Aspects of Microbiology 9

Extracellular Enzymes

Priest

Aspects of Microbiology 9

Extracellular Enzymes

Fergus G. Priest

Van Nostrand Reinhold (UK) Co. Ltd

First published in 1984 by
Van Nostrand Reinhold (UK) Co. Ltd
Molly Millars Lane, Wokingham, Berkshire, England

Photoset in Times 9 on 10pt by Kelly Typesetting Limited
Bradford-on-Avon, Wiltshire

Printed and bound in Hong Kong

ISBN 0 442 30588 5

Fergus Priest is currently a Lecturer in Microbiology at Heriot-Watt University, Edinburgh. He is a member of the Society for General Microbiology, Society for Applied Bacteriology, Institute of Brewing and the American Society for Microbiology. His research interests include the regulation of extracellular enzyme synthesis, protein secretion in Gram-positive bacteria, and novel enzymes of biotechnological potential.

Preface

The experience gained over the past 50 or 60 years in the manufacture of industrial enzymes from microorganisms encompasses microbiology, biochemistry and chemical engineering and is assuming considerable importance in the development of biotechnology. Most industrially important enzymes are extracellular. A detailed picture of the molecular biology of protein secretion is emerging from the concerted efforts of both biochemists and geneticists and it is becoming apparent that the secretion process is essentially the same in both prokaryotic and eukaryotic cells. These studies are providing the exciting prospect of the secretion of foreign proteins, such as insulin, by microbial cells containing cloned genes. The commercial implications include simple and efficient product recovery and increased yields. Moreover, yields of extracellular enzymes have been steadily improved by genetic manipulation. Although this has not yet provided a detailed understanding of the regulation of extracellular enzyme synthesis—largely due to the lack of exploitable genetic systems for the relevant microorganisms—the approaches that have been adopted should be of interest and value to those involved in the manufacture of a variety of microbial products.

Scale-up of laboratory scale procedures to the pilot plant and into commercial practice is causing considerable problems in many microbial processes. Here again, the wealth of experience gained from producing bacterial and fungal enzymes on an industrial scale should be invaluable to those venturing into similar microbiologically based industries.

Finally, the enzyme industry itself is worthy of attention. From its inception early this century it has expanded into food technology, waste product utilization and pharmaceuticals. Two major boosts to the industry were the inclusion of alkaline proteases from *Bacillus* strains in household washing detergents in the 1960s, and the development of enzymes immobilized on solid supports later that decade. Immobilized glucose isomerase is used for the conversion of glucose (derived from the enzymic hydrolysis of starch) into the sweeter-tasting fructose. The replacement of sucrose in many foods and beverages by these high fructose corn syrups has helped to promote the enzyme industry into multi-million dollar markets which promise to expand further as new enzymes are discovered and processes invented.

This book deals with both the commercial and academic aspects of extracellular enzyme synthesis. It describes those enzymes that are produced on an industrial scale and outlines their uses and how they are manufactured. It also provides detailed coverage of the molecular biology of protein secretion, the regulation of protein synthesis and current approaches to increasing enzyme yield. It should therefore be of value to advanced undergraduate and graduate students in microbiology, biochemistry and related disciplines who are seeking a concise account of this branch of industrial microbiology. It should also provide an up-to-date and straightforward account of the molecular biology of extracellular enzyme synthesis for those involved in the microbiologically based industries. Thus it is an attempt to bridge one of those gaps between academic and industrial microbiology that now comes under the umbrella of biotechnology.

