Journal of Bioenergetics and Biomembranes, Vol. 22, No. 3, 1990

MINI-REVIEW

Signal Peptidases and Signal Peptide Hydrolases

Inderjit K. Dev[1] and Paul H. Ray[1]

Received November 22, 1989

Abstract

Signal peptidases, the endoproteases that remove the amino-terminal signal sequence from many secretory proteins, have been isolated from various sources. Seven signal peptidases have been purified, two from *E. coli*, two from mammalian sources, and three from mitochondrial matrix. The mitochondrial enzymes are soluble and function as a heterogeneous dimer. The mammalian enzymes are isolated as a complex and share a common glycosylated subunit. The bacterial enzymes are isolated as monomers and show no sequence homology with each other or the mammalian enzymes. The membrane-bound enzymes seem to require a substrate containing a consensus sequence following the -3, -1 rule of von Heijne at the cleavage site; however, processing of the substrate is strongly influenced by the hydrophobic region of the signal peptide. The enzymes appear to recognize an unknown three-dimensional motif rather than a specific amino acid sequence around the cleavage site. The matrix mitochondrial enzymes are metallo-endopeptidases; however, the other signal peptidases may belong to a unique class of proteases as they are resistant to chelators and most protease inhibitors. There are no data concerning the substrate binding site of these enzymes.

In vivo, the signal peptide is rapidly degraded. Three different enzymes in *Escherichia coli* that can degrade a signal peptide *in vitro* have been identified. The intact signal peptide is not accumulated in mutants lacking these enzymes, which suggests that these peptidases individually are not responsible for the degredation of an intact signal peptide *in vivo*. It is speculated that signal peptidases and signal peptide hydrolases are integral components of the secretory pathway and that inhibition of the terminal steps can block translocation.

Key Words: Canine signal peptidase (SPC); Hen oviduct signal peptidase (HOSP); leader peptidase (SPase I); prolipoprotein signal peptidase (SPase II); secretory proteins; hydrophobic core; cleavage site; translocation.

[1]Division of Molecular Genetics and Microbiology, Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709.

0145-479X/90/0600-0271$06.00/0

Introduction

The majority of proteins that are exported, from their site of synthesis in the cytoplasm across the plasma membrane or the endoplasmic reticulum to their site of function, have amino-terminal signal sequences that are removed during or shortly after the translocation event (Blobel and Dobberstein, 1975). The enzymes that catalyze the removal of the signal peptides are termed signal peptidases. They are endoproteases in that the signal sequences (15 to 30 residues) are removed intact (Mollay *et al.*, 1982; Jackson and Blobel, 1977; Zwinzinski and Wickner, 1980; Dev and Ray, 1984). Precursor proteins are translocated and processed in heterologous systems (Watts *et al.*, 1983; Muller *et al.*, 1982) with fidelity in that the mammalian or bacterial systems can effectively translocate and process precursor proteins from either system. These unique enzymes are essential proteins (Date, 1983; Wu and Tokunaga, 1986; Scheckman, 1985; Verner and Schatz, 1988) whose function is required for the proper secretion of proteins and cell viability.

The signal peptide hydrolase(s), the enzyme(s) that degrades the signal peptide, is probably an integral part of the translocation pathway and suggests that the enzymes and proteins that are part of this pathway function as a unit, even though the bacterial proteins involved are being isolated as individual components.

Processing Site

What parameters in the signal sequence or mature protein does the signal peptidase recognize that allows processing to ensue? There is very little known about the catalytic properties of signal peptidases since there are no known site-specific inhibitors and kinetic studies with full-length precursors have been difficult (Caulfield *et al.*, 1989). A number of signal peptides, deduced from the sequenced DNA, were analyzed to determine if there were common structural features (Austen, 1979; Perlman and Halvorson, 1983; Watson, 1984; von Heijne, 1983, 1984, 1985, 1986; von Heijne and Abrahmsen, 1989). Even though there is little primary sequence homology, signal sequences from bacteria to mammals appear to have certain features in common, particularly concerning the conservation of residues at positions -1 and -3 relative to the cleavage site. As defined by von Heijne (1985), the signal peptide can be divided into three distinct regions: (1) the *n*-region is positively charged and variable in length and amino acid composition; (2) the central *h*-region, known as the hydrophobic core, is enriched in hydrophobic amino acids, is variable in length (7–20) residues), and probably exists in an α-helical configuration (Gierasch, 1989); (3) the *c*-region, the carboxyl-terminal segment

of the signal peptide, is smaller (5–7 residues) and more polar than the *h*-region and contains half of the cleavage site.

