of length of signal peptides and the procedure employed is described below

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(i) Calculate the length (AL) of the signal peptide

(ii) Divide the length to which the sequence should be normalized (NL) by the actual length of the sequence (AL). Round up the obtained value (OL) to the nearest integer.

(iii) Identify the first residue in the sequence to be normalized. Using this residue, make a string containing the same residue till the length of the string (S$) is OL.

(iv) Multiply the obtained length (OL) by the (AL) and subtract the value obtained from the normalized length (NL) and calculate the remainder (RL).

(v) Add the remainder to the normalized length (NL) and assign the obtained value to the new normalized length (NNL).

(vi) Divide the new normalized length (NNL) by the actual length (AL) and once again obtain the integer nearest (OL) to the new normalized length. Identify the next residue make a new string and append it to the previous string to obtain a new string

(vii) Continue with steps (iii) to (v) every time using the next residue in the sequence till the last residue.

(viii) Calculate the length of the appended string and subtract it from the normalized length. Using the last base make a string whose length is equal to the remainder.

In this way with a slight approximation one can normalize the sequence to any required length with each integer length value corresponding to an amino acid. The structural evaluation can now be done by calculating the positional preferences for groups of amino acids.

7.2.2 Calculation of positional preferences

The preference of helix inducers (leucine and alanine) and helix destabilizers (valine, proline, glycine, methionine, cysteine, phenylalanine and isoleucine) to occur at a position "i" along the normalized length is calculated as follows :

$$
P_{o_{(a,i)}} = \frac{F_{(a,i)}}{N} \frac{T}{F_{(a)}}
$$

where $F_{(a,i)}$ is the frequency with which an amino acid or a group of amino acids "a" occurs at a position "i"; $F_{(a)}$ is the total frequency of occurrence of that amino acid or group of amino acids at all positions; "T" is the total number of amino acid residues studied and "N" is the total number of residues occurring at any position "i".

Such procedures for calculating positional preferences have been used earlier (Chou and Fasman, 1973; Richardson and Richardson, 1988).

7.3 Results and Discussion

The length of signal peptides is highly variable. Furthermore, the primary sequence of peptides that translocate proteins across the same membrane in the same species may have no primary sequence homology. Despite numerous efforts to find well conserved patterns, only the overall gross design seems to be common to these sequences (Von Heijne, 1989). Being typically between 15-30 amino acids long, all signal peptides have three physico-chemically distinct regions; a positively charged amino terminal called the n-region, a central hydrophobic h-region and a more polar carboxy terminal called the c-region (Von Heijne, 1985). These three regions have been well characterized. The n-region can vary considerably in length (one to twenty) and amino acid composition, but always has a net positive charge,

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both in prokaryotic as well as eukaryotic species (Von Heijne, 1984). The h-region is rich in nonpolar amino acids and varies from seven to sixteen residues. The c-region has a more distinct pattern of amino acids, in particular, in positions close to the cleavage site. All naturally occurring cleavage sites confirm to the *-1,-3* rule (Von Heijne, 1986) which states that the residue in the position -1 and -3 must be small and uncharged. In contrast, the position -2 should be occupied by large, bulky or charged residues.

The responses of signal peptides to the environment may be of vital importance in protein translocation (Batenburg *et al*, 1988a, b). Characterization of isolated signal peptides has revealed what critical properties are, particularly if functional signal peptides are compared to variants that are non-functional. However, a potential limitation in the study of isolated signal sequences is that they are not likely to have strongly preferred conformations. Linear peptides of fewer than 30 residues generally sample over several conformations in aqueous solutions (Wright *et al*, 1988). Even in structure promoting environments, most short poly-peptides are likely to be interconverting between various different structures, with at best show a bias towards one conformation. Characterization of such a dynamic state is extremely difficult. On the other hand, the biological roles of signal peptides may require them to be conformationally dynamic and respond to different environments by conformational changes (Randall *et al*, 1987).

Assessing the importance of these preferred conformations of isolated signal peptides in terms of their function *in vivo* is not straight forward. It is difficult to mimic the micro-environments likely to be encountered in the export process, and it is still not clear which particular conformational propensity is required for function (Gierasch, 1989).

The main basis for the proposed *Helix Reversion Hypothesis* is provided by a statistical analysis of the positional preference of amino acids both in eukaryotic as well as prokaryotic leader peptides.

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Figure 7.1 depicts plots of $P_{o_{(a,i)}}$ versus $"i"$ for helix inducers and helix destabilizers.

N_P denotes the theoretically expected preference value if there was no positional bias in the occurrence of an amino acid. Any value of $P_{o_{(a,i)}}$ greater than N_P implies that the amino acid or group of amino acids ($"a"$), show a preference to occur at that position ($"i"$), while any value less than N_P implies a bias of that amino acid or a group of amino acids not to occur at that position. The percentage occurrence of amino acids in leader peptides is shown in table 7.1. It was observed that certain helical conformation inducers like leucine and alanine (Chou and Fasman, 1973), which constitute 38% of the total composition (table 7.1), show a high preference to occur in the interior of the leader peptide (Figure. 7.1). Leucine, due to its abundance could be postulated to play an important role in inducing a conformation that is important in the interaction with the SRP and the membrane. Furthermore the amino acids valine, isoleucine, phenylalanine, methionine, cysteine, proline and glycine, which are helix destabilizers, show a bias to occur at 3 particular positions (Figure. 7.1). The centrally weak zone is sandwiched by two potentially strong helical zones and this positional specificity can give rise to the "molten globule" state as postulated by Bychkova *et al* (1988).

An analysis using the chi-square test was performed to check the statistical significance for the difference in frequency of occurrence of helix formers and destabilizers for every 5% of the normalized length of the leader peptide. These results indicate that with 19 degrees of freedom and $X^2_{0.05}$ there is significant variation in the occurrence of helix formers and desta-bilizers along the normalized length of leader peptides. Furthermore this trend is clearly observed in both prokaryotes as well as eukaryotes (Shinde *et al*, 1989).

All leader peptides are known to be highly hydrophobic, which means that the con-formation taken will be one such that the hydrophobic forces act to reduce the surface area exposed to the aqueous cytoplasm. This conformation will be only marginally stable, devoid
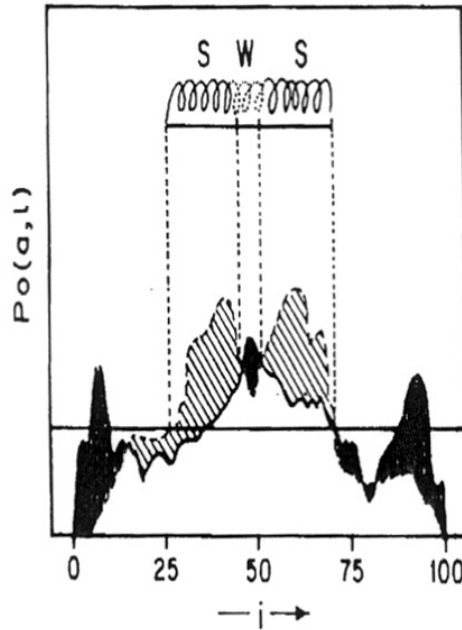
FIGURE 7.1 Preferential occurrence of helix formers (- - -) and helix weakeners (------) along the normalized length of signal sequences

 depicts strongly helical regions

 depicts weakly helical regions

TABLE 7.1

Percentage Composition of Amino Acid in various leader peptides

Amino Acid	% at N-Terminal	% at C-Terminal	% in hydrophobic Core	Total % in peptide
Leucine	2.77	2.20	18.20	23.17
Alanine	1.95	4.00	8.58	14.53
Serine	1.22	1.85	5.15	8.22
Valine	0.96	1.10	5.57	7.63
Methionine	5.44	0.21	1.15	6.80
Glycine	0.63	1.62	2.93	5.18
Phenylalanine	0.83	0.56	3.68	5.07
Threonine	0.56	0.73	3.13	4.42
Cysteine	0.25	0.71	2.08	3.04
Isoleucine	0.98	0.64	3.37	4.99
Proline	0.56	0.69	1.83	3.08
Arginine	1.99	0.32	0.00	2.31
Tryptophan	0.37	0.20	1.15	1.72
Lysine	3.06	0.40	0.00	3.46
Glutamine	0.22	0.39	1.11	1.72
Tyrosine	0.32	0.12	0.80	1.24
Glutamic acid	0.33	0.54	0.00	0.87
Aspargine	0.44	0.26	0.44	1.14
Aspartic acid	0.34	0.20	0.00	0.54
Histidine	0.28	0.59	0.00	0.87
Grand Total	**23.50**	**17.33**	**59.17**	**100.00**

of any stable tertiary structure mainly due to the lack of any disulphide bonds and will depend entirely on the environment (Kaiser and Kerdy, 1984). Furthermore, strong evidence exists that protein translocation requires the involvement of a non-native or denatured state essentially in the form of a compact but flexible *molten globule* state (Bychkova *et al*, 1988). This state could be a hairpin like structure, with the bend occurring at the helix destabilizing zones in the interior of the hydrophobic core. Both the arms are, however, contributed by the leader peptide alone.

Blobel and coworkers (Walter *et al*, 1981) have shown the involvement of a cytoplasmic protein called the signal recognition particle (SRP) in the initial stages of export to form a 70-mer translation arrested ribosome complex. As no significant primary sequence homology exists between the various leader peptides, implies that the SRP binds to the leader peptide and the ribosome in an unconventional way, possibly via conformational recognition (Randall *et al* 1987). As the average length of leader peptides is about 22 amino acids and about 40 amino acid residues are shielded in the ribosome (Bernbeau and Lake, 1982). This indicates that in the 70-mer complex, the leader is fully exposed to the cytoplasm and will try to attain conformation that is stable in the aqueous cytoplasmic phase. The only possible conformation with the least exposure of the hydrophobic surface will be the hairpin-like helix. This is because, compared to an extended helix, approximately one-fourth of the total surface area exposed to the cytoplasm is reduced when the conformation taken is a hairpin-like one (Engelman and Steitz, 1981). We propose that this is the conformation that is recognized by the SRP. The stability of this conformation will further increase by intramolecular hydro-phobic interactions and bifurcating hydrogen bonds (Figure 7.2). From the evidences of Blobel and coworkers and Lodish and coworkers (Kaiser and Kerdy, 1984; Gilmore and Blobel, 1982; Walter *et al*, 1981; Walter and Blobel, 1981a, b) we can conclude that the SRP binds to the leader sequence and the ribosome in a way so as to:

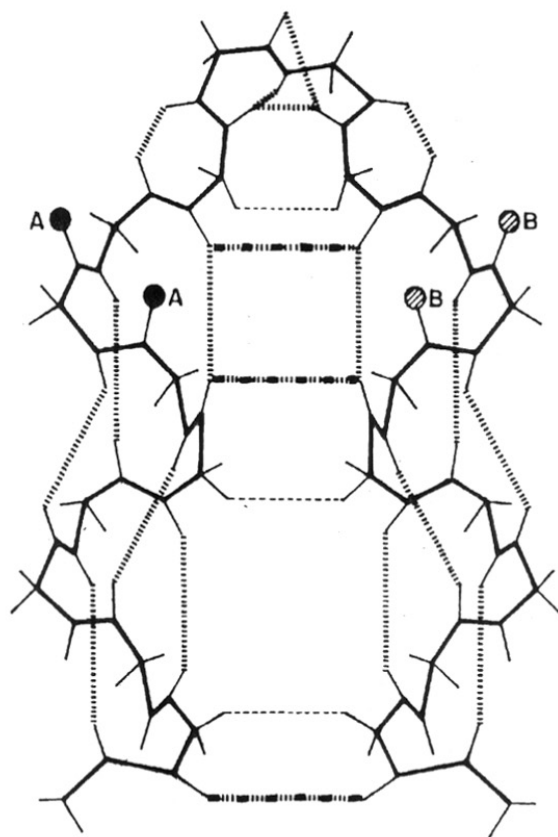**FIGURE 7.2** A schematic representation of the proposed hairpin like conformation.

- - - - -   Hydrophobic interactions

||||||||||||||| Hydrogen bonding

■||| ■ ||■   Bifurcating hydrogen bonds

A and B   represent unsaturated groups

(i) sterically hinder the ribosome-membrane binding site located on the exit domain

(ii) sterically hinder the A site of the ribosome so as to arrest chain elongation

(iii) hinder the charged N-terminal sequence of the nascent polypeptide from binding to the membrane

(iv) position the signal sequence in a hairpin structure so as to facilitate penetration and

(v) to be in a position so as to attach to the SRP receptor on the membrane.

High resolution X-ray techniques have shown the exit and the translation domain are well separated on the ribosomal surface. The distance between the exit domain and the A site is estimated to be about 150 A (Bernbeau and Lake, 1982). This means that the SRP should take dimensions to span this gap so as to satisfy the above conditions. Electron microscopy studies of SRP have shown the length to be approximately 240 A (Andrews et al, 1985). When the SRP of the 70-mer complex binds to the SRP receptor it would relax the arrest on the elongation probably by a conformational change in the SRP, effecting the release of the signal sequence-ribosome complex. However, with this release, SRP would in turn lose its conformation for which the SRP receptor has high affinity, and hence dissociate away from the receptor.

After the release of elongation arrest, the ribosome binding site as well as the charged N-terminal of the sequence would be free, thus effecting an immediate binding of the ribosome-polypeptide chain to the membrane. The model is schematically shown in figure 7.3. The hydrophobic hairpin is now exposed to the cytoplasm. However, due to the strong tendency for apolar amino acids to partition into the nonaqueous interior of the lipid bilayer, the peptide will tend to adopt a structure that maximizes the clustering of its apolar residues. This indicates that the hairpin conformation which it had acquired in the cytoplasm to reduce exposure of the hydrophobic groups, will now essentially change to an extended helix. This
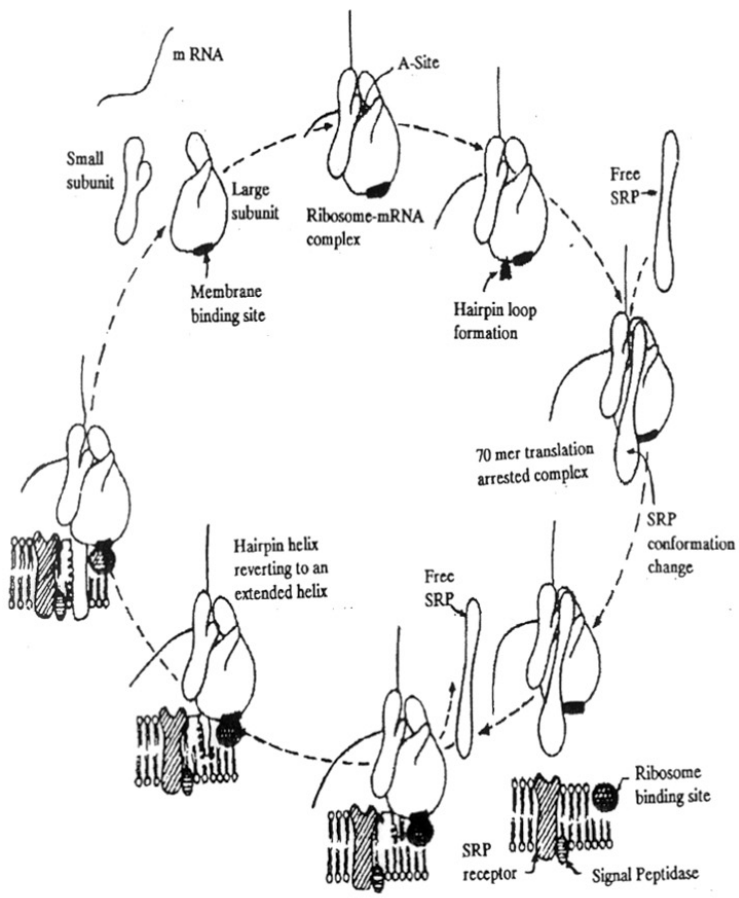
FIGURE 7.3 A schematic representation of translocation of proteins across membranes as postulated in the **Helix Reversion Hypothesis.**

structure reversion could be the key factor in the initiation of export. Hence the leader peptide in this state can accommodate to both polar and nonpolar environments. Furthermore, the hairpin-like conformation, with a less polar surface, would be translocated through the bilayer with a small free energy of activation. Energy studies have shown that such a reversion could be thermodynamically favoured due to the increase in the hydrophobic interactions (de Vrije *et al*, 1990). The subsequent transfer could then take place in a completely lipophilic atmosphere (Von Heijne and Blomberg, 1979). Alternatively, the transport could continue via the translocator protein as postulated by Singer and coworkers (1987). For post-translational translocation the precursor should be devoid of a stable tertiary structure. Hence in most cases post-translational translocation does not occur. However, if the stable tertiary structure can change to an unstable one, possibly via energy supplied by NTP, translocation can occur (Randall *et al*, 1987).