1984 F. G. PRIEST, *Edinburgh*

Contents

1 Introduction

Microorganisms are responsible for the recycling of much of the organic material in the environment. As animals and plants die, they are attacked by small animals and microorganisms. Their constituent molecules are liberated and used by these saprophytes as a supply of energy and to make new cell components. The low-molecular weight, water-soluble materials are readily assimilated but most of the original organism comprises macromolecules. In plants, cellulose, hemicellulose, lignin, pectin and starch predominate; in animals, proteins, glycoproteins, glycogen and chitin are major constituents. Microorganisms contain specialized cell wall polymers such as peptidoglycan and all organisms contain nucleic acids. These macromolecules are a major food source for heterotrophic microorganisms but, since they are so large, they cannot be readily utilized. Microorganisms have adopted essentially two strategies to enable them to metabolize these compounds. The compound can be engulfed by the cytoplasmic membrane to form a vacuole within the cytoplasm. Enzymes are secreted into this vacuole and the polymeric substrates degraded and subsequently metabolized. Uptake of water and aqueous solutions by this method is referred to as pinocytosis; uptake of particulate matter is termed phagocytosis. Since the prokaryotic membrane is unable to carry out these processes, pinocytosis and phagocytosis are restricted to those eukaryotic microorganisms that lack a cell wall. The major group of such organisms is the protozoa. Those eukaryotes and prokaryotes that possess a cell wall have adopted an alternative strategy for the assimilation of macromolecular nutrients. Enzymes are liberated by the cell, or colony of cells, degrade polymeric material in the environment and the low-molecular weight products are assimilated. Consequently, extracellular enzymes are common in those microorganisms that inhabit soil and decaying plant and animal matter. Amongst the bacteria, strains of *Bacillus, Clostridium, Cytophaga* and many actinomycetes, in particular streptomycetes are prolific producers of extracellular enzymes. Moreover, Gram negative bacteria such as vibrios, aeromonads and pseudomonads are common in decomposing seaweeds and other marine habitats and often secrete agarases and similar enzymes. Filamentous fungi and yeasts also secrete a variety of extracellular enzymes.

It will be apparent that most extracellular enzymes are depolymerases acting on polysaccharides, proteins and nucleic acids. The majority are hydrolases, although exceptions do occur such as the pectin lyases which are in fact *trans* eliminases (see Chapter 4). Some extracellular enzymes have low-molecular weight substrates. A notable example is penicillinase (β-lactamase) which hydrolyses the β-lactam ring of penicillin and renders the antibiotic harmless. Since penicillin inhibits cell wall synthesis, it is essential that it should be inactivated in the environment before it can bind to the cell surface.

It will be useful at this point to define the term 'extracellular' as it pertains to enzymes, since this term has caused confusion in the past. It is now generally agreed that extracellular refers to any enzyme that crosses the cytoplasmic membrane. Stricly speaking, digestive enzymes within the phagocytic vacuole of

the protozoa, or enzymes liberated into the environment by a bacterium, are all extracellular since they have crossed the cytoplasmic membrane. The final location of an extracellular enzyme will therefore be determined by the structure of the cell.

Cell wall structure and enzyme location

Bacteria are traditionally divided into two groups depending on their reaction to the Gram strain. This in turn reflects the chemical composition and structure of the cell wall (Rogers, 1983). Gram positive bacteria have a relatively simple cell wall comprising a thick coat (about 20 nm) of peptidoglycan containing covalently bound teichoic acid. This net-like molecule bounds the cytoplasmic membrane and provides structural strength to the cell. Extracellular enzymes cross the membrane and may be temporarily restricted by the cell wall but eventually diffuse into the environment. Some enzymes, however, remain attached to the outer surface of the membrane. Since these molecules have crossed the membrane they are considered to be extracellular but, on forming protoplasts (by enzymic removal of the cell wall in isotonic medium) they are partially or completely released from the membrane. Under certain growth conditions such enzymes may be naturally released from the cell, the alkaline phosphatase and α-glucosidase of *Bacillus licheniformis* being two examples. A third location for an enzyme in the Gram positive cell is anchored to the inner surface of the membrane. Strictly speaking this is not an extracellular location since the enzyme does not traverse the membrane.

The envelope of Gram negative bacteria is a complicated structure comprising two membranes (Rogers, 1983). The cytoplasmic membrane is bounded by a thin layer of peptidoglycan. This is surrounded by the outer membrane and between these two hydrophobic barriers lies a hydrophilic space, the periplasm. The periplasm may account for 20 to 40% of the total cell mass and contains a variety of proteins including specific amino acid and sugar binding proteins and hydrolytic enzymes. There are therefore several locations for enzymes in Gram negative bacteria: cytoplasmic, anchored to the inside or outside of the cytoplasmic membrane, in the periplasm, fixed to the inner or outer surface of the outer membrane, or secreted into the environment. Since all proteins except those in the cytoplasm or on the inner surface of the cytoplasmic membrane have crossed the membrane and are released by osmotic shock treatment or by conversion of the cells to sphaeroplasts (osmotically fragile cells derived by lysozyme treatment), these enzymes are considered to be extracellular. Many of the conventional extracellular enzymes of Gram positive bacteria may have their counterparts in the periplasmic enzymes of Gram negative bacteria. Consequently extracellular enzymes *sensu stricto* are relatively rare in Gram negative bacteria but do occur particularly in pseudomonads, aeromonads and some enterobacteria. Indeed, the enterotoxin of *Vibrio cholerae* is secreted into the surrounding medium.