Perlman and Halvorson (1983) and von Heijne (1983) proposed that the signal peptidase recognizes a consensus cleavage site. This consensus cleavage site follows the (−3, −1) pattern and is usually 5–7 residues distal to the *h*-region. Small, neutral amino acids are found at residues −3 and −1 (from the cleavage site) but are usually not found at −2 (von Heijne, 1984). Other larger, polar or aromatic residues are absent at positions −1 and −3 but are found at −2. From the analysis, Perlman and Halvorson (1983) noted that the amino acids distal to the hydrophobic core showed a tendency to form a β-turn that would be necessary to bring the cleavage site into contact with the signal peptidase located on the external surface of the plasma membrane or on the lumal side of the endoplasmic reticulum. Overall, mutational studies around the cleavage site have confirmed the importance of the β-turn and the residues at −1 and −3 for the processing reaction (Duffaud *et al.*, 1985; Ghrayeb *et al.*, 1985., Pluckthun and Knowles, 1987; Folz *et al.*, 1988; Cioffi *et al.*, 1989; Fikes and Bassford, 1989; Fikes *et al.*, 1990). However, the interpretation of these data is complicated by the observation that a number of mutations in this region affect translocation and hence cleavage, as do certain mutations in the *h*-region (Cioffi *et al.*, 1989; Caulfield *et al.*, 1989). Moreover, it has been obseved that a number of mutations of pMBP (pro-maltose-binding protein) in the *h*-region affect the rate of processing by signal peptidase I (Ray *et al.*, 1986). Caulfield *et al.* (1989) have demonstrated, utilizing a synthetic cleavable peptide, that a modification within the *h*-region twelve amino acids from the cleavage site directly influenced the binding of the hen oviduct signal peptidase.

It is difficult to envision the processing step as a traditional enzyme/substrate interaction. Both the substrate and the enzyme are membrane bound and processing does not appear to be a rate-limiting step in translocation (Randall, 1983). The model put forth by Perlman and Halvorson (1983) suggests that the positively charged amino-terminus binds to some component(s) on the inner surface of the membrane, and the *h*-region first inserts as an α-helix and then extends through the membrane. As the protein precursor extends through the membrane, the region at −5 to −7 emerges and a β-turn ensues to position the cleavage site for correct processing by the membrane-bound signal peptidase. This model still seems an appropriate way to envision the initiation of the processing step.

It has been stated that processing takes place during translocation or immediately after translocation. There is no direct evidence concerning when processing takes place during translocation or the overall conformation of the substrate. In accordance with the above model, processing should take place during translocation. If the signal sequence is inserted into the membrane

as a loop or "reverse hairpin" structure as proposed by Inouye and Halegoua (1980), then the substrate would have at least two very different conformations for a water-soluble protein like MBP. If processing occurs during translocation, the substrate would be constrained by the insertion of the signal sequence imbeded in the membrane and the mature domain emerging through the membrane. If processing occurs after translocation, the substrate would be only tethered by the signal peptide. Wickner and Blobel and their co-workers have shown that signal peptidases are orientated in the membrane so that the catalytic site is on the exterior surface of the plasma membrane or in the lumen of the endoplasmic reticulum. It has been shown that nascent pMBP can be processed when 80% of the protein is synthesized (Randall, 1983) and pro-OmpA and pMBP can be processed by trypsin-treated liposomes containing internally orientated SPase I (Ohno-Iwashita *et al.*, 1984), yet the mature proteins do not sediment with the liposomes. A recent mutation in pMBP (designated MBP177 by Collier and Bassford, 1989) that extends the *h*-region by seven amino acids, but does not affect the processing site, is translocated but not processed *in vivo*. *In vitro* studies have shown that MBP177 is processed with SPase I (J. Weiss, unpublished results). These data at least indicate that (1) processing can take place before translocation is complete, (2) that the processing site can extend through liposomes while the bulk of the mature protein remains exterior to the liposome, and (3, 4) that if the processing site is not in juxtaposition with the signal peptidase, processing does not occur *in vivo* even though *in vitro* the substrate can be processed. These data suggest that processing takes place before translocation is complete.