It may be doubted that leader peptides in eukaryotes are actually exposed to water. They may simply be buried into a hydrophobic pocket of SRP as soon as they emerge from the ribosome. This would imply that the SRP and ribosome would be physically associated with the nascent polypeptide chain even before the translation arrest. However, as per our knowledge, no such experimental evidence is available. It has also been shown by Rapoport and coworkers (1987) that a time lag exists between the synthesis of the leader peptide and its binding to the SRP. Furthermore, by *in vitro* studies Ainger and Meyer (1986) have shown that the SRP can arrest nascent chain elongation at various times during a synchronous translation, thus allowing the generation of nascent chains of increasing length. The SRP is added to the system after a time lag, indicating that the signal sequence is completely exposed to the aqueous cytoplasm. Even so the conformation adopted in the cytoplasm is still recognized by the SRP. If the signal sequence was to enter directly inside the SRP, the conformation which it would acquire in the hydrophobic pocket of the SRP would be expected to

be radically different from that which it would acquire in the aqueous cytoplasm. This is because the conformation adopted by a peptide is substantially affected by the environment (Kaiser and Kerdy, 1984). This advocates a proposal that the SRP can recognize not only different leader peptides but also different conformations, which implies that the SRP could interact not only with leader peptides, but also with certain other proteins. However, as per our knowledge no such experimental evidence has been reported. Thus the possibility that the leader peptide may be simply buried into the hydrophobic pocket of the SRP seems remote.

It may be argued that the normalization or averaging of the sequences may disguise several signal sequences where the pattern of residues is quite different. This possibility was eliminated by repeatedly drawing out sequences, randomly, one hundred at a time, and plotting the positional preference of the residues in these sequences and comparing them with that of the total data bank. The trend observed in all the cases was similar to each other and the overall trend for all the sequences is depicted in figure 7.1. The amino acids V, P, G, M, C, F and I have been classified as helix destabilizers mainly because the ratio of their alpha to beta forming potentials as calculated by Chou and Fasman (1973) is less than one.

7.4   Biochemical Evidence in Support of Proposed Model

7.4.1   *Leucine is essential in conformation induction, protein-protein and protein-lipid interactions.*

Circular dichroism studies on random copolymers in aqueous solutions have shown that an increased helical stability is coincident with the incorporation of a larger amount of L-leucine in these polymers (Chou and Fasman, 1973). Furthermore it is shown that leucine

is thermodynamically a stronger helix former than most other amino acids. Alanine also shows the ability to increase the helical content of peptides. In view of this point the 27 % occurrence of these amino acids in leader peptides would be expected to induce a helical conformation.

Incorporation of β-hydroxy leucine in place of leucine abolishes *in vitro* translocation, which could occur due to a high change in hydrophobicity and inability to induce a helical conformation (Walter *et al*, 1981). This could probably be due to an increase in the steric hindrance coincident with the incorporation of a bulky hydroxyl group in case of β-hydroxy leucine.

7.4.2   *The hairpin like conformation of the leader peptide is stabilized in the vicinity of the ribosome and the SRP probably recognizes this conformation*

The SRP has been shown to interact with the leader peptide alone with a weak affinity (Rapoport *et al*, 1986). If the hairpin like conformation is acquired by this peptide in the absence of the ribosome, it would then be expected to bind strongly to the SRP. However, this is not the case. Furthermore, the binding of the SRP with the ribosomes alone has also been shown to be very weak (Rapoport *et al*, 1987). In the context of the ribosome, the nascent polypeptide - SRP affinity is increased by approximately 6000 times. This could probably imply that the hairpin like conformation of the leader peptide is stabilized in the context of the ribosome. Secondly, the post translational translocation of polypeptides has been shown to depend on the concentration of ribosomes (Roitsch and Lehle, 1988). This indirectly implies that the export conducive conformation of the leader peptide is attained only in the presence of the ribosome.

One of the possible reasons for not being able to observe a hairpin-like helical conformation in isolated signal peptides is the environment in which the studies are conducted.

It is very difficult to mimic the micro-environment which the peptide is exposed to when it is in the vicinity of the ribosome. Furthermore, it has been observed *in vitro* experiments, post-translational translocation of synthesized proteins is induced by increasing the free ribosome concentration.

Generally, a specific interaction between two proteins involves complementary steric fit of the surfaces, thus requiring particular amino acids at precise positions. However, as no primary sequence homology is observed in various leader peptides, it is postulated that the conformation could play an important role in recognition. In many cases, it has been shown that most amino acids in proteins are only structural in nature and their replacement by residues of similar lipophilicity do not impair biological activity (Kaiser, 1986). Thus various leader peptides, with absolutely no primary sequence homology whatsoever, could by virtue of their composition give rise to similar conformations. The SRP, therefore, may recognize the conformational backbone, irrespective of the side chains. Although such interactions may seem improbable, a precedent for this proposal is the binding by the protease La (product of the lon gene) of its denatured substrate (Randall *et al*, 1987; Waxman and Goldberg. 1986).

7.4.3   *Conformational change with change in environment*

The helical content of signal sequences has been shown to increase with an increase in hydrophobicity of the medium (Rosenblatt *et al*, 1980). Circular dichroism studies of the leader peptide in an aqueous medium have also shown that conformational changes are possible upon the association and integration with the hydrophobic membrane (Briggs *et al*, 1986).

The essential features of this model are in accordance with recent experimental results on protein export. Experimental work and energy calculations supporting this hypothesis was

carried out simultaneously by de Virje *et al*, (1990) which lead to their proposal of the *unlooping model* for protein translocation. The essential features of this model are similar to the helix reversion hypothesis (Shinde *et al*, 1989)

The salient features of the unlooping model are :

(i) Insertion of the signal peptide in the hydrophobic core of the membrane which depends upon the state of packing of the lipids and by electrostatic charges between the positively charged N-terminal and the negatively charged phospholipids head groups in the membrane.

(ii) Conformational and topological changes in the signal peptide upon insertion inside the membrane.

(iii) Changes in the local lipid structure as a result of signal peptide-lipid interaction.

In the first stage of this process, the positively charged N-terminus of the signal peptide initially interacts electrostatically with the negatively charged phospholipids. In the second stage, the signal peptide inserts as a loop into the hydrophobic core of the membrane. Unlooping of the signal peptide in stage three moves the N-terminus of the mature sequence across the bilayer. During the stages two and three, lipid organization is locally disturbed and this could facilitate passage of the protein. Finally after unlooping, signal peptidases cut the precursor to give the mature sequence.

CHAPTER 8

ROLE OF SURFACE TOPOLOGY
IN THE RECOGNITION OF
MEMBRANE SPANNING ALPHA HELIX
IN SIGNAL PEPTIDES
BY SIGNAL PEPTIDE PEPTIDASES

Signal peptides are selectively identified and degraded over other transmembrane sequences by membrane associated proteases. This cleavage is initiated only after the precursor protein has been cut by signal peptidases on the trans side of the membrane. This is more significant when one realizes that the overall amino acid sequence composition, in both, transmembrane sequences and signal sequences is similar. The possible reasons for this are investigated in this chapter. Results indicate that when the surface topology of the membrane spanning sequences is studied, remarkable differences are observed. Possible implications of this analysis are also discussed.

CHAPTER 8

8.1   Introduction

In the previous chapter, an alternative model for initiation of protein translocation is proposed. After the export of the protein across a particular membrane, the signal peptide is selectively recognized and degraded by a class of integral membrane proteins known as signal peptide peptidases. The reasons for this selectivity have been investigated in this chapter.

It is a well established fact that signal peptides are responsible for triggering the translocation of secretory proteins across membranes (Blobel, 1980; Milstein *et al*, 1972; Randall *et al*, 1987). These peptides, which are highly hydrophobic in nature and mostly located at the N-terminal of a precursor protein, are cleaved off after initiating the translocation of that particular protein across the membrane (Ray *et al*, 1986). Although these sequences have no obvious primary sequence homology, they do display common characteristics as a group (Perlman and Halvorson, 1983; von Heijne, 1983; Shinde *et al*, 1989). Several lines of evidence argue that the signal peptides, both from prokaryotic and eukaryotic sources, work much in the same way (Gierasch, 1989).

Even though considerable efforts have been made in the last one and half decades in elucidating the mechanism of protein translocation (Shinde *et al*, 1989; Verner and Schatz, 1988; Batenburg *et al*, 1988), the intricate details of this process still remain ambiguous. After initiating the process of translocation, the signal peptide is cleaved off from the precursor by a protease called the signal peptidase (Dalbey and Wickner, 1985; Dev and Ray, 1984). This cleavage is then followed by rapid hydrolysis of the peptide by specific proteases termed as signal peptide peptidases (Zwizinski *et al*, 1984). However, the detailed mechanism and subsequent steps of degradation of the peptide are still unclear. Whatever is the mode of degradation, it is extremely efficient and a rapid

one. The reasons for this rapid hydrolysis have been elucidated by Austen *et al* (1984). Their data suggests that the signal peptide itself may be involved in the regulation of protein translocation. This therefore makes rapid degradation of the signal peptide essential for maintaining proper export.

Novak *et al* (1986) have shown that the signal peptide is degraded only after it is cleaved from the precursor and this degradation is at least 300 times more rapid than that of other cellular proteins (Novak, *et al*, 1986). Furthermore, this degradation is specific only for signal peptides, and the hydrolases which bring about the degradation do not act on transmembrane protein sequences (Ichihara *et al*, 1984).

The transmembrane domains of proteins generally consist of 22 to 23 hydrophobic and neutral amino acids, followed by one or more charged residues, preceding the cytoplasmic domain (Eisenberg, 1984). There are no differences, within limits of statistical errors, in the overall amino acid content between uncleaved signal peptides and membrane spanning sequences (von Heijne and Gavel, 1988). Furthermore, both signal peptides and membrane spanning sequences adopt similar conformations upon integration into membranes (Briggs and Gierasch, 1984). Even then the signal peptide peptidases are highly specific in their action on signal peptides. In view of the similarities between signal peptides and transmembrane sequences, it is interesting to recognize clues which could throw light on the mechanism by which the signal peptides are specifically differentiated from transmembrane sequences and degraded only after their cleavage from the precursor proteins.

It has been shown here that there exist remarkable topological differences between transmembrane sequences and signal peptides when studied from a structural point of view. The positional preferences on the alpha helical structure indicate that specific regions of the signal peptide must interact with components of the translocation apparatus. Such regions, which are absent in transmembrane sequences, may be responsible for the specificity of signal peptide peptidases. The possible implications of these topological differences on the surface of the membrane spanning region on the structure-function relationship of both signal peptides and membrane spanning sequences have also been discussed.

8.2 Materials and Methods

All signal peptides were selected from the current version of SIGPEP data base (von Heijne, 1987), which holds a total of about 200 prokaryotic and 900 eukaryotic sequences. In the present analysis, only the signal peptides with completely known primary sequences were included. Transmembrane sequences were extracted from the EMBL DNA library (version 14.0, January 1988). All transmembrane sequences were analyzed by taking into consideration their orientation in the membrane.

8.2.1 Identification of the Membrane Spanning Core

The identification of the origin of the membrane spanning core in signal peptides was done by scanning the hydrophobicity on the Kyte-Doolittle scale, using a window of three residues (Kyte and Doolittle, 1982). The selected hydrophobic core was further searched for charged amino acid residues. If a charged residue occurred at the N-terminal of the selected core then the residue after the charged residue was considered as first amino acid residue in the membrane spanning region. This is because no charged amino acid residues can be accommodated in the membrane spanning core (von Heijne, 1986). In most of the cases the hydrophobic core identified by the above procedure tally with those identified by the method of von Heijne (von Heijne, 1985). In addition to the hydrophobic core, the C-terminal of the signal peptide was also assumed to be in the membrane spanning region (i.e. H + C region as identified by von Heijne, 1985). This is because in many cases, the signal peptidases have been shown to occur as integral membrane proteins (Yadeau and Blobel, 1989). Secondly, this region of the peptide has to traverse through the membrane even if it is cut by a soluble signal peptidase located trans to the cytoplasmic side of the membrane (Yadeau and Blobel, 1989).

8.2.2 Evaluation of Topology of Membrane Spanning Sequences

Isolated signal peptides upon integration into the membrane have shown to adopt an alpha helical conformation (Singer, 1971). As the present aspect of this work has been addressed to the initiation of degradation of the signal peptide in the interior of the membrane, after it has been cleaved off the precursor by the signal peptidase, both the H and C regions have been analyzed as the membrane spanning core in signal peptides. The distribution of amino acids on the surface of this membrane associated alpha helix may play an important role in the structure-function relationship of signal peptides and transmembrane sequences. To analyze the topology of the surface, the residues constituting the hydrophobic region were arranged in a helical wheel (Schiffer and Edmundson, 1967) with an angle (θ) of 100 degrees between each. An angle of 30 degrees was chosen to be the window size and any residue occurring within +/- 15 degrees of an angle under consideration was assigned that angle. This is a method similar to that employed by Kyte and Doolittle (1982) for reducing the extent of noise in the hydrophobicity plots. The normalization of the sequences and calculation of the positional preferences was carried out as described earlier (Shinde *et al*, 1989; Richardson and Richardson, 1988). The positional preferences of residues were analyzed by plotting the ratio of the preferential occurrence of polar to nonpolar amino acids as a function of length as well as the angle that the residue makes with the axis of the alpha helix.

It may be argued that since the above analysis is statistical in nature and as all sequences have been lumped together, it may disguise several other signal sequences where the pattern of residues may be very different. This possibility was eliminated by drawing sequences, randomly, one hundred, at a time and plotting the positional preference of the residues in these sequences and comparing them with that of the total data bank. Similar trends were observed in all cases and the overall trends are depicted in the figures.

An analysis using the chi-square test was performed to check the statistical significance of the above analysis. The results indicate that with $X^2_{0.05}$ (19 degrees of freedom), there is a significant difference between transmembrane sequences and signal peptides.