Structurally the fungal cell wall resembles the Gram positive bacterial wall. It is largely comprised of 1,3-α- and 1,3-β-glucan with chitin and varying amounts of cellulose and protein. It is not, however, a homogeneous mixture of these constituent polymers but appears to be a structured and complex assembly. Although little is known about the secretion of proteins by fungi, it is generally assumed that the molecules diffuse through the wall once released from the cytoplasm. With

regard to enzyme secretion, the fungal cell wall is therefore similar to the Gram positive bacterial wall.

Commercial enzymes

The exploitation of enzymes is not a recent development: they have been used throughout the ages in leather tanning and cheese making, in the preparation of malted barley for beer brewing and in the leavening of bread. These processes used enzymes in the form of animal and plant tissues or whole microorganisms. The birth of commercial enzymes as partially-purified preparations from living cells is more recent, and can be traced to the end of the last century. Jokichi Takamine, a Japanese scientist living in the USA, filed the first patent for an enzyme in 1894. In this process, *Aspergillus oryzae* was grown on moist rice or wheat bran and the secreted amylase was extracted with water or salts. This 'Takadiastase' is still used as a digestive aid today. The use of bacteria, in particular *Bacillus* strains, for enzyme production followed some twenty years later and again involved growing the microorganism as a surface pellicle in trays of semi-solid medium. The usefulness of extracellular enzymes was readily appreciated. Extracellular enzymes are easier to recover and purify than their cytoplasmic counterparts; in particulr, cell breakage is unnecessary and problems involving removal of nucleic acids are absent. Secondly, it is easier to obtain very high yields of extracellular enzymes because the yield is not restricted by the biomass obtainable. Consequently, the submerged culture techniques developed by the antibiotic industry in the 1940s were readily adopted by enzyme manufacturers, and the increase in productivity provided by improved control of growth conditions boosted the industry considerably. There followed slow but steady growth in the 1950s that was dramatically amplified the following decade by the introduction of enzyme washing powders containing alkaline protease from *Bacillus licheniformis*. Microbial rennets for cheese manufacture and the enzymic conversion of starch into a mixture of glucose and fructose for use as a food sweetener have since been developed and represent two recent growth areas of industrial enzyme usage. Furthermore, current interest in the efficient utilization of renewable resources and the pressure on industry to work within environmentally acceptable limits has stimulated wider interest in enzymes. These factors have combined to produce a world market for industrial enzymes in 1981 variously estimated at between $150 million and $400 million. It is predicted that this will rise to some $600 million by 1985. The bulk of this market comprises proteases and carbohydrases which together account for about 90% of the total; the remainder includes technical and pharmaceutical products. Considering the thousands of enzymes known, this is a very restricted sample but it emphasises that it is much easier to discover a new enzyme than to identify a profitable market.

This book deals with the industrial enzymes (Table 1). The production trend over the past 30 years has been away from animal and plant sources and towards microorganisms to the extent that new products are almost invariably derived from bacteria or fungi. There are several reasons for this: (1) microorganisms grow rapidly and are ideal for intensive cultivation, (2) medium constituents are cheap and generally comprise agricultural products available in bulk and (3) choice of producer-organism is wide and can be improved by genetic manipulation. Thus the often variable and unpredictable sources of animal and plant enzymes are

Table 1 Commercial enzyme products

Source/name	Commercially available before: 1900	1950	1980	Production in 1980: tonnes/year	as % of total
Animal					
Rennet	X			2	0.15
Trypsin		X		15	1
Pepsin		X		5	0.4
Plant					
Papain		X		100	8
Microbial					
Fungal amylase	X			10	0.8
Bacterial amylase		X		300	23
Glucoamylase			X	300	23
Fungal protease	X			10	0.8
Bacillus protease		X		500	38
Pectinase		X		10	0.8
Glucose isomerase			X	50	4
Fungal rennet			X	10	0.8

gradually being replaced by microbial equivalents, although for some applications animal and plant enzymes have retained their market share.