Preparation of Substrates and Assay of Signal Peptidase Activity

Signal peptidases are usually assayed by the cleavage of a radiolabelled precursor protein to its mature (lower molecular weight) form as determined by their migration on SDS-polyacrylamide gels (Date and Wickner, 1981; Dev and Ray, 1984; Jackson and Blobel, 1977; Waters *et al.*, 1986). The radiolabelled precursor proteins can be prepared both *in vivo* and *in vitro* (Ray *et al.*, 1986); however, the most common and convenient method of preparation is by the *in vitro* translation of the appropriate m-RNA in the presence of a radiolabelled amino acid (Erickson and Blobel, 1983; Ehen *et al.*, 1985; Chen and Tai, 1985; Silver *et al.*, 1981, Zwizinski and Wickner, 1982; Crooke *et al.*, 1988).

Prolipoprotein, a substrate that requires modification before cleavage, can be labelled *in vivo* in the presence of globomycin and purified by antibody affinity chromatography. The cleavage of prolipoprotein by signal peptidase II can be monitored by extraction of the labelled signal peptide with 80%

acetone (Dev and Ray, 1984). Recently, two assays have been developed using synthetic peptides as substrates for either the bacterial enzyme or the hen oviduct enzyme (Novak *et al.* 1989; Caulfield *et al.* 1989). The cleavage of a nine amino acid peptide (−7/+2) derived from pMBP can be monitored by an automated HPLC system. Labelled synthetic peptide analogs of pre-proparathyoid hormone have been prepared by Caulfield *et al.* (1989) and processed by the hen oviduct signal peptide. The cleavage products were initially separated by HPLC but later it was noted that the products of the cleavage reaction were soluble in 5% trichloroacetic acid. SPase I can cleave a synthetic pentapeptide (−3/+2) derived from the sequence of pMBP, but the hen oviduct enzyme cannot cleave the same substrate (P. Novak, unpublished).

With the mammalian signal peptidase(s) it has long been noted that not all full-length precursor proteins are processed with equal efficiency and the substrates are not completely processed (Jackson and Blobel, 1977; Jackson, 1983; Baker and Lively, 1987; Evans *et al.*, 1986, YaDeau and Blobel, 1989). As noted by Baker *et al.* (1986), the processing of preplacental lactogen, made *in vitro* in the presence of optimal concentrations of specific antibody to the mature region, was done in a linear fashion with respect to enzyme concentration only up to 20% even though the enzyme was present at a concentration much higher than the substrate. Uncleaved substrate was resistant to added fresh enzyme. Previously, it was noted that antibody-bound preHPL was a better substrate for the hen oviduct signal peptidase (HOSP). A synthetic peptide of 31 amino acids is processed only 70% by purified HOSP (Caulfield *et al.*, 1989). These data, together with the finding that preprolactin is a better substrate for the purified canine signal peptidase complex (SPC) after treatment with SDS (Evans *et al.*, 1986), suggest that the substrate conformation for the mamalian enzymes plays a critical role in the cleavage reaction. In contrast, the bacterial enzymes appear to completely process their substrates (Watts *et al.*, 1983; Ito, 1982; Dev and Ray, 1984). The rate of processing of precursor proteins *in vitro* is very slow and usually the reactions contain a higher concentration of enzyme than substrate. This suggests that the mammalian signal peptidases, which are isolated as a complex, bind and process only the substrate in a particular conformation and that the hydrophobic region is very important for processing *per se* or in the initial binding of the substrate to the complex. The data concerned with the complete processing of the precursors by the bacterial signal peptidases, which are not isolated as a complex, could indicate that these enzymes only recognize residues around the cleavage site and are not as influenced by the hydrophobic region as the mammalian complex appears to be. Alternately, the bacterial enzyme could slowly bind to the substrate and upon binding confer a conformation to the substrate that is consistent with the cleavage reaction.