8.3   Results

8.3.1   *Comparison of positional preferences for helix formers and helix weakeners in membrane spanning sequences and signal peptides*

Figure 8.1 shows the preferential occurrence of helix formers and helix weakeners in transmembrane sequences and the hydrophobic core of signal peptides. A weakly helical region flanked on either side by a stretch of strongly helical residues can give rise to a helical hairpin-like structure in signal peptides (Shinde *et al*, 1989; de Virje *et al*, 1990). However, no such preferential occurrence is observed in transmembrane sequences. Therefore, a difference in the positional preferences is clearly noticed. This is interesting as it has been shown by Von Heijne (von Heijne and Gavel, 1988) that there is no difference, within limits of statistical errors, between membrane spanning sequences and signal peptides. The implications of such positional preferences in the initiation of peptide degradation in the membrane have been discussed later. No significant difference is observed when prokaryotic and eukaryotic sequences are considered separately (Shinde, 1990a).

8.3.2   *Clustering of Leucine residues in signal peptides*

Both signal peptides and membrane spanning sequences show a large occurrence of apolar amino acids. This has also been reported by earlier workers (Singer, 1971). However, there is a clear clustering of leucine residues in signal peptides as depicted in figure 8.2. The occurrence of leucine preferentially on a side of the helical surface, one which is relatively more polar than the rest of the surface, has not yet been reported. Leucine has already been established as the most abundant residue in the inner helical regions of proteins. The importance of this residue can be attributed to its branched, fork like hydrophobic side chain (Chou and Fasman, 1973). The possible role of this clustering may be attributed to the conformational flexibility required in proper functioning of the signal peptides and its possible interaction with other membrane associated proteins.
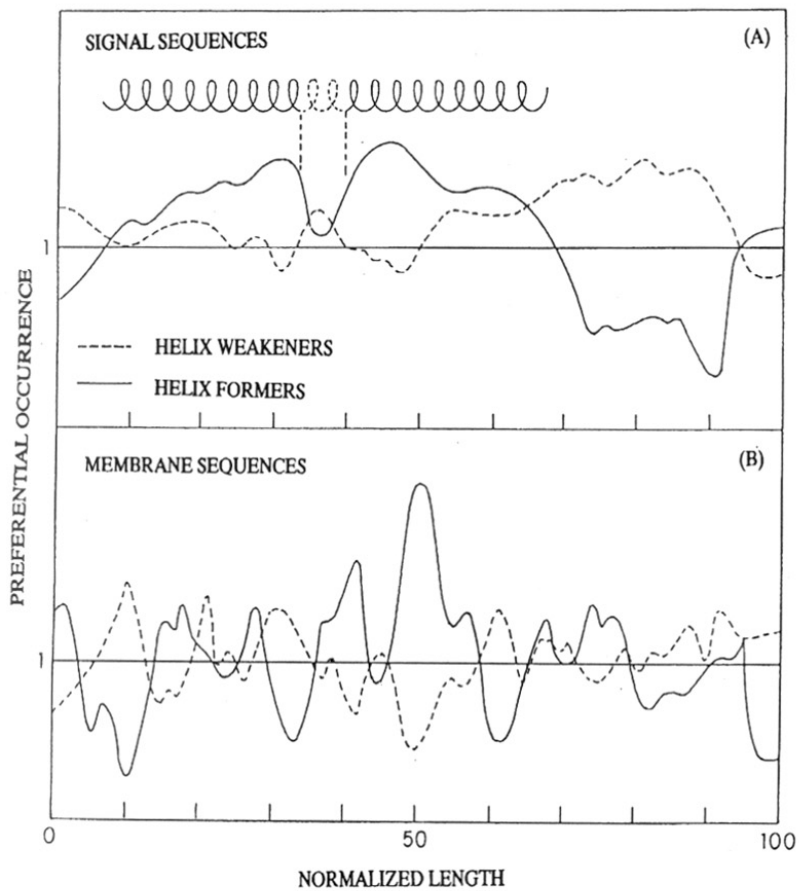
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FIGURE 8.1   Variation in the preferential occurrence of helix formers and helix destabilizers along the normalized length.
(A) Membrane spanning core of signal peptides
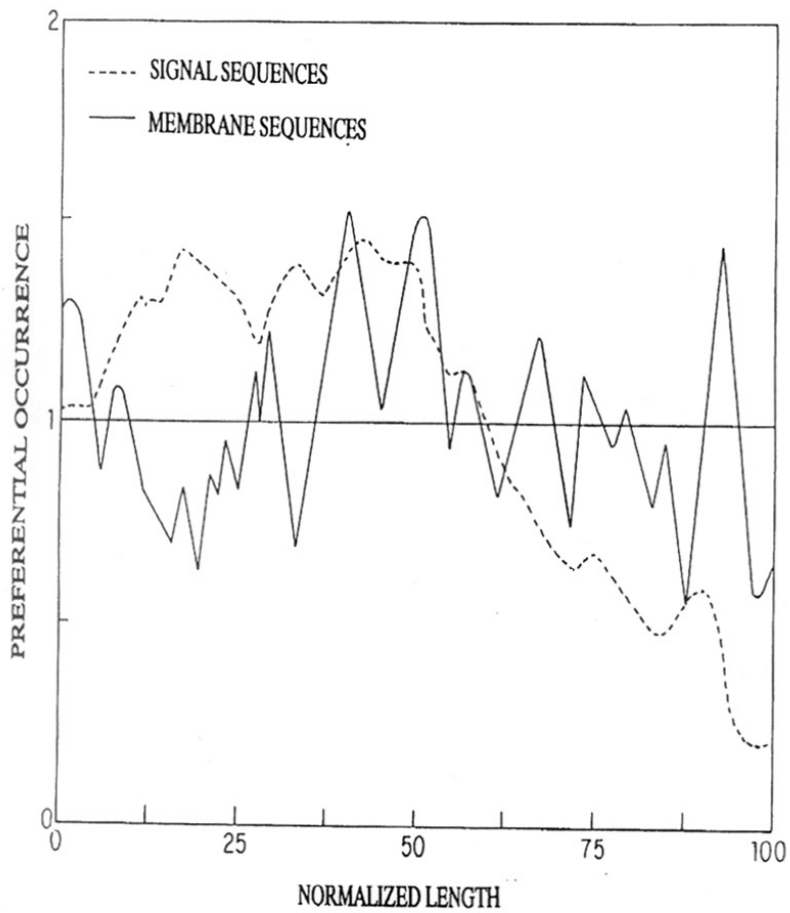(B) Transmembrane sequences in proteins.

FIGURE 8.2   Profiles depicting the variation in occurrence of the amino acid leucine along the normalized length.

This is proved by the fact that incorporation of β-hydroxy leucine in place of leucine abolishes *in vitro* translocation, which could occur due to inability to induce a helical conformation and possibly an inability to interact with some proteins due to a change in hydrophobicity and loss of the forked side chain (Walter *et al*, 1981).

8.3.3 *Positional preference for surface distribution of nonpolar and polar residues*

Charged amino acids cannot occur within the hydrophobic core of signal peptides (von Heijne, 1985), They, however, may at times be accommodated in some membrane spanning sequences. Such sequences are then associated with other similar sequences, thus shielding the charged amino acids from the hydrophobic interior of the membrane. Some polar amino acid residues may also be accommodated in the hydrophobic core. The interest is in elucidating the role, if any, of polar amino acids in these sequences. Figure 8.3A qualitatively depicts the ratio of preferential occurrence of polar to nonpolar amino acids in the interior of the core. The nonpolarity in signal peptide is almost uniformly distributed in their interior. This preference is, however, only in the case of signal peptides. No such bias was found in transmembrane sequences which show a more random distribution of residues on the surface of the alpha helix (Figure 8.3B).

The positional bias was more noticeable when the ratios of positional preferences between polar and nonpolar residues was plotted as a function of the angle (θ) made by a residue between the axis of the alpha helix and the first residue in the alpha helix (Figure 8.4A). As one moves along the surface of the helix, a distinct aggregation of polar and nonpolar residues is observed. This indicates that most signal peptides may probably be some what *amphiphilic* in nature. The maximum polar and nonpolar regions are, however, not exactly on opposite sides of the helix. Amphiphilic structures can be evaluated by using hydrophobic moment analysis. Its direct application in the present study may not be possible as the above conclusions are made statistically over a large sample of signal peptides whose lengths have been normalized.
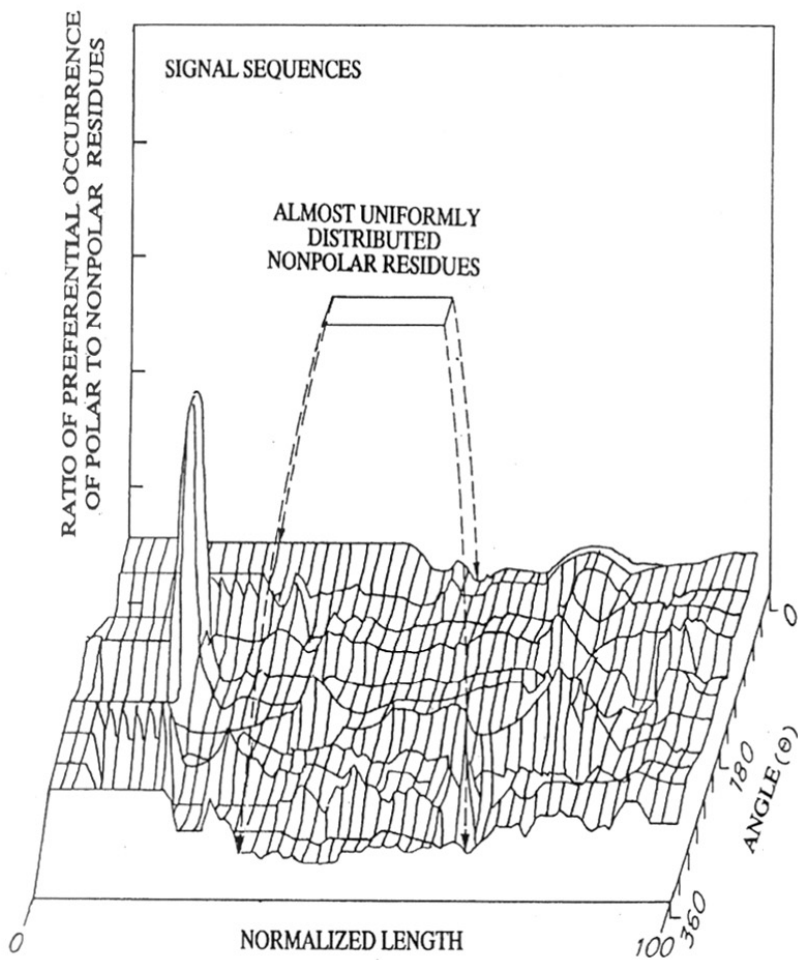
FIGURE 8.3A The ratio of preferential occurences of polar to nonpolar residues in signal peptides along the entire surface of the membrane spanning region.
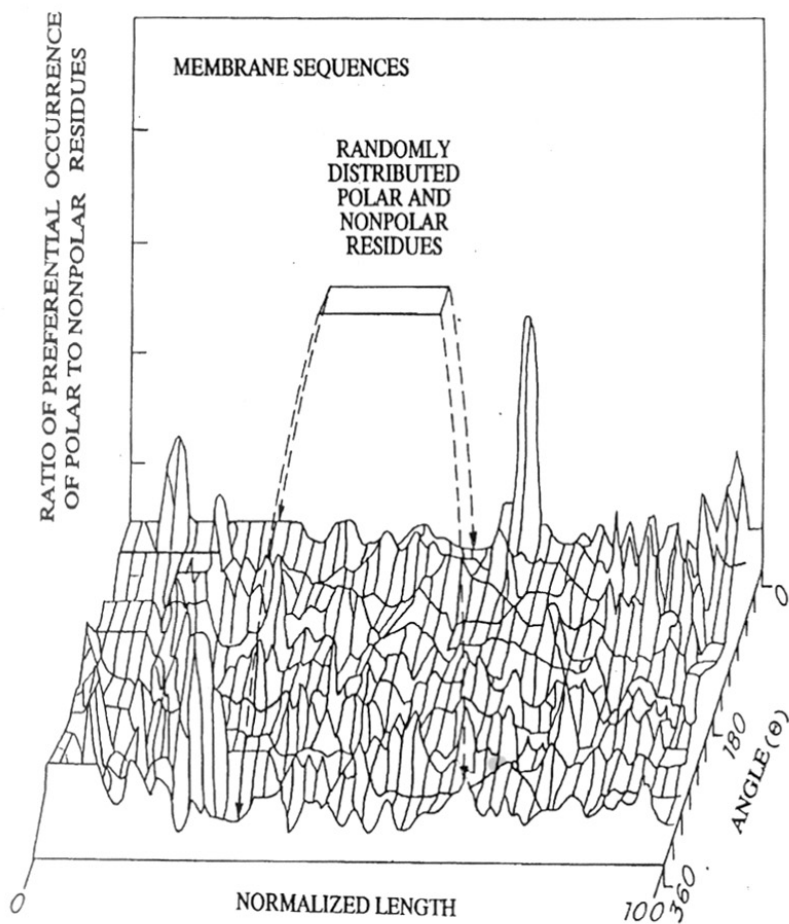
FIGURE 8.3B The ratio of preferential occurences of polar to nonpolar residues in transmembrane sequences of proteins along the entire surface of the membrane spanning region.

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The peptidase which initiates the hydrolysis of the signal peptides in the membrane in the case of *E. coli*, have been shown to be endoproteases (Novak and Dev, 1988). The ability of these membrane associated signal peptide peptidases to hydrolyze the signal peptide only after it is cleaved from the precursor implies that there may be some structural changes in the sequence after its cleavage. These structural changes may be attributed to the topological distribution of the amino acids on the surface of the helix. The clustering of polar residues on a particular face of the alpha helix of the signal peptide indicates that the polar side of the alpha helix may be in close vicinity of a protein with a similar surface. This is because, in an aqueous medium the polar side can extensively interact with the medium via protein-water H-bonds (Arnold, 1988). The situation in a hydrophobic environment is quite different and the hydrophilic forces would dominate the folding. The polar surface will therefore try to achieve stability by interacting with hydrophilic moieties in some other protein. This protein may be a peptidase similar to Protease IV as found in *E. coli,* as preliminary conformational studies have shown that a partly polar alpha helical surface may also exist in this peptidase (Shinde *et al*, 1990, a) and has been dealt with in the next chapter.

Thus, in conclusion, the distribution of amino acids on the surface of the alpha helical membrane spanning core along with other post translational modifications (Hare, 1990) may play an important role in degradation specificity of signal peptide peptidases. The above analysis depicts the finer differences between signal peptides and membrane spanning sequences. These differences may go unnoticed when amino acid compositions or sequence homologies are studied. However, the secondary structure, and positional preferences in relation to this secondary structure, provide clues about the differences between the two types of sequences. It is speculated that these topological differences may, along with other post translational modifications, play an important role in differentiation between signal peptides and transmembrane sequences. The above analysis can be useful in designing mutation experiments for elucidating the exact mechanism of the specificity and selectivity in signal peptide degradation.

The distribution of polar and nonpolar amino acid residues on the surface of membrane spanning sequences is however random. The randomness of the distribution was verified using the tests based on runs at a 0.05 level of significance (Freund and Walpole, 1987). Furthermore, Figure 8.4B clearly indicates that even though the region between the angle θ of approximately 225-360 degrees is significantly more nonpolar than the rest of the surface, the preference of leucine and alanine to occur in this zone is relatively lower than that observed in the zone of θ 0-180 degrees. This implies that leucine residues are clustered to a large extent on the same side of the alpha helix as the polar residues, but are distinctly separated along the length of the axis (Figure 8.3A).