In this book, those enzymes produced on a commercial scale from micro-organisms and their uses are described. However, the field of extracellular enzymes is approaching a revolutionary phase. As more is learnt of the process of protein secretion across membranes and the techniques of genetic engineering become more sophisticated, the prospect emerges that virtually any protein may be made extracellular. Thus, a process that originated as a means of scavenging nutrients from the environment will be exploited on a large scale to engineer microorganisms that can secrete high yields of valuable proteins. To understand how this will come about, we must first consider the process of protein secretion and the regulation of protein synthesis. This will be followed by an account of the current enzyme products and their uses. The penultimate chapters will focus on screening strains for potentially useful enzymes and the development of genetically engineered strains that secrete large amounts of protein. Finally, some engineering aspects of industrial scale production and purification of proteins will be considered.

Reference

ROGERS, H. J. (1983). *Bacterial Cell Structures*. Van Nostrand Reinhold (UK), England.

2 Enzyme secretion

Signal hypothesis

The key feature of an extracellular enzyme is that it is transported across a membrane. The central question to the understanding of protein export is therefore, how does the cell distinguish between cytoplasmic proteins and those destined either for incorporation into the membrane or across it to some other location? Precursor forms of secretory proteins usually contain an NH_2-terminal extension of some 15 to 32 amino acids. It has been proposed that this 'signal' or 'leader' peptide, as it emerges from the ribosome, directs the ribosome to the membrane. According to the original model, the signal peptide recruited other membrane proteins to form a pore or tunnel in the membrane that was stabilized by attachment of the ribosome. As the protein was synthesized, it was exported through this pore in a process termed cotranslational secretion. Once part, or all, of the protein had been exported, the signal peptide was removed by a specific protease (signal peptidase). The elements of the signal hypothesis are shown in Figure 1, and, although it has been substantially refined in recent years the underlying theme remains correct.

Five important principles have emerged from the signal hypothesis: (1) there is no difference beteen membrane bound and cytoplasmic ribosomes, (2) secreted proteins are generally synthesized in a larger, precursor form, (3) proteins are secreted cotranslationally (although not invariably), (4) there can be a stop-secretion sequence within the protein that halts secretion giving rise to an integral, membrane protein, (5) some form of export machinery in the membrane is required and (6) the process of protein export has been highly conserved throughout evolution to the extent that prokaryotic cells recognize eukaryotic signal sequences and *vice versa*. These aspects of protein secretion will be examined in detail in this chapter.

Precursor forms of exported proteins Following formulation of the signal hypothesis, nascent secretory proteins from various systems were characterized and the signal peptides analysed. This was fairly straight-forward since the precursor form of the protein was generally larger than the mature form and could be separated from the mature protein by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). However, the precursors are very short-lived which complicates their detection. In eukaryotes this was overcome by using cell-free translation systems which, in the absence of the membranes that contained the processing enzyme, manufactured precursor proteins. For example, the first study of this kind used mRNA from myeloma cells which was translated in an *in vitro* reticulocyte lysate system into the precursor form of the light chain of immunoglobulin G. This molecule contained an additional peptide of molecular mass 3000. When membranes were added to the translation system, however, the precursor form was converted to mature IgG. This initial work by Milstein and his colleagues in 1972 was rapidly exploited in other eukaryotic systems since it was