8.4 Discussions

The statistical analysis presented above makes it possible to draw a number of conclusions regarding the structure-function relationships in signal peptides. The preferential occurrence of helix formers and weakeners along the length of signal peptides gives rise to a centrally weak zone sandwiched by two potentially strong helical zones which can result in a *molten globule* state as postulated by Bychkova *et al* (1988). Such positional preferences are, however, not identified in transmembrane sequences. It has already been proposed by Shinde *et al* (1989) in the *Helix Reversion Hypothesis* and de Vrije *et al* (1990) via the *Unlooping model* that conformational and topological changes which occur in the signal peptide, as it traverses through the membrane are very important in initiation of protein translocation. The weakly helical region flanked by potentially strong helical residues on either side, in the interior of the hydrophobic core of signal peptides, can give rise to high flexibility in the conformation acquired by the peptide in either the aqueous cytoplasm or the hydrophobic interior of the membrane (Shinde *et al*, 1989). Flexibility is an important factor in proteolysis. Unfolded or partly folded polypeptides are often more sensitive to proteases than fully folded proteins. It has also been shown that a loop like structure may be an ideal site for action of proteases (Leszczynski and Rose, 1986).
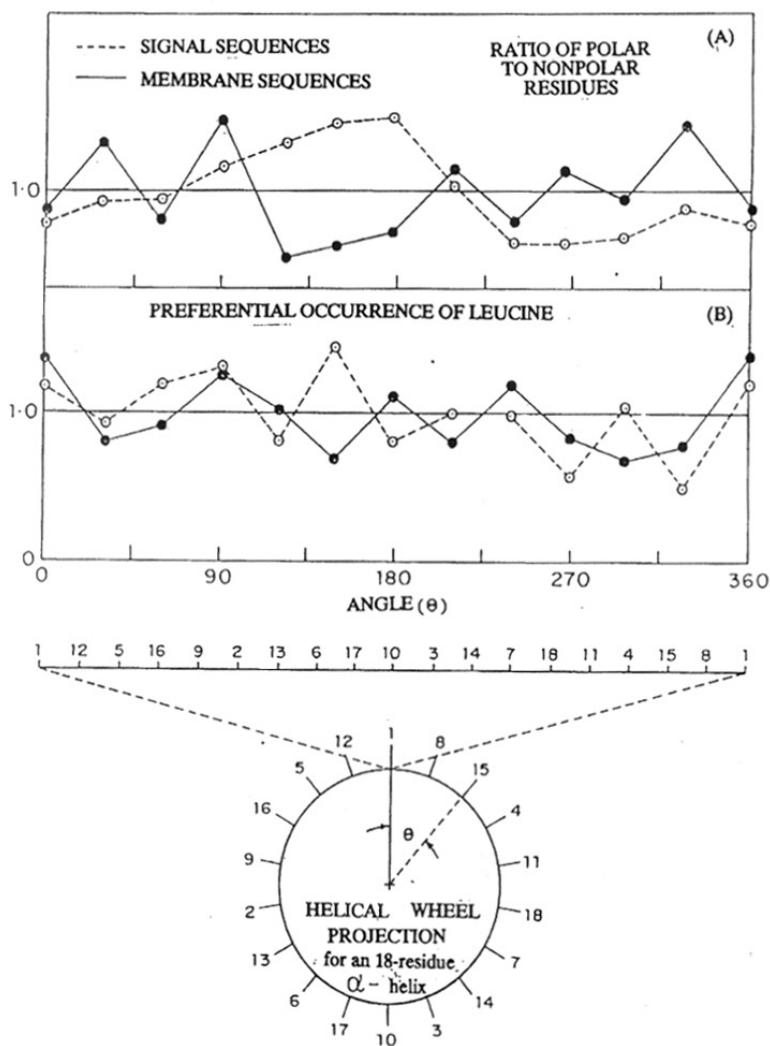
FIGURE 8.4 A The ratio of the preferential occurence of polar to nonpolar residues in signal peptides and transmembrane sequences of proteins about the axis of the membrane spanning alpha helix.

B The preferential occurrence of leucine residues about the axis of the alpha helix.

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The peptidase which initiates the hydrolysis of the signal peptides in the membrane in the case of *E. coli*, have been shown to be endoproteases (Novak and Dev, 1988). The ability of these membrane associated signal peptide peptidases to hydrolyze the signal peptide only after it is cleaved from the precursor implies that there may be some structural changes in the sequence after its cleavage. These structural changes may be attributed to the topological distribution of the amino acids on the surface of the helix. The clustering of polar residues on a particular face of the alpha helix of the signal peptide indicates that the polar side of the alpha helix may be in close vicinity of a protein with a similar surface. This is because, in an . aqueous medium the polar side can extensively interact with the medium via protein-water H-bonds (Arnold, 1988). The situation in a hydrophobic environment is quite different and the . hydrophilic forces would dominate the folding. The polar surface will therefore try to achieve stability by interacting with hydrophilic moieties in some other protein. This protein may be a peptidase similar to Protease IV as found in *E. coli,* as preliminary conformational studies have shown that a partly polar alpha helical surface may also exist in this peptidase (Shinde *et al,* 1990, a) and has been dealt with in the next chapter.

Thus, in conclusion, the distribution of amino acids on the surface of the alpha helical membrane spanning core along with other post translational modifications (Hare, 1990) may play an important role in degradation specificity of signal peptide peptidases. The above analysis depicts the finer differences between signal peptides and membrane spanning sequences. These differences may go unnoticed when amino acid compositions or sequence homologies are studied. However, the secondary structure, and positional preferences in relation to this secondary structure, provide clues about the differences between the two types of sequences. It is speculated that these topological differences may, along with other post translational modifications, play an important role in differentiation between signal peptides and transmembrane sequences. The above analysis can be useful in designing mutation experiments for elucidating the exact mechanism of the specificity and selectivity in signal peptide degradation.

CHAPTER 9

MECHANISM
FOR INITIATION OF DEGRADATION
OF SIGNAL PEPTIDES IN THE MEMBRANE
BY PROTEASE IV FROM ESCHERICHIA COLI

Signal peptides are rapidly degraded in the membrane by membrane associated proteases known as signal peptide peptidases. The signal peptide peptidase (Protease IV) from *Escherichia coli* has been analyzed to obtain a possible mechanism for this rapid degradation. The membrane associated regions of Protease IV have been predicted. Furthermore, regions in the protease which could probably interact with the signal peptides in the membrane have also been identified. Based on the analysis, a mechanism for the initiation of protein degradation in the membrane has been proposed.

CHAPTER 9

9.1   Introduction

As already discussed in chapter 8, the signal peptide after its cleavage from its precursor remains in the membrane for an extremely short period of time. The free signal peptide in the membrane is selectively and efficiently identified by integral membrane proteases known as signal peptide peptidases. The role of topological distribution of amino acids on the membrane spanning helix may play an important role in this process (Shinde, 1990 a). In the present chapter, Protease IV, a signal peptide peptidase from *Escherichia coli*, has been analyzed structurally in order to investigate the mechanism of its interaction with signal peptides. Based on the analysis, a model which explains the reasons *why only signal peptides are degraded, that too only after their cleavage from the precursor protein* has been proposed. Hydrophobic forces which play an important role in surface interactions (Mawal *et al*, 1989) have been shown to be extremely important in signal peptide function (Randall *et al*, 1987).

As stated in the previous chapter, the amino acid composition of the membrane-spanning sequences and signal sequences are similar within the statistical limits of error (von Heijne and Gavel, 1988). Furthermore, both these sequences acquire similar conformations upon association with the membrane bilayer (Briggs *et al*, 1986). However, only signal sequences are selectively degraded by the signal peptide peptidases (Ichihara *et al*, 1984). One of the possible reasons for not recognizing transmembrane sequences could be post-translational modifications on them.

Efforts to arrive at a consensus on conformations of signal peptides by computational methods have not been very successful often giving conflicting results (Duffaud *et al*, 1985). Yet to find structural patterns, to supplement patterns in physico-chemical properties and the consensus regions, would be very useful and might help in the design and analysis of mutation experiments (Klein *et al*, 1988). Structure prediction of membrane-associated sequences has always proven to be a difficult

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task. Nevertheless, algorithms giving a fair idea of the possible structure of a transmembrane sequence have been developed (Eisenberg, 1984). In the present work, the signal peptides and the signal peptide peptidase from *Escherichia coli* have been analyzed with the view of identifying regions responsible for recognizing differences between signal sequences and transmembrane sequences.

Identification of helical regions in Protease IV and signal peptides, and understanding the helix-helix interactions between them may help clarify, in general, the mechanism of signal peptide degradation. The prediction algorithm of Argos *et al* (1982), based on physical characteristics of the twenty amino acids, was utilized to identify likely membrane-buried regions in the primary sequence of Protease IV. These results were compared with those obtained using the method of Engelman *et al* (1986). The analysis indicates clustering of hydrophobic and hydrophilic residues, which are distinctly separated along the primary sequence of the protease, on the same side of the alpha helix. Similar regions have also been identified in signal peptides. The implications of such an analysis have also been discussed.

9.2   Materials and Methods

All signal peptides were selected from the current version of SIGPEP data base (von Heijne, 1987), which holds a total of about 200 prokaryotic and 900 eukaryotic sequences. In the present analysis, only the signal peptides (from *E. coli*) with completely known primary sequences were included. Transmembrane sequences were extracted from the EMBL DNA library (version 14.0, January 1988). All transmembrane sequences were analyzed by taking into consideration their orientation in the membrane. Protease IV was cloned and sequenced by Ichihara *et al* (1986).

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9.2.1   Identification of the Membrane Spanning Core

The identification of the membrane spanning core in both signal peptides, transmembrane sequences and the Protease IV sequence of *E. coli* was carried out as described in section 8.2.

9.2.2   Algorithms for Structure Prediction of Membrane Associated Proteins

In membranes only two backbone structures are possible. Since water molecules are not available to saturate the hydrogen bonds of the polypeptide backbone atoms, hydrogen bonds must be formed among the backbone themselves. This requires the backbone to fold in an ordered fashion, either in an alpha helix or a beta-strand. In the case of alpha helices, the hydrogen bonds can be formed within one helix so that a single helix may represent a stable structure in the membrane. To do so its entire surface must be hydrophobic. The identification of membrane buried sequences may be done using the ever popular Kyte-Doolittle method. This method, however, only predicts the hydrophobic sequences not differentiating between alpha helices or beta strands.

Methods for predicting transmembrane helical regions from amino acid sequence data rely on scanning the sequence for stretches of residues long enough to span the nonpolar region of a lipid bilayer as alpha helices and hydrophobic enough to be expected to be at lower free energy across a membrane than in an aqueous environment (Engelman *et al*, 1986). Only two structures are known with enough certainty to provide a test of these predictions: *bacterial photosynthetic reaction centers* and *bacteriorhodopsin*. The existence and approximate position in the sequence of the eighteen transmembrane helices in these two structures are well predicted by a simple hydrophobicity analysis (Michel *et al*, 1986; Engelman *et al*, 1982; Ovchinnikov *et al*, 1985). This success is all the more striking given that the helices involved, predicted to be stable as independent entities in the bilayer, actually have more contact with other helices and pigments than they do with lipids.

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The membrane-spanning regions of Protease IV were recognized by the algorithms of Argos *et al* (1982) and Engelman *et al* (1986). The plots of weighted parameters (Figure 9.1a) and the hydropathy plots (Figure 9.1b) versus residue number are shown. The sequences of regions coinciding by both methods, which exhibit amino acid segregation, are shown in figure 9.2. Clustering of polar and nonpolar amino acids on the surface of the membrane spanning regions of the signal peptides are shown in figure 9.3. Figures 9.2 and 9.3b show zones which are rich in leucine.

9.2.3   *Evaluation of Surface Topology of Membrane Associated Helical Regions*

The topographical studies were carried out using the procedures outlined in section 8.2. The membrane buried preference of the sequences was analyzed using the Engelman scale (Engelman *et al*, 1986).

9.3   Results and Discussions

The above analysis makes it possible to draw a number of conclusions regarding the possible structural interactions between Protease IV and signal peptides of *E. coli*. The peptidases which initiate the hydrolysis of the signal peptides in the membrane of *E. coli* have been shown to be endoproteases (Novak and Dev, 1988). The signal peptide peptidases hydrolyze the signal peptide only after it is cleaved from the precursor. The signal peptide would therefore need to undergo some conformational change that would initiate its degradation.

Protease IV from *E. coli* has been well characterized. Its amino acid sequence has been determined by Ichihara *et al* (1986). For this reason, it was considered to be an ideal sample to study possible interactions between signal peptide peptidases and signal sequences. Furthermore, using one particular species (*E. coli* in this case) would avoid arguments that the structural organization of signal peptides in other species may be different. The analysis may then be extrapolated to signal peptides as a whole.
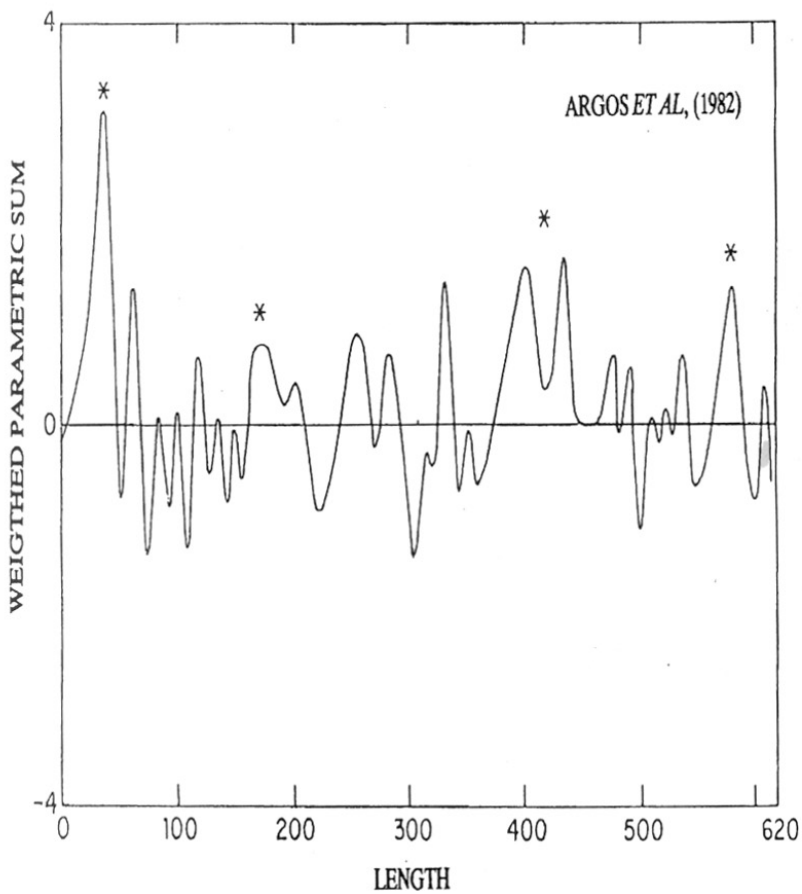
FIGURE 9.1A Profile of weighted parameter (Argos *et al*, 1982) as a function of length of Protease IV. "*" depicts probable transmembrane helices

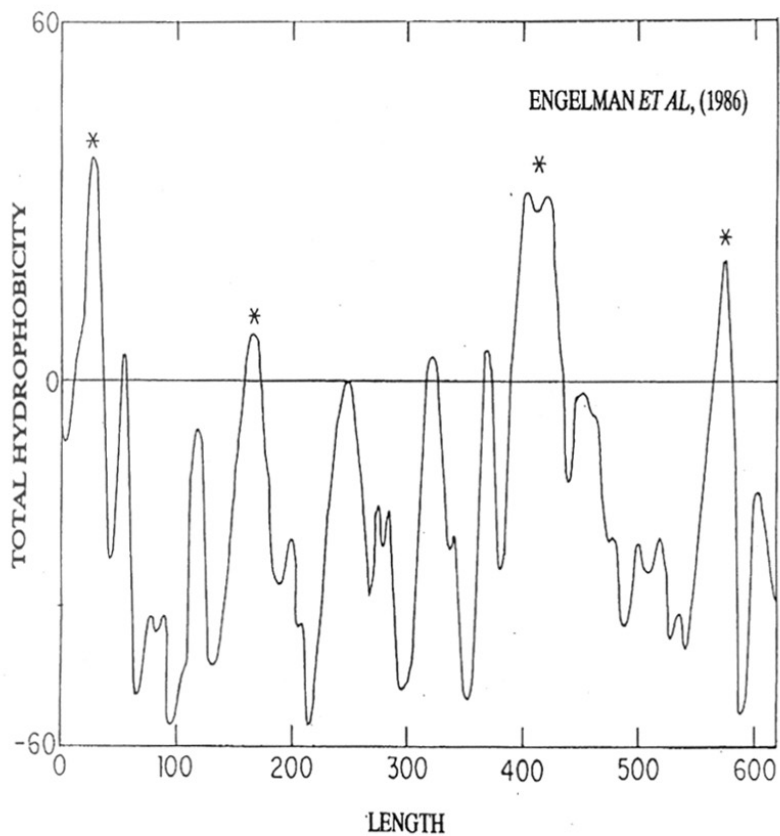**FIGURE 9.1B** Profile of hydrophobicity (Engelman *et al*, 1986) as a function of length of Protease IV. "*" depicts probable transmembrane helices
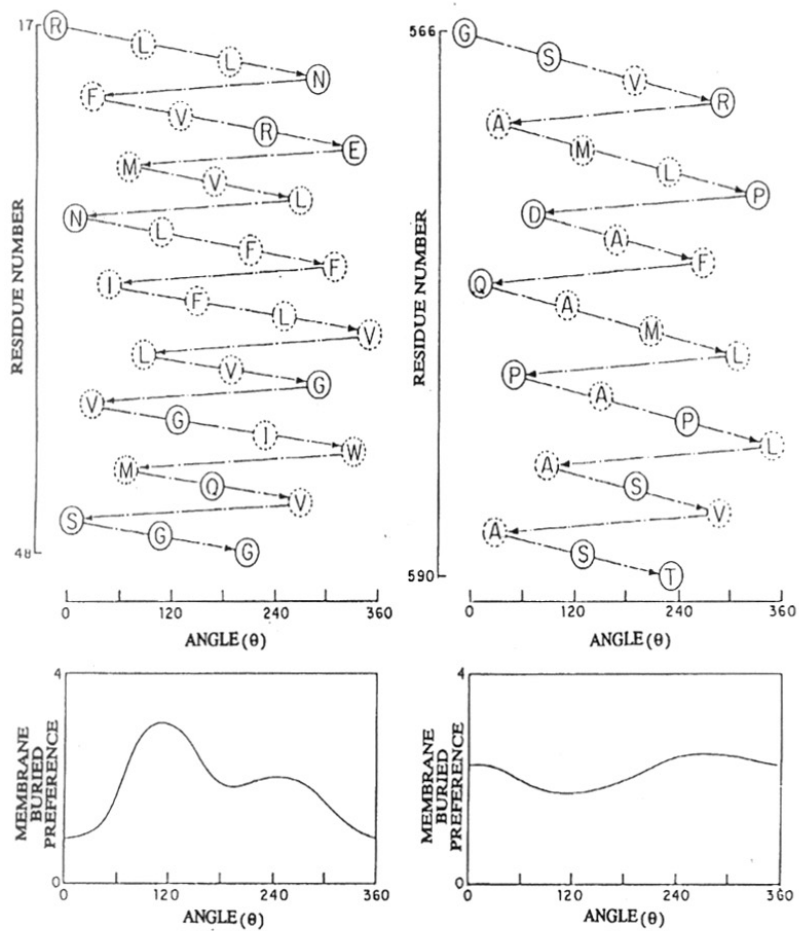
FIGURE 9.2 Proposed transmembrane regions of Protease IV arranged into an alpha helix with the corresponding membrane buried preference as a function of angle θ. Solid circles depict polar amino acid residues while dotted circle depicts nonpolar amino acid residues.

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In the previous chapter, regions have been identified in signal sequences which show a distinct topological distribution of nonpolar and polar residues. This bias is noticed when the ratio of the positional preferences of polar to nonpolar residues is plotted as a function of the angle between a residue in the alpha helix and the first residue in that helix (figure 9.3a). The results show a distinct segregation of polar and nonpolar residues on the surface of the helix implying that most signal peptides may probably be somewhat *amphiphilic* in nature. The maximum polar and nonpolar regions are, however, not exactly on opposite sides of the helix. Such a bias is however absent in transmembrane sequences.

Furthermore, figure 9.3b clearly indicates that even though the region from 80 to 225 degrees is significantly more polar than the rest of the surface, the preference of leucine to occur in this zone is relatively higher than that observed in other regions. This implies that the more polar face of the alpha helix incidently contains more leucine than the rest of the surface. It has been shown earlier (Shinde *et al*, 1989) that the interior of the signal peptide is leucine rich and very few polar amino acids may occur in it. This region exposed to the lipid bilayer will try to orient itself in a way so as to shield the polar region from the lipophilic interior of the membrane. It would therefore be interesting to identify whether such complementary regions also exist in the signal peptide peptidase. Conformational changes may be induced due to the positional preferences of the amino acids on the surface of the helix.

Helical structure is known to be induced in polypeptides in nonaqueous environments (Singer, 1962, 1971). The large free energy cost of transferring an unsatisfied hydrogen-bond donor or acceptor from an aqueous to a nonpolar environment or of breaking of such a bond in a nonpolar environment suggests that most hydrogen bonds must be satisfied when the peptides are inserted into the membrane environment. Alpha helices accomplish this in a systematic fashion, in that it links nearby parts of the chain. The nonpolar helical structure is a very stable one and can accommodate several polar groups without becoming unstable. The insertion of polar groups into the bilayer may be necessary for proper functioning of the protein.
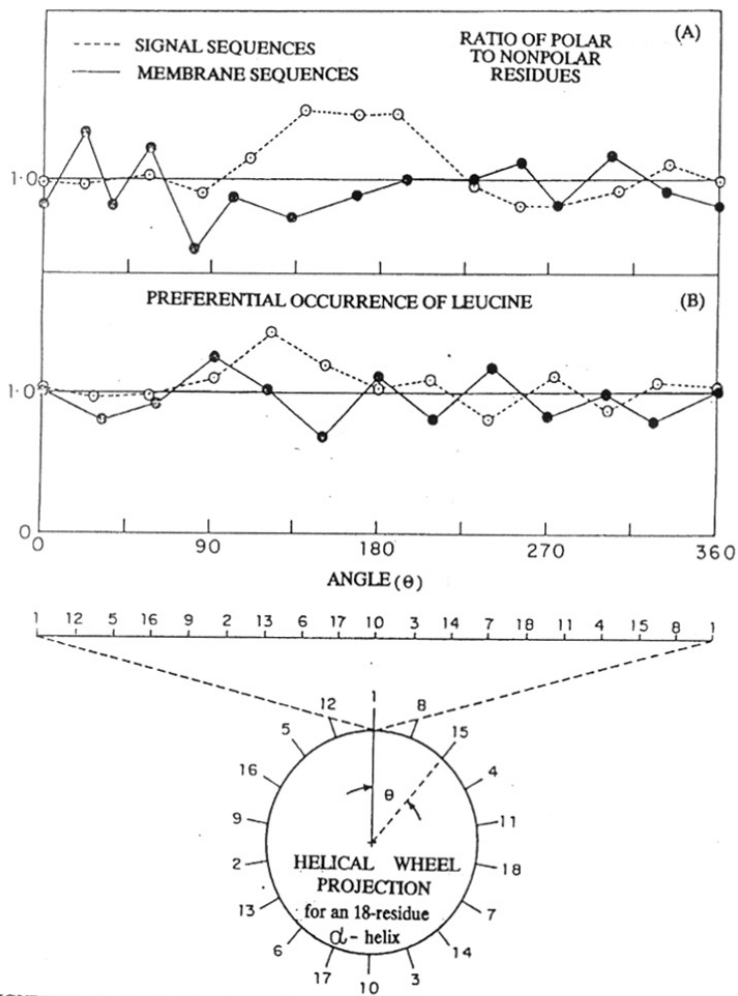
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FIGURE 9.3 A Ratio of preferential occurrence of polar to nonpolar residues in signal peptides (*E coli*) and transmembrane sequences in proteins about the axis of the alpha helix.

B Preferential occurrence of leucine in signal peptides (*E coli*) and transmembrane sequences about the axis of the alpha helix.

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The possible helical regions in Protease IV are shown in figure 9.1. These regions were analyzed for amphiphilicity similar to that found in signal sequences. Such identified regions are shown in figure 9.2. It can clearly be seen from figure 9.2 that the polar and nonpolar regions lie on the same side of the alpha helix but are distinctly segregated along the length of the alpha helix. This clustering of residues on a particular face of the alpha helix of the signal peptide suggests that the polar side of the alpha helix may be in close vicinity of a protein with a similar surface. It has been shown that the formation of oligomeric complexes of proteins in membranes frequently involve the side to side interaction of transmembrane helices (Popot and Engelman, 1990; Popot and de Vitry, 1990). Examples of this type are seen in the oligomer of the photosynthetic reaction center and in the trimeric association of bacteriorhodopsin molecules. Association of single transmembrane helices is encountered in complexes of the photosynthetic and respiratory chains (Popot and de Vitry, 1990) and may play some role in the dimerization of some anchored proteins (Bormann *et al*, 1989). In these instances, the packing of the helices at the subunit interfaces is like that within the subunit themselves. Thus, understanding the helix-helix interactions in the bilayers may clarify both the folding of individual molecules and factors involved in oligomerization.

We propose that a *side to side helix interaction* is possible between the signal peptide integrated in the membrane and a protein similar to Protease IV. This could be important in initiating signal peptide degradation. After its cleavage from the precursor, the signal peptide in the membrane may be relatively more free to undergo conformational changes inside the membrane. Thus the signal peptide may now change its conformation, with its polar region moving closer to the signal peptide peptidase. This flexibility may be very important for cleavage as it has been shown that flexibility in the conformation is a necessity for degradation of proteins and peptides (Parsell and Sauer, 1989).
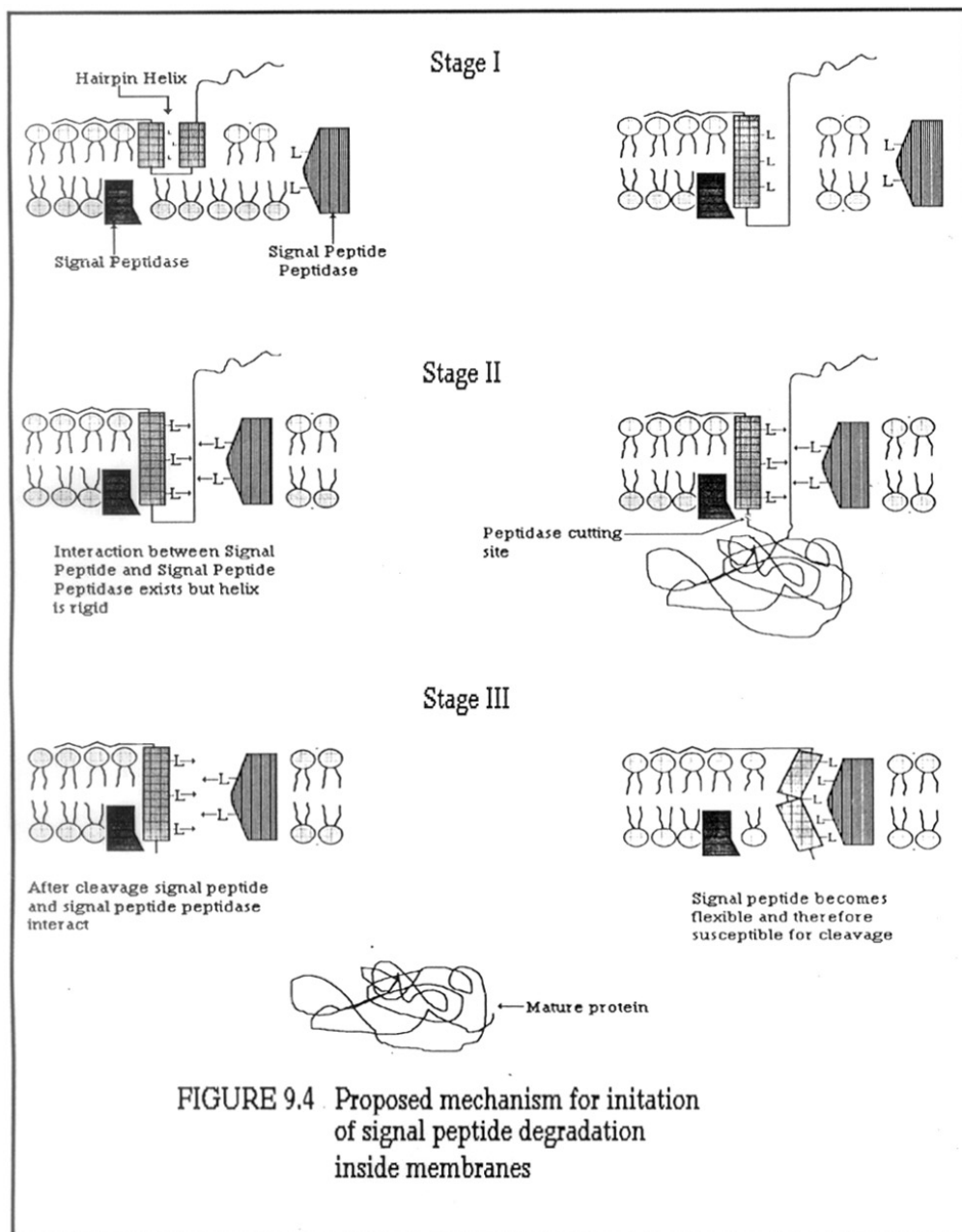
The proposed mechanism for initiation of signal peptide degradation is depicted in the figure

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9.4. The individual steps are described below in detail.

Stage I : The signal peptide penetrates inside the membrane in a hairpin like helical form. After entering, it reverts back to an extended alpha helix by the mechanism described in chapter 7.