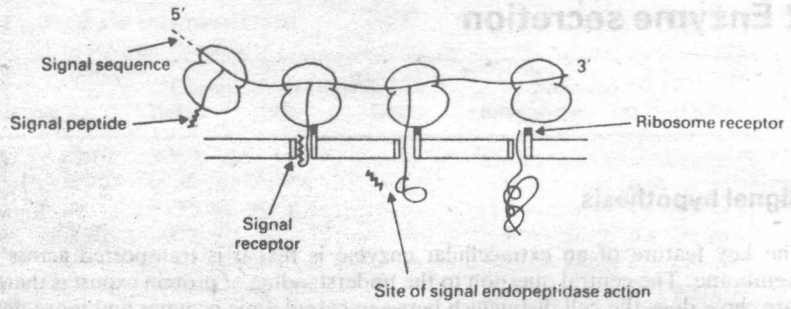

Fig. 1 Schematic diagram of the signal hypothesis for the transport of proteins across membranes (after Blobel *et al.*, 1979). The signal sequence is translated into a signal peptide that recruits one or more receptor proteins in the membrane to form a pore. Similarly, the ribosome binds to a receptor protein. The nascent polypeptide chain is transferred through this pore and the signal sequence is removed by the endoproteolytic action of the signal peptidase. On completion of cotranslational transport, the receptor proteins are free to diffuse in the plane of the membrane.

relatively straight-forward to isolate specific mRNA molecules from specialized eukaryotic cells and to translate them in heterologous cell-free systems based on rabbit reticulocytes or wheat germ cells. The list of eukaryotic secreted proteins known to contain a precursor signal sequence is now substantial (40 to 50; Kreil, 1981).

In bacteria, the same approach could not be used because it was not possible to isolate mRNAs for specific proteins. This stems from the absence of specialized secretory cells devoted to single or relatively few proteins which contain ample quantities of specific mRNAs and from the instability of prokaryotic mRNA. Alternative strategies were therefore adopted to demonstrate precursor forms of secreted proteins. In chain-completion experiments, membrane-bound polysomes are separated from cytoplasmic ribosomes by sucrose gradient centrifugation or gel filtration and then incubated in a suitable medium for the *in vitro* synthesis (completion) of the partially-synthesized polypeptide chains. The products are then identified serologically and characterized by SDS-PAGE. In some instances, coupled transcription and translation systems using specialized transducing phage or cloned DNA templates have also been used to generate precursor forms of secreted proteins. Secondly, *in vivo* procedures have been successful for the study of outer membrane proteins in *E. coli*. Various chemicals that partially disrupt the envelope structure of *E. coli* allow protein synthesis to continue but inhibit the processing of exported proteins. Thus cells treated with toluene or phenethyl alcohol accumulate precursor forms of lipoprotein which can be extracted from the total envelope proteins by precipitation with antilipoprotein antiserum and characterized by SDS-PAGE. Finally, in several instances the presence of a signal sequence has been inferred by comparison of the DNA sequence of the gene with the amino acid sequence of the mature protein.

Using such techniques, many bacterial secreted proteins have been shown to be synthesized as a larger precursor form of the mature protein. In *E. coli* these include some phage-coded, cytoplasmic membrane proteins (phage M13 major and minor coat proteins), several periplasmic proteins including alkaline phosphatase, various binding proteins involved in the transport of small molecules

and the plasmid-coded TEM β-lactamase, and some outer membrane proteins including *lamB* protein and lipoprotein. In *Bacillus*, α-amylase and β-lactamase of *B. amyloliquefaciens* and *B. lichenformis*, respectively, are synthesized in larger precursor form.

Structure and function of signal sequences The primary structures of many eukaryotic and prokaryotic signal sequences have now been established by protein or DNA sequencing. They vary considerably in size (15 to 32 amino acids) and superficially there is little homology. However, when the distribution of hydrophobic and polar amino acid residues is compared, it is apparent that all signal sequences contain two particular domains; an NH_2-terminal hydrophilic segment followed by a central core of predominantly hydrophobic amino acids (Figure 2). The hydrophilic region is positively charged and accounts for the variable length of signal sequences. It is generally between one and seven amino acids long and its exact structure does not appear to be too critical since *E. coli* exports rat preproinsulin even when the signal sequence is preceded by 18 additional amino acids at the NH_2-terminus.