Stage II : The topological distribution of amino acids on the surface of the membrane spanning alpha helix induces it to interact with an integral membrane protein. This protein, namely Protease IV, has a similar surface. It is proposed that transmembrane sequence of the Protease IV contain not only the structural information necessary for insertion and anchoring but specific binding sites which are responsible for identification and initiation of protein degradation. The side to side interactions between the polar surfaces of the both the signal peptides and the Protease IV membrane spanning region would be favoured. This is because, integral membrane proteins are partially buried in the nonpolar environment of the lipid bilayer, where the hydrophobic effect is absent and the intrachain hydrogen bonds and polar interactions take a much greater significance than in water, since the lipid is unable to form them. This balance has led to the expectation that the transmembrane region of membrane proteins would consist of predominantly hydrophobic segments with regular secondary structure and, more specifically, of bundles of hydrophobic alpha helices (Henderson 1975, 1977). Hydrogen bonds and ion pairs can be used to drive the association of local regions of a pair of helices. Van der Waals interactions would then promote detailed close packing and further stabilize the association. The strength of one hydrogen bond in a nonaqueous medium is 4-6 kcal/mol (Allen, 1975). Thus a single hydrogen bond, in principle could match the entropic factor. Ion pairing could provide even larger association energies but would depend on the local environment of the ion pair (Honig *et al*, 1986). This expectation has been largely fulfilled, although some exceptions do exist.

The contribution of packing effects deserves close examination. It may be difficult for lipid chains to pack well against the surface of an alpha helix given the irregular contour of presented by the protruding side chains. The magnitudes of such contributions are difficult to calculate but are presently being attempted.

FIGURE 9.4 Proposed mechanism for initation
of signal peptide degradation
inside membranes

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The *leucine-like zippers* could also play an important role in this interaction. It is proposed that the alpha helical signal peptide, when attached to the precursor protein, is a rigid rod like structure even though the a strong interacting force can exist between the two helices. The rigidity may be attributed to the protein on the trans side of the membrane. For the alpha helix to bend or be flexible would require some amino acids translocated on the other side of the membrane to be pulled inside the membrane. The amount of energy required to pull polar or charged amino acids from a hydrophilic to a hydrophobic medium would be exorbitant (Singer, 1971). This therefore induces rigidity in the transmembrane alpha helix. Some time during or after the process of translocation, the signal peptide is cut from the precursor by the signal peptidase, to yield the mature protein.

Stage III : After the cleavage to yield the mature protein, the signal peptide becomes flexible. This is because there is nothing that can stop the C-terminus from being pulled inside the interior of the membrane. The polar regions between the signal peptide and Protease IV now interact and the signal peptide due its flexibility can undergo rapid degradation in the interior of the membrane.

CHAPTER 10

CAN MOBILE SHAPES OF FRACTALS
CAUSE RATE ENHANCEMENTS ?

The concept of mobile fractal shapes *(fractiles)* is introduced and applied to biological processes. Systems like biological membranes, proteins and nucleic acids, which can be described as fractals, exhibit significant dynamism in their shapes. Numerous such examples in other physical and chemical systems also exist. The effects of this dynamism or mobility on the overall physical, chemical and biological process have been studied. Results indicate that mobility can drastically affect the rate constants of such processes.

Chapter 10

10.1   Introduction

Signal peptides, after they are cut by membrane associated signal peptide peptidases, are free to move inside the lipid bilayer of the membrane. The fluidity of the membrane has been shown to affect the rates of variety of biological processes. The implications of this fluidity, which membranes can pose over rates of reactions, have been considered in this chapter. Initially in this chapter, the concept of *fractal structure* and *fractal dimension* has been introduced. The applications of these concepts to naturally occurring shapes and effect of such surfaces on reaction kinetics has also been considered and applied to physical, chemical and biological systems.

10.2   The Concept of Fractal Structures and Fractal Dimension

Investigators who study bulk matter face a problem of bewildering complexity. Each macroscopic bit of the world contains gigantic numbers of atoms and molecules that are often arranged in complicated, disorderly patterns. In case of a perfect crystal or a smoothly flowing liquid, the pattern is uniform on large scales. Even though a great deal can be understood about such systems, the vast majority of complicated natural phenomena such as turbulent flows of liquids or air, the accretion of metal particles in an electrolytic bath and the formation of mountain ranges etc. have virtually defied understanding (Sander, 1987).

In the past 10 years both scientists and mathematicians have made much progress towards gaining such comprehension. Central to many of the new insights is the revolutionary concept of a *fractal* (Mandelbrot, 1977). A fractal is an object with a sprawling, tenuous pattern (Figure 10.1). As the pattern is magnified it reveals repetitive levels of detail, so that similar structure exists on all scales. A fractal might, for example, look the same whether viewed on the scale of a meter, a millimeter or a micrometer (Figure 10.2). Mandelbrot (1982, 1984,) pointed out that many disorderly
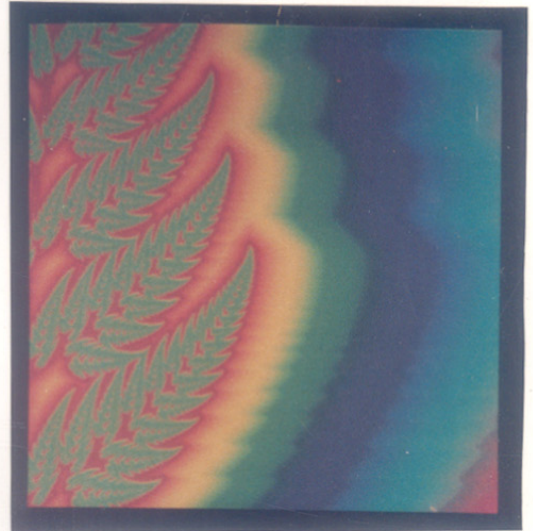
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FIGURE 10.1 Mandelbrot's concept of *fractals* provides a descriptive and mathematical way to describe many seemingly complex forms found in nature. The figure shows some examples of fractal shapes that can be generated on a computer (Barnsley, 1989; Mandelbrot, 1977 & 1990).

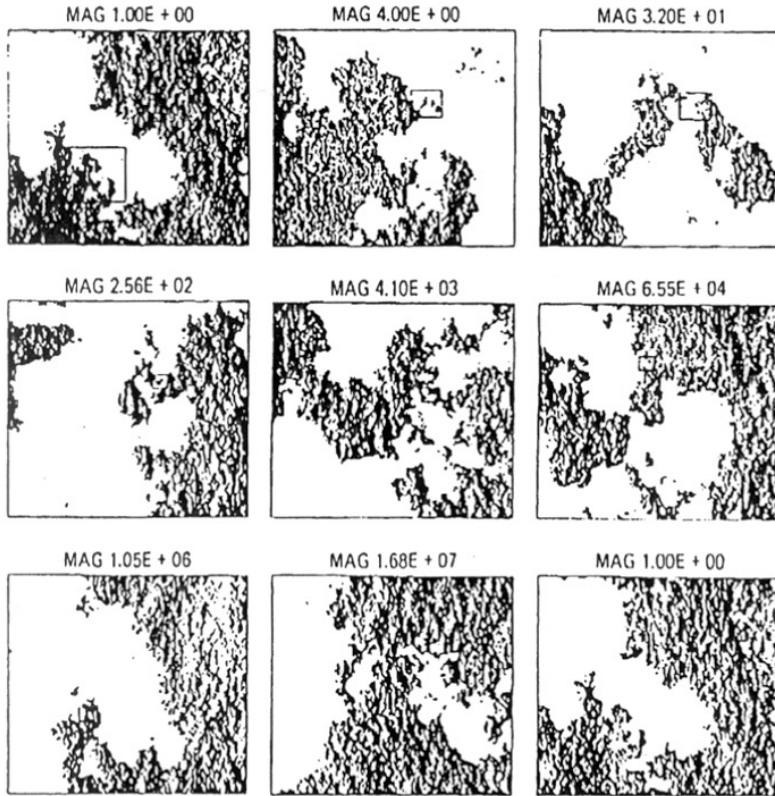| MAG 1.00E + 00 | MAG 4.00E + 00 | MAG 3.20E + 01 |
| MAG 2.56E + 02 | MAG 4.10E + 03 | MAG 6.55E + 04 |
| MAG 1.05E + 06 | MAG 1.68E + 07 | MAG 1.00E + 00 |

FIGURE 10.2   Zoom sequences of a coastline of a statistically self-similar fractal landscape of dimension (D) 2.2. Each succeeding picture shows a blowup of the framed portion of the previous image. As the surface is magnified, a small portion looks similar to (but not exactly the same as) a larger portion. The total magnification corresponds to 16 million (Barnsley et al, 1988).

objects in nature have this property. In other words, fractal structures are shapes that cannot be defined by Euclidean geometry. They are often remnants of chaotic nonlinear dynamics (Goldberger et al, 1990). Because a fractal is composed of similar structures of ever fine detail, its length is not well defined, as a result of which mathematicians calculate the *dimension* of a fractal structure to quantify how it fills space (Sander, 1986). The familiar concept of dimension applies to Euclidean geometry. Lines have one dimension, circles have two and spheres have three dimensions. Fractal dimensions, however, can sample between one to three and it need not have an integral value. The greater the dimension of a fractal, the greater the chance that a given region of space contains a piece of that fractal. The fractal dimension of an object is an *universal property* of the system and is independent of how the object was formed. The fractal dimension and the other universal properties are related to the behavior at larger scales, where the particular details average out. As a consequence, a simple model that neglects most of the complexity of the real system nonetheless describes the scaling properties of the system correctly is obtained. Abstract discussions of objects now called as fractals were given long before Mandelbrot by other mathematicians (Mandelbrot, 1974). *Scale invariance* is a symmetry of fractals. Just as round objects are symmetric under conditions of rotations, fractals are symmetric under conditions of dilations, or changes of scale.

10.3   Examples of Fractals in Nature

An increasing amount of evidence suggests that nature's love of fractal shapes is a deep one (Figure 10.3). Fractals known as percolation clusters (Figure 10.4) have been identified with pattern of a fluid flowing through a solid matrix, such as water seeping through a soil or coffee through ground coffee beans. Soot, colloids and some polymers seem to be fractals. Fractals also occur in the motion of air bubbles in oil, the growth of some crystals and the behavior of electrical discharges resembling lightning bolts. The growth of bacterial colonies also results in fractal shapes (Sharma et al, 1990) The random patterns of clouds and coast lines are almost certainly fractals as well (Stauffer, 1986).
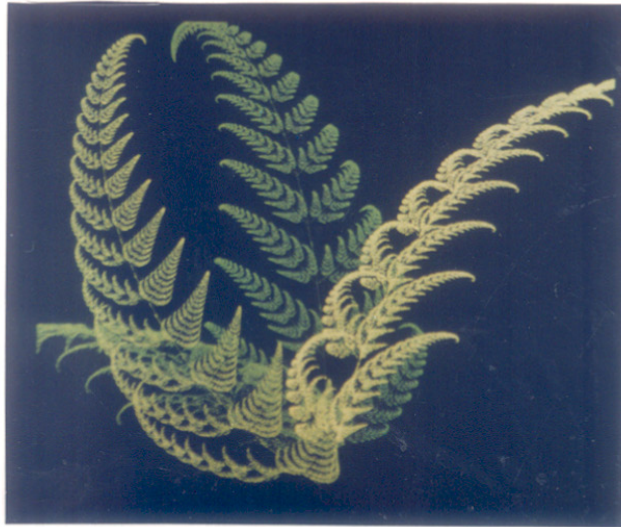
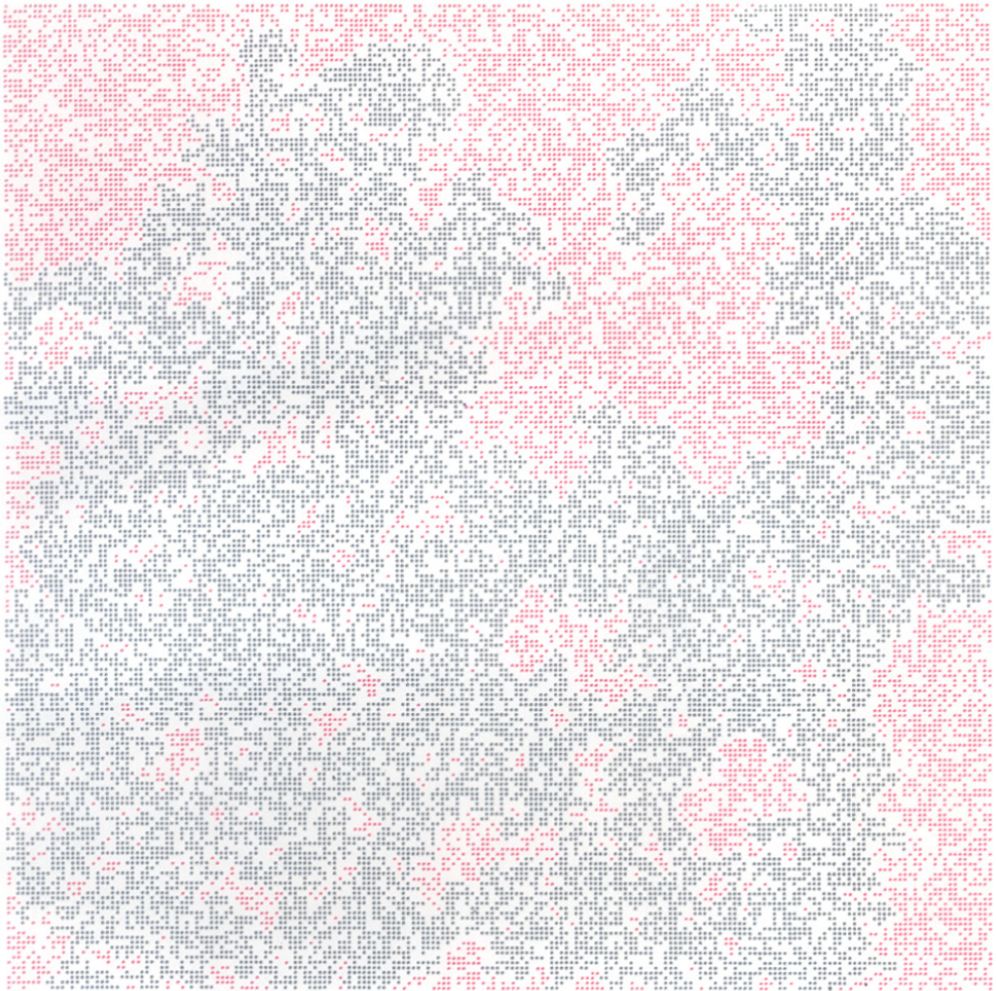FIGURE 10.3   Examples of fractal shapes that occur in nature (Mandelbrot, 1977 & 1990).

FIGURE 10.4   A 200 X 200 percolation cluster at its threshold. The pink dots indicate/depict finite clusters whereas the black points represent the infinite cluster.

In biological systems, one can expect the formation of fractal structures at interfaces between two or more phases (Smirnov, 1990). Several biological patterns contain many components, which are found in a physical solution in states of different phases. It can lead to particle formation at the interface and further to their sticking into a fractal cluster. An example of such a type concerns the behavior of some phospholipids in a watery solution at the air-water interface (Miller *et al*, 1986). The phospholipid is collected in some domains which form a fractal structure by sticking to each other. The domains aggregate due to interactions and a change in the surface tension of the water through the action of these domains. The fractal dimension of such self-similar structures of crystalline domains is equal to 1.5 +/- 0.1.

In the human body fractal like structures abound in networks of blood vessels, nerves and ducts. The most carefully studied fractal in the body is *the systems of tubes that transport gas* to and from the lungs (Goldberger and West, 1987). Many other organs also appear to be fractal in nature, though their dimension has not been quantified. Such structures have tremendous advantages; they can increase the surface area available for absorption and distribution of nutrients or substrates. Fractal structures by virtue of their redundancy and irregularity are robust and resistant to injury. Fractal like structures play a very important role in healthy, mechanical and electrical dynamics of the heart (Goldberger *et al*, 1988)

Other examples of fractals in biological systems include proteins and nucleic acids. Much work has been done in applying the concept of fractals to such systems (Sharma *et al*, 1990; Lewis and Ress, 1985; Wako, 1989; Isogai and Itoh, 1984; Pfeifer *et al,* 1985; Dubuc *et al*, 1989).

10.4   Fractal Reaction Kinetics

Among the most important chemical reactions are those called as *heterogeneous*. These reactions take place at the interfaces of different phases, for example, gas-solid or liquid-solid

boundaries, and include industrial surface-catalysis and electrode reactions, as well many bioen-zymatic reactions and membrane reactions and some geochemical and atmospheric reactions (Kopelman, 1988). In addition there are many heterogeneous *non-chemical* reactions: in solid-state physics there are electron-hole, soliton-antisoliton, exciton-exciton *recombinations* as well as aggregations of excitations, defects and so forth (Kopelman, 1986; Klymko *et al*, 1982, 1983; Kopelman *et al*, 1986). Charge and excitation recombination, as well as excitation quenching, are also found in biological systems, such as photosynthetic units (Kopelman, 1988)

The universally found instruction in chemical synthesis is to *stir well*. However, convective stirring cannot be achieved for reactions in or on media that are solid, viscous, porous, otherwise unstructured. In absence of convective stirring, there is still diffusive stirring, which is called as *self stirring*. However, under dimensional constraints (surface reactions) or topological constraints (surface reactions), self stirring may be highly inefficient. Fractal spaces such as percolation clusters are ideal testing grounds for *understirred* reaction kinetics and such studies are called as *fractal-like reaction kinetics* (Kopelman, 1988).

10.5   The Concept of Fractiles or Mobile Fractal Shapes

It is known that different fractal sets with different appearances or textures may have the same fractal dimension (Voss, 1988). However, if a reaction occurs on a fractal shape whose lacunarity evolves with time, the rates of the reactions may be drastically different. We define *fractiles* as fractal objects which change their shapes, but maintain the same fractal dimension (Shinde *et al*, 1990 c).

10.6   Applicability of Fractiles in Real Systems

Fractile systems do occur in practice frequently and the example of cell membranes may help

to familiarize the concept in simple terms. Biological membranes are organized sheet-like assemblies consisting mainly of proteins and lipids. The functions carried out by them are indispensable for life. Membranes are highly selective semipermeable barriers which contain specific molecular gates and pumps. These transport systems regulate the molecular and ionic composition of the intracellular medium. Cell membranes are frequently described as fractals with non-integer dimensions. Though membranes are fractal in nature, their fractal dimension has not been calculated to date. The protein and lipid units in the membrane plane are known to undergo diffusion and the rates of their movement are dependent upon various factors such as the composition of the lipids and the protein to lipid ratio. Furthermore, external factors like temperature, pH etc also affect the diffusion of the components (Tocanne *et al*, 1990). The protein and lipids which form the lattice points that describe a fractal shape can themselves be in motion. This leads one to the notion of a mobile fractal. The activity of certain membrane associated proteins is known to be affected by the lipid composition around it. As mobility of the membrane lipids is high, the local concentration around any protein may undergo a constant change as a result of which the activity of the protein can be affected.

Other examples include DNA-protein complexes (von Hippel *et al*, 1989) and reactions on catalytic surfaces. During the DNA-protein interaction (which depends on the environment (Shakked *et al*, 1989) intermediate complexes are formed. It is known that the regulatory protein in such complexes can bind to sites that are quite far apart along the DNA contour. However, they are brought together transiently by the segmental diffusion of individual loops of the DNA double helix (Berg *et al*, 1981). This again suggests that a fractal describing DNA can experience changes in its shapes (for pictorial representation see (Stryer, 1988)). In a broader sense, mobility of the individual components of the membrane will amount to a change in fractal shape.

A closer look at most systems reveals mobility in one form or another (McCammon, 1984; Duprez *et al*, 1990; Hopkins *et al*, 1990). It is known that a number of enzymes have mobile surface

loops that change conformations to allow ligand entry and to secure the ligand in place (James and Sielecki, 1983; Anderson et al, 1979; McCammon and Northrup, 1981). X-Ray analysis of several enzymes also indicate large scale structural changes upon sorption of substrate (Anderson et al, 1978; Bennet and Steitz, 1978). The presence of external fields, indirectly inducing mobility, is also known to enhance the rates in some other systems (Vayenas et al, 1990; Weaver and Astumain, 1990).

The past decade has witnessed vigorous activities in applying the concept of fractals to various problems in as diverse areas as chemistry, biology, geology, solid state physics, astrophysics and atmospheric sciences (Sander, 1986, 1987; Smirnov, 1990; Kopelman, 1988; Goldberger et al, 1990). The literature is replete with information regarding the results of several different types of experimental systems and theoretical tools to compute the rates of transport and reactions on fractals. Despite this fact, the effects of mobility of the fractal shapes have not so far been investigated. This becomes more relevant when one notices that a large number of experimental systems with rate exceeding the limit set by the diffusion controlled nature of the process by orders of magnitude are known (see experiments of Riggs et al (1970)). In fact the question of how systems of different sorts might transcend the rate limits set by three dimensional diffusional control has been an unresolved question despite the flurry of theoretical investigations and the experimental papers coming from as varied disciplines as mathematics to physical chemistry to biology. The possible explanations offered and the progress in some of these areas has been recently reviewed (von Hippel et al, 1989).

10.7   Simulation Studies of Elementary Reactions on Fractiles

The present work undertakes to explore the effects of mobility of the fractal on the behavior of the system. The consideration of mobile fractals leads to the question as to whether the fractal dimension of the system itself would change or not. Such a question should be answered on a case

to case basis. Our interests here are to first establish the qualitative effects and thus for the time being we begin with a fractal object of specified dimension and allow its shape to change (at different speeds) while maintaining the same fractal dimension.

We consider a system for which extensive Monte-Carlo simulations have been carried out (two-particle annihilation process, A + B --> P, with *initial pair-correlated* and *random distribution* (Ben-Avraham, 1987)) and the results known. This will help in clearly identifying those effects that come about due to mobility of the fractal and, in the limiting case of no mobility, will also provide a test for the validity of simulated results.

In a random type of distribution, for two-particle annihilation on a fractal surface, the particles A and B are allowed to adsorb on the surface in a way such that there exists absolutely no relation between the position acquired by a particle A and the position of a particle B. In the case of distribution of the initial pair-correlated type, the distribution af a particle A is such that it is within a short and defined distance of a particle B and *vice versa*. Generally, this is achieved by distributing species A and B as pairs on the fractal surface.

10.7.1   Generation of the Percolation Cluster

For simulation purpose we begin with a percolation cluster at its threshold value p_c (0.5982). The percolation cluster was generated essentially by the way of Stauffer (1985). In the usual process, each point on the lattice is occupied randomly with the probability p and left unoccupied with a probability $(1-p)$, independently of what its neighbor is. A *cluster* is a group of neighboring occupied sites. For some probability p, larger than some threshold p_c, one *infinite cluster* appears besides many *finite* clusters, whereas for p below p_c all clusters are finite in the thermodynamic limit. A infinite cluster will therefore touch all four sides of the lattice grid that contain it. In all the simulations only the infinite cluster, generated at p_c was utilized. A typical *infinite cluster* along with the *finite clusters* are depicted in the figure 10.4.

10.7.2 Movement of Cluster

The next step is to identify particles which can move. The possible attachments of particles and allowed movements are shown in figure 10.5. Four pivots at the four edges are identified. These are not allowed to move to ensure same fractal dimension all through the simulation. Each particle capable of movement, except for the singly attached ones, is identified and allowed to move to a neighboring site not occupied by the cluster but being itself a nearest neighboring site to a point on the cluster. The singly attached particles are allowed to random walk and attach to a cluster site with a certain probability. The evolution of the fractal surface with time is shown in figure 10.6.

10.7.3 Reaction Simulation and Random Walk on Percolation Clusters

To simulate diffusive annihilation on a 2D percolation cluster, sites on the cluster are *picked up* and *occupied* with *particles,* either A or B, in a way avoiding multiple occupancy until a desired concentration of the *particles* has been achieved. The *particles* are then selected at random and then moved to a randomly selected *nearest-neighbor* site. The reaction occurs if two antiparticles are nearest-neighbors of each other. The simulations were thus carried out using standard procedure (Meakin and Stanley, 1984) and the results were compared and validated with the results obtained for the reaction on a static fractal using a large lattice size (1000 X 1000) (Figure 10.7). However, for computational reasons and for relative ease in engineering fractal mobility, we preferred conducting the simulations on a smaller lattice. The effect of smaller lattices on reaction profiles have been noted by earlier workers (Toussaint and Wilczek, 1983). These would not affect the qualitative results as finite size effects would be observed in case of both the static and mobile fractals. Additionally periodic boundary conditions were employed in the simulations. Hence a comparison between the two would still give a reasonable indication of the effects of fractal mobility on the reaction kinetics.
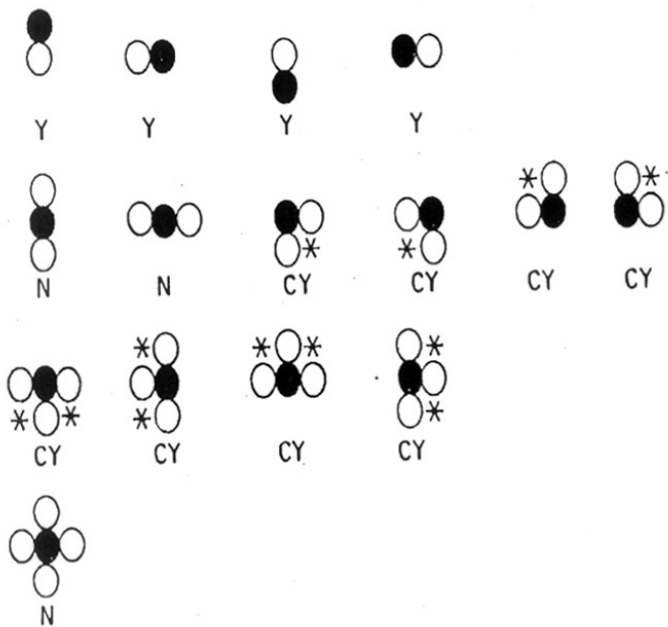
FIGURE 10.5 Schematic showing possible ways of particle attachment and conditions for movement. Solid circles indicate the particle being considered for movement.

N ----- cannot move at all

Y ----- can move randomly anywhere on the lattice but the position where it moves should have at least one of its neighbors as a part of the fractal.

CY ----- can move a distance of one lattice point if
a) position marked * is/are occupied by the fractal lattice points
b) if the position where it moves has at least one of its neighbors as a part of the fractal

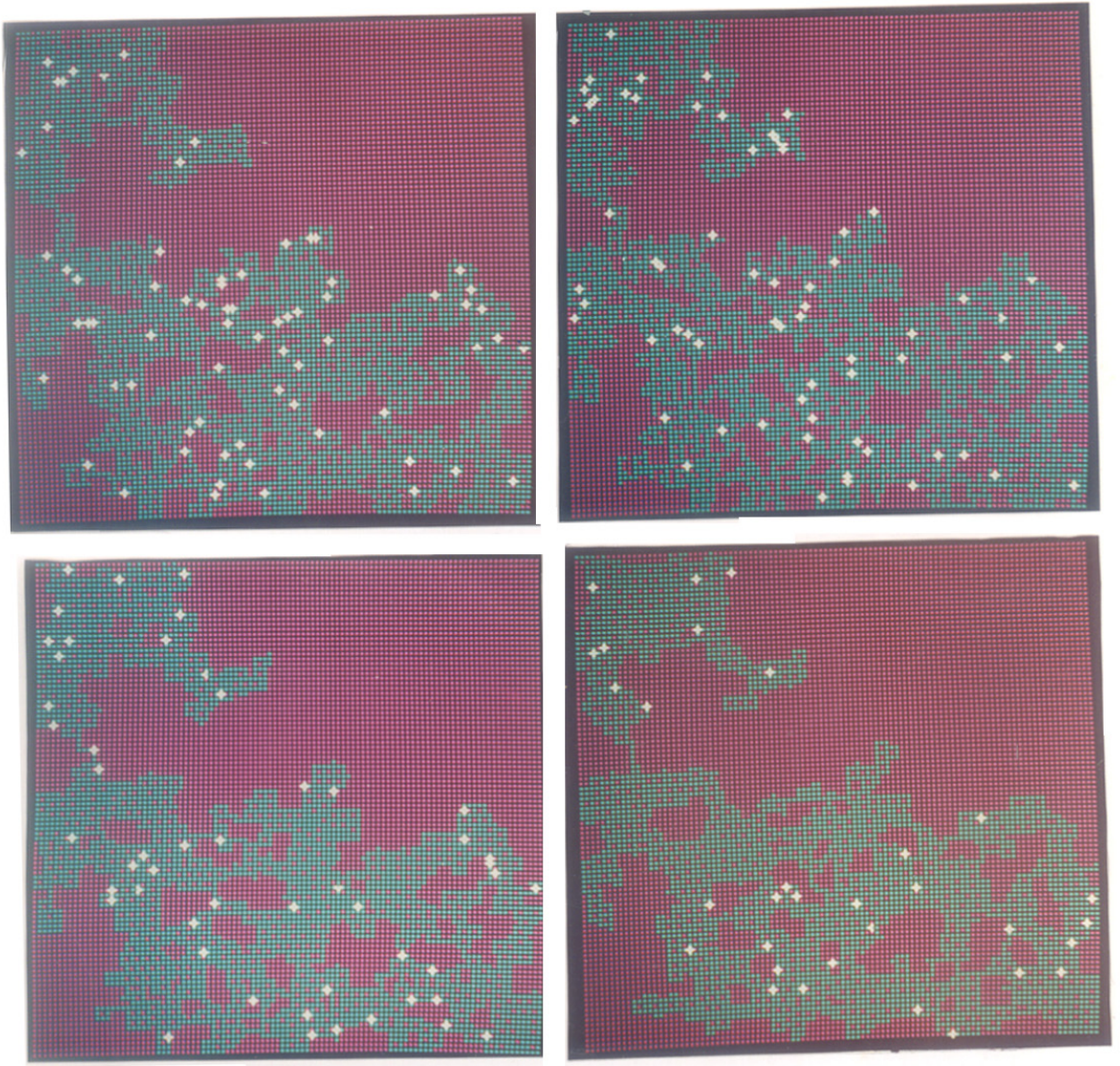**FIGURE 10.6** Evolution of fractal shape with time
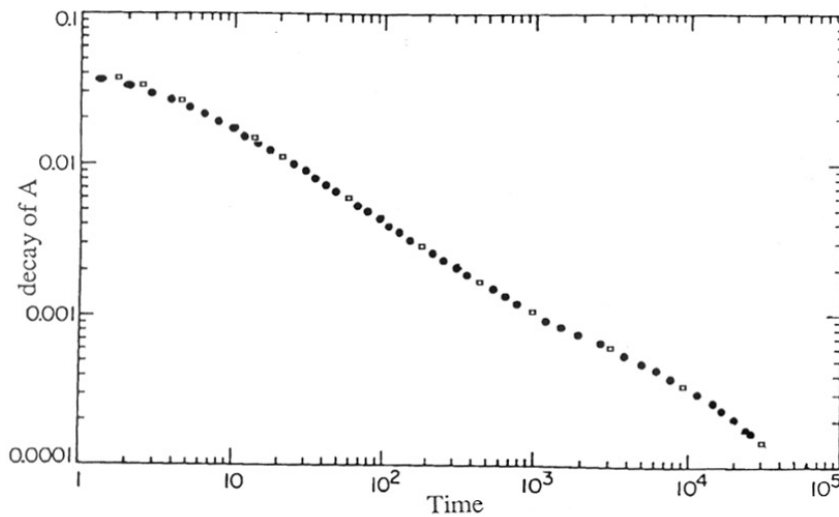
FIGURE 10.7   Particle density in the particle-antiparticle model (A + B --> Inert) in two dimensions for an initial particle density of 0.05. The solid circles depict the results of Toussaint and Wilczek (1983), whereas the empty boxes show our results. The lattice size in both the cases is 1000 X 1000.