The central, hydrophobic core of the signal sequence plays a crucial part in secretion. This section is about 55 Å long (15 to 19 amino acids) and terminates in an amino acid before the cleavage site which has a short side chain (for example alanine or glycine). The hydrophobic region usually contains a proline or glycine residue in the centre (about position 17). Using rules for the prediction of protein secondary structures, it has been determined that these peptides most probably exist in highly ordered conformations and it appears that the ability of the hydrophobic core to form two areas of α-helix split by the polar residue around position 17 is critical for protein export. Indeed mutations of the *lamB* signal sequence that would be expected to destroy these α-helices do inhibit export, and suppressor mutations that should re-establish the secondary structure, allow export.

Several models have been proposed to relate the structure of the signal peptide to its function. Inouye and Halegoua (1980) suggested the loop model in which the basic hydrophilic domain of the signal sequence, as it emerges from the ribosome, attaches to the negatively-charged inner surface of the cytoplasmic membrane by ionic interaction. As the protein is translated, the hydrophobic core of the signal sequence is progressively inserted into the membrane until the cleavage site emerges as a loop on the outside of the membrane. Signal peptidase then cleaves the signal sequence from the nascent, polypeptide chain allowing the protein to be cotranslationally secreted (Figure 3). According to this model, if the charge at the

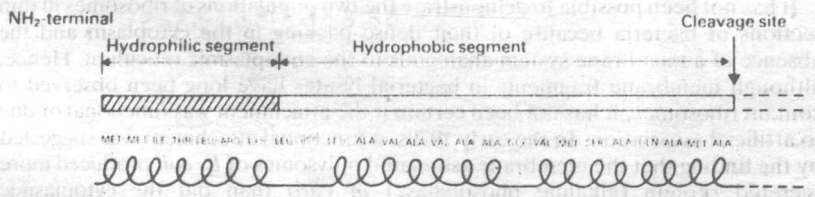

Fig. 2 Structure of the signal peptide of the *lamB* protein of *Escherichia coli*. Signal sequences typically display a hydrophilic NH_2-terminal section of variable length and an internal hydrophobic region of 15 to 19 amino acids. Areas of predicted α-helix (loops) or random coils (straight line) are indicated.

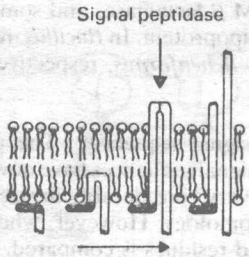

Fig. 3 Loop model for the translocation of secretory proteins across membranes (from Inouye & Halegoua, 1980). The solid portion represents the basic region of the NH-terminal end which attaches to the inner surface of the membrane. The following blank portion represents the hydrophobic region which is progressively inserted into the membrane. When the cleavage site emerges on the outside of the membrane, it is hydrolysed by the signal peptidase (arrow) allowing the protein to be secreted through the membrane. (By permission of CRC Press, Florida).

NH_2-terminus of the signal peptide was negative instead of positive, initiation of export would not occur and the protein would accumulate in the cytoplasm. Such mutants with net negative charges have been prepared by *in vitro* mutagenesis and behave as predicted, but it seems that this model may be a simplification since recent evidence suggests that there may after all be an export machinery in the membrane which is involved in protein translocation.

Cotranslational secretion The early influential studies of protein transport used specialized secretory cells of animal origin (mainly pancreas and liver cells) in which electron microscopy disclosed two distinct populations of ribosomes; some were apparently attached to the inner surface of the endoplasmic reticulum, while others were free in the cytoplasm. Palade observed a parallel between the abundance of membrane bound ribosomes and the secretion of proteins and suggested that proteins were transported across the membrane as they were synthesized by these ribosomes in a process termed cotranslational secretion. The soluble ribosomes would be responsible for cytoplasmic protein synthesis. Although it was rapidly established that cotranslational secretion was the predominant mode of protein export, if should not be inferred that this is the only process. Post-translational secretion (transfer across the membrane of a completed protein) occurs in both eukaryotic and prokaryotic systems.

It has not been possible to demonstrate the two populations of ribosomes in thin sections of bacteria because of their dense packing in the cytoplasm and the absence of a membrane system analogous to the endoplasmic reticulum. Hence, although membrane fragments in bacterial lysates have long been observed to contain ribosomes, it has not been certain if the attachment was functional or due to artificial association. In the early 1970s, a functional attachment was suggested by the finding that the membrane associated polysomes of *E. coli* produced more secreted protein (alkaline phosphatase) *in vitro* than did the cytoplasmic ribosomes. Improved methods of separating the two populations of ribosomes using sucrose gradient centrifugation or gel filtration achieved more definitive results, and it has since been demonstrated that α-amylase in *B. subtilis* and *B. licheniformis* and numerous periplasmic proteins in *E. coli* are synthesized

exclusively by membrane associated ribosomes while the cytoplasmic elongation factor EFTu is made only on soluble ribosomes. It would seem, therefore, that most secreted proteins are transferred across the membrane cotranslationally from membrane bound ribosomes. Nevertheless, these findings could be interpreted as the elongating chain folding against the membrane surface with subsequent engulfment by the membrane after release of the protein from the ribosome.

Direct evidence for cotranslational secretion Cotranslational secretion of a protein could be unambiguously demonstrated if the end of the growing chain protruding from the membrane could be labelled, while the other end remained attached to a ribosome as peptidyl-tRNA. To achieve this, *E. coli* was chilled and treated with chloramphenicol which stabilizes the polysomes. The cells were converted to spheroplasts and labelled with ^{35}S-acetylmethionyl methylphosphate sulphone which reacts with free amino groups of proteins but does not penetrate membranes. The spheroplasts were then washed, osmotically lysed and the membrane associated ribosomes purified by sucrose gradient centrifugation. After removal of the membrane with detergents, a substantial amount of the initial label remained in the polysomes (Figure 4). Moreover the label was presumably attached via the nascent polypeptide chain, since it was released by chain completion *in vitro*. Amongst the translation products, the secreted protein alkaline phosphatase could be identified. These experiments have since been refined and used to establish cotranslational secretion of α-amylase in *B. subtilis*, β-lactamase in *B. licheniformis* and toxin in *Corynebacterium diphtheriae* (Davis & Tai, 1980).

Post-translational secretion Several proteins are incorporated into or transported across membranes after they have been synthesized. This has been demonstrated for proteins located in chloroplasts and mitochondria, which are made on cytoplasmic ribosomes and subsequently imported into the organelle. In bacteria there are also several examples of post-translational secretion. Subunit A of cholera toxin is synthesized *in vitro* by soluble and not by membrane bound

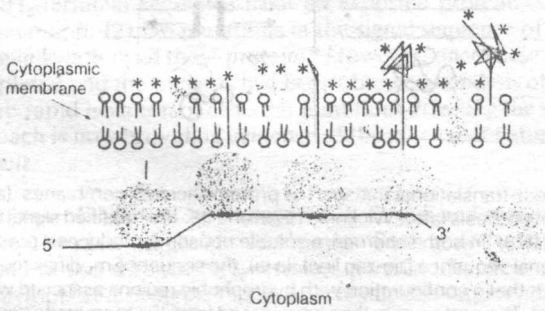

Fig. 4 Scheme for extracellular labelling of secreted proteins. The nascent polypeptide chain emerges from the membrane and is labelled with [^{35}S] acetyl methionyl methylphosphate sulphone (asterisks). Subsequent demonstration of label attached to polysomes indicates cotranslational secretion. (From Smith *et al.*, 1977.)

ribosomes and the toxin can be detected immunologically in cell-free extracts of *V. cholerae*. Moreover, in some *Bacillus* strains, α-glucosidase may accumulate in the cytoplasm before being secreted into the environment.

The signal hypothesis can be modified to accommodate post-translational secretion as shown in Figure 5. It is proposed that the protein to be translocated has a signal sequence which remains exposed and interacts with receptors in the membrane to form a pore. Passage through the membrane would be accompanied by unfolding during transfer with subsequent re-folding.

An alternative scheme is the 'membrane trigger' hypothesis. Again a signal sequence is involved, but the function of this peptide is to promote the folding of the protein in such a way that it interacts with the membrane and export is triggered into and across the membrane. Thus no specific export machinery is required (Figure 5). Once on the outside of the membrane, removal of the signal peptide would make the process irreversible. This model is supported by the demonstration that phage M13 coat protein correctly inserts into liposomes comprising nothing but phospholipid and purified processing enzyme.