The lattice points describing the fractal are moved to gauge the effect on the reaction. After all the particles have attempted to move once and A and B are found to be nearest neighbors, the number of product formed is incremented by $N_A * k1$, where N_A is the number of A molecules with B as nearest neighbors (pair) and $k1$ is the rate constant. Each time this value exceeds an integer one pair of A and B is selected, at random, and converted to inert product. The fractal is made to move from one to about 60 times during one catalytic turn over (this is defined as the number of substrate molecules converted into product per unit time, when the fractal is fully saturated with the substrate). This order of mobility is in the practical range. For instance in the case of cell membranes it is known that integral membrane proteins can diffuse about 60 nm during the time it takes for approximately one catalytic event or more of the integral redox proteins during the electron transfer activity (Sowers and Hackenbrock, 1981).

10.8   Results and Discussions

Figure 10.8 shows that the mobility of the fractal leads to greater product formation (case of a random initial distribution) in the 'two-particle annihilation' process with spatial inhomogeneities. The mobility of fractal causes 'surface renewal' resulting in bringing more and more of A and B particles together and facilitating the reaction. In the case of a pair-correlated initial distribution, however, a lower amount of product is formed when compared to a reaction on a static fractal surface. The mobility of the surface in this case tends to restructure the pairing and thus losing A to B contact and results in low product formation. Figure 10.9 shows decay of reactants as a function of time.

Figure 10.10 shows the effect of fractal mobility on the amount of the product formed as a function of initial concentration of A and B. Profile IV in this figure corresponds to behavior on a static fractal (rate constant = 1) while profiles II, III and V show the behavior on a fractal surface with different mobilities. If we were to reproduce the profile 'II' say on a static fractal, we observe that we would need a rate constant which is about 100 times larger. This is equivalent to saying

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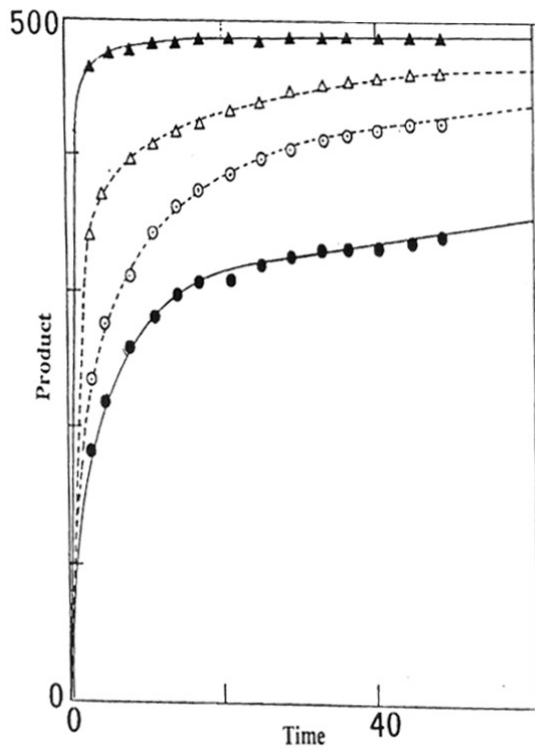
FIGURE 10.8   Product versus time curves with a random initial distribution on a static (●),
on a mobile fractal (O), with a pair-correlated initial distribution on a static
fractal (▲) and a mobile fractal (△).

179
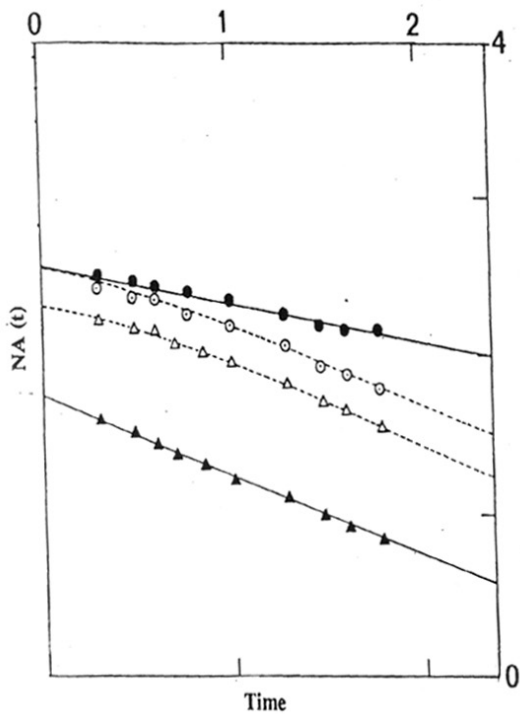
FIGURE 10.9   Profiles depicting the decay of A with time with a random initial distribution on a static fractal (●), on a mobile fractal (O), with a pair-correlated initial distribution on a static fractal (▲) and a mobile fractal (△).

180
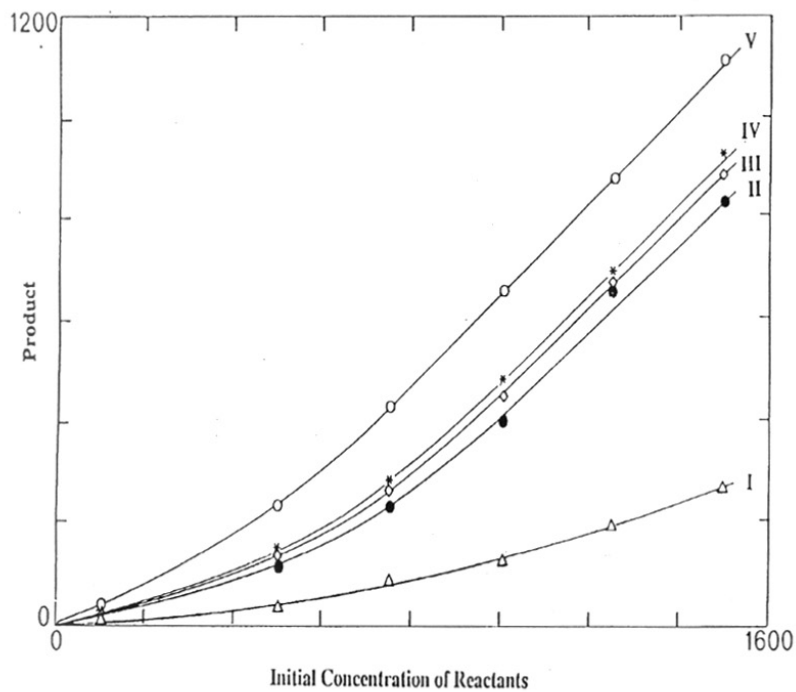
FIGURE 10.10 Profiles showing the extent of product formation as a function of initial concentration of reactants

I ----- reaction on a static fractal with reaction rate constant 0.1

II ----- reaction on a mobile fractal (moved forty five times) with a reactant rate constant 0.01

III ----- reaction on a mobile fractal (moved five times with a reaction rate constant 0.1

IV ----- reaction on a static fractal with reaction rate constant 1.0

V ----- reaction on a mobile fractal (moved once) with reaction rate constant 1.0

that on a mobile fractal corresponding to profile II, we have observed a 100-fold increase in the rate. Additionally, simulations for A + B --> *either* A *or* B (Ben-Avraham, 1987) and the complex reaction scheme (Meakin, 1986) with an added step for the formation of C when two surface adsorbed A particles come to the same site, were also performed. The results of the former show an enhancement in reaction rates on a mobile fractal. Similarly, higher selectivity is observed for reaction on a mobile fractal.

In summary, the general results of analysis verify the known results for the case of static fractals, thus validating the methodology, and provide a measure of the effects of the mobility. It is interesting to note that rate enhancements to the tune of several hundred-fold seem possible, although in certain cases (pair-correlated distribution) the overall effect may be deleterious. The mobile fractal shapes seem to provide another way for explaining rate enhancements in the range observed in some systems and can have wide implications in understanding several biochemical as well as physical and chemical systems. The concept of fractiles when applied to real problems may have drastic effects on reaction rates and reaction profiles.

Argyrakis and Kopelman (1989) have studied the effect of stirring on decay rates on fractal surfaces. In their simulations, either a part of the reactants or the complete reactants are removed and randomly redistributed over the fractal surface. Similarly, Harison and Zwanzig (1985) in their model for a dynamic percolation cluster, randomly redistribute points on the percolation cluster. In both the above cases, though some amount of dynamism does exist, the past, present and the future states of the system are totally unrelated. However, in most systems, such assumptions may be incorrect. In the present case, when the fractal surface is made mobile, certain constraints on the mobility arise due to the intrinsic quality of the system. These result in a time dependent evolution of the system where the present state of the system has its origin in the past and the future state will evolve only from the present state.

10.9 Implication of Fractiles on Protein Translocation

During the last years much attention has been focussed on elucidating the process of protein translocation across biological membranes. Essential for this process is the presence of a temporal N-terminal extension called as the signal sequence. To understand the functioning of signal peptides in the translocation process, knowledge of the interactions that signal peptides have with components of the secretion machinery is of extreme importance. A direct interaction of the signal peptide with signal peptidases which cleave signal peptides (Zwizinski and Wickner, 1980) is supposed to occur. Due to their basic and amphiphilic character, signal sequences seem to be very well suited for intercalating into membranes and a major part of the translocation process involves, either directly or indirectly, interactions with membrane lipids.

The overall organization of phospholipids in *E. coli* is that of a liquid crystalline bilayer and lowering the temperature or incorporation of higher melting fatty acids, using unsaturated fatty auxotrophs results in the formation of gel state lipids in the membrane and derived liposomes. This disorder -> order phase change in the bilayer has many functional consequences (de Virje *et al*, 1990). The change in order can modulate, not only the activity of membrane associated proteins (namely, the signal recognition particle, the docking protein, signal peptidases and other proteases) but also the encounter probabilities between cleaved signal peptides and the signal peptide peptidases. This would in turn affect the rate of degradation of the signal peptide in the membrane. Thus the concept of fractiles or mobile fractal shapes can at least in part explain rate changes in biological reactions.

CHAPTER 11

CONCLUSIONS

CHAPTER 11

Purification of proteins from complex mixtures, especially when the desired protein is present in extremely small quantities, is often a very laborious task. Various techniques using diverse properties of biological molecules have therefore been developed over the past few decades for this purpose. The need for cost effective separation procedures is now the need of the hour.

Biological recognition is an important property associated with nature. Utilization of this property is the principle of affinity separations. Engineering biosystems, using biological recognition, is another way of bringing about cost effective separations. The classical example is the use of certain properties of biomembranes to make them selectively permeable to desired proteins. This can be achieved by understanding the apparatus of the biosystem and then tailoring it to do what is required of it. This thesis aims at the investigation of some of the aspects of biological separation processes.

In the initial parts, certain aspects of immunoaffinity chromatographic separations are considered: the raising of polyclonal antibodies against proteins (galactosyltransferase and rice prolamin), their subsequent purification, quantitation and characterization followed by attachment to suitable matrices to achieve separation of the antigens that have elicited their synthesis. After being certain about the antigenicity of the protein and the purity of the antibodies, these antibodies were attached to suitable matrices (Sepharose CL-4B, Sepharose CL-6B and silicalite zeolites) to achieve separation of the antigens.

Sepharose is the most commonly used matrix for immobilization of antibodies. However, the exorbitant cost of using sepharose for large scale separations is a principle bottle-neck in modern biotechnology. Furthermore, derivatisation of sepharose, either using Cyanogen Bromide or Divinyl Sulphone, before attachment of the antibodies makes one forfeit any chance of reusing the matrix.

Moreover, the matrix is easily degraded by bioorganisms. Thus, alternate matrices which are robust, cheap and reusable should be identified. Some part of the present thesis is devoted to aspects of reusability of immunoadsorbents. Effect of deactivation of adsorbed antibodies on the performance of the reactor are analyzed. Clues to minimize this deactivation have also been obtained. After accepting deactivation as an intrinsic part of the separation procedure, aspects of reactor design have been considered. The results obtained from this study give important clues for enhancing the life of the matrix and increasing the efficiency of the entire process.

Also considered in the present thesis is the utilization of *Silicalite* as a potential matrix for chromatographic separations. The results indicate that *Silicalite* is an ideal matrix for separation of hydrophobic proteins, with rice prolamin as a case study.

The latter part of the thesis is devoted to the aspect of protein translocation across biological membranes. Engineering the cell to extrude a desired protein into the growth medium may be a newer way to look at separations. The advantages of this are mainly maintaining the viability of the cell and, at the same time, reducing, to a certain extent, the amounts of other proteins from which the desired protein may have to be isolated. Though this thought seems to be an impressive one, being able to achieve it in reality is difficult. This is because before any attempt to this effect can be undertaken, a thorough understanding of the system has to be achieved. The role of biological recognition is again evident in this case. *How are certain proteins allowed to pass through while others are not? ... What are the signals (signal peptides in this case) responsible for this process?... How do these signal peptides interact with the membrane or certain other components present in the membrane?...* are few of the many dominant questions that have been answered atleast partly, if not totally. The results provide possible mechanisms for the initiation of protein export across the membrane followed by ways by which signal peptides are selectively identified and degraded preferentially over other very similar proteins and peptides.

Towards the end of the thesis, the concept of mobile shapes of fractals or *fractiles* has been introduced in order to explain how rates of reactions can cross the limits set by three-dimensional control. The implications of fractiles on protein translocation have also been portrayed.

The work in this thesis therefore addresses and evaluates certain theoretical and practical aspects which can directly or indirectly affect biological separation processes.

LITERATURE CITED

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14) 5' End of Copia Element of *Drosophila Melanogaster* is Highly Repetitive in *Aedes Aegypti*
Joyce Nair, Asha Nayak, Ujwal Shinde and M.V. Hegde
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M.R. Mawal, Y.R. Mawal, U.P. Shinde and P.K. Ranjekar

2) Effect of Deactivation on Efficiency of Immunoaffinity Separations :
An Experimental Study.
U.P. Shinde, Y.R. Mawal and B.D. Kulkarni

3) Effect of Deactivation on Efficiency of Immunoaffinity Separations :
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4) Mathematical Modelling of Penicillin Fermentation.
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5) Effect of Changing Fractal Dimension on Reactions
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6) Highly repetitive DNA from *Aedes Aegyepti* is Homologous to Transposable Elements from *Drosophila Melanogaster*
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