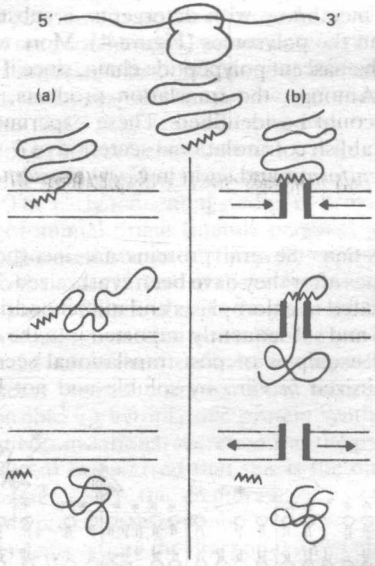

Fig. 5 Models for post-translational transport of proteins across membranes. (a): membrane trigger hypothesis (after Wickner, 1979) and (b): the modified signal hypothesis (after Blobel *et al.*, 1979). In both schemes, a soluble ribosome produces a precursor protein bearing a signal sequence (zig-zag line). In (a), the sequence modifies the folding of the polypeptide such that a configuration with hydrophobic regions associatd with the membrane is formed. The protein may then be released from the membrane possibly with endoproteolytic removal of the signal sequence. In (b), the signal sequence of the completed polypeptide chain recruits receptor proteins in the membrane to form a pore. The protein unfolds and translocation proceeds, followed by removal of the signal sequence.

Processing the precursor In the original signal hypothesis, the precursor protein is processed by removal of the signal peptide during or immediately after the synthesis of the protein. Recent studies in *E. coli* have provided insights into the stages at which processing occurs. In these experiments, proteins are pulse-labelled and immunoprecipitated to obtain a particular protein. When this precipitate is analysed by SDS-PAGE, an array of molecules is obtained comprising precursor and mature species and incomplete peptides. Since precursor is observed, it indicates that at least some post-translational processing occurs. After limited *in situ* proteolysis of the polypeptides in the gel, the digestion products can be electrophoresed in a second dimension and peptides characteristic of the NH_2-terminus of both precursor and mature forms of the protein can be obtained. It is therefore possible to demonstrate that among the incomplete nascent chains, some still have their signal sequence attached whereas others have been processed and contain an NH_2-terminus characteristic of the mature form (Josefsson & Randall, 1981). In this way, it has been established that the maltose and arabinose binding proteins and alkaline phosphatase of *E. coli* are processed both cotranslationally and post-translationally, while others are processed either during or after translation. For all these proteins, processing is a relatively late event occurring after the protein has been elongated to at least 80% of its final length.

Comparison of the cleavage sites in different precursor proteins reveals little specificity; the peptide is hydrolysed between an amino acid residue with a short side chain (generally glycine or alanine) and the adjacent residue (Figure 2). In eukaryotes, the signal peptidase activity is located in the membrane of the endoplasmic reticulum. An *E. coli* processing enzyme has been purified from both the cytoplasmic and outer membranes in which it is present in roughly equal amounts. This is the first example of such a dual distribution of a membrane protein in *E. coli*.

Genetic studies

The effectiveness of a combined genetic and biochemical approach to the analysis of a process such as protein secretion has been ably demonstrated in *E. coli*. Such studies initially focussed on two questions. (1) If a cytoplasmic protein is provided with an NH_2-terminal sequence from an exported protein, is this sufficient to promote secretion. (2) Do mutations in the signal sequence of secreted proteins alter the final locations of these proteins? However, once these initial studies had been completed and the mutants had been obtained, analysis of their phenotypes led to more rapid isolation of different mutations affecting the secretion process. This approach is now providing evidence of the molecular nature of the secretion process itself.

Protein fusions DNA can be transposed in *E. coli* and two genes brought into juxtaposition by classical genetic techniques. Such gene fusions code for hybrid proteins and in this way the NH_2-terminal portion of a secreted protein can be fused to a cytoplasmic protein. The genetic manipulations involved are outside the scope of this book and have been fully described by Silhavy *et al.* (1979).

Strains of *E. coli* have been developed in which the *lacZ* (β-galactosidase) gene has been fused to various loci in the *mal* (maltose) utilization operon; *malF*, which codes for the maltose transport protein located in the cytoplasmic membrane;