Signal Peptidase I or Leader Peptidase

The characterization of signal peptidase (SPase I) or leader peptidase is a direct tribute to William Wickner and his co-workers (Zwizinski and Wickner, 1980; Date and Wickner, 1981; Wolf *et al.*, 1982, 1983, 1985; Dalbey and Wickner, 1986, 1987; and others reviewed in Wickner, 1988). SPase I was first purified from a wild-type strain of *E. coli*, its gene was identified from the Clark and Carbon library, and the structural gene was cloned (Date and Wickner, 1981; Wolfe *et al.*, 1983a and codes for an essential enzyme (Date and Wickner, 1981; Dalbey and Wickner, 1986). Treatment of right-side-out and inverted membrane vesicles with proteases (Moore and Miura, 1987), and site-directed mutagenesis (Dalbey and Wickner, 1987; von Heijne *et al.*, 1988) along with fusions of the Pho A gene to various amino-terminus fragments of the signal peptidase gene have defined the orientation of this protein in the membrane (San Millan *et al.*, 1989).

SPase I is coded for by the *lep* gene, which is separated from its promoter by a region of DNA that can code for a protein with a molecular weight of 67 kDa, and maps near 54 min on the *E. coli* chromosome (Date and Wickner, 1981; Silver and Wickner, 1983; March and Inouye, 1985). The gene codes for a protein of 323 amino acids (37 kDa) and does not have an amino-terminal cleavable signal sequence (Wolfe *et al.*, 1983). SPase I is a transmembrane protein that requires *SecA* and *SecY* and the membrane electrochemical potential for correct membrane assembly (Wolfe and Wickner, 1984; Wolfe *et al.*, 1985). Dalbey and Wickner (1988) have designated SPase I as an oligotopic membrane protein containing three apolar regions (H1–H3) and two polar regions (P1 and P2). Starting from the amino-terminus, segment H1 (residues 1–22) is a hydrophobic domain extending through the membrane so that the amino-terminus is facing the periplasmic space; P1 (residues 23–61, containing 15 charged residues) follows H-1, lies within the cytoplasm, and has been shown to be a translocation poison (von Heijne *et al.*, 1988); H-2 (residues 62–76) spans the membrane and appears to be the internal signal sequence (Dalbey and Wickner, 1987); H-3 (residues 83–98) is a slightly hydrophobic segment but is part of the enzyme's large periplasmic domain. The carboxyl-terminal portion of the enzyme (residues 141–323) is located in the periplasm and probably contains the catalytic site (Dalbey and Wickner, 1987). This periplasmic domain is susceptible to digestion by trypsin in spheroplasts (Moore and Miura, 1987) and yields an 11-kDa amino-terminus fragment protected by the membrane. It is not known if any part of the immediate amino-terminus of SPase I is involved in substrate binding or in the cleavage reaction.

Substrate Specificity

From *in vivo* studies, SPase I is thought to process all the nonlipoprotein amino-terminal signal sequence containing precursor proteins in *E. coli*. SPase I catalyzes the removal of heterogenous signal peptides at a specific site even though the proposed site is not unique in itself. The requirements for cleavage are vague except that the majority conform to the -1, -3 motif, with the sequence . . . ala-X-ala . . . being the most common (von Heijne, 1986). There are no kinetic data available for SPase I.

In vivo, a number of mutations around the cleavage site affect the rate of processing (Fikes and Bassford, 1989; Kuhn and Wickner, 1985a). *In vitro*, studies by Kuhn and Wickner (1985b) have shown that the conserved residues at -1, -3, and -6 in procoat are essential for processing but do not appear to affect translocation. A substitution in procoat of threonine at -1 in place of alanine was poorly processed *in vivo* or *in vitro*. Other mutations in procoat (-6, -3) from the cleavage site adversely affected processing. Dierstein and Wickner (1986) examined the requirements for substrate recognition by cleaving native procoat with proteases or chemical cleaving reagents to isolate well-defined peptides that included the cleavage site. Their results indicated that a 16 amino acid peptide (-9, $+7$) was slowly cleaved by purified SPase I but that the charged amino-terminus and the bulk of the hydrophobic core was not required for correct processing. Another 23 amino acid peptide extending from residue -15 to $+8$ was comparable to procoat as a substrate. Utilizing various synthetic peptides around the cleavage site of pMBP (-7 to $+5$) Dev *et al.* (submitted) show that a pentapetide (-3 to $+2$) can be cleaved by SPase I and that by extending length towards the amino-terminus one amino acid at a time from $-3/+5$ to $-7/+5$ the rate of hydrolysis increases while the K_m remains relatively constant (1.4–2.6 mM). There is no data on the conformation of the synthetic substrates.

Properties of SPase I

SPase I has a subunit molecular weight of 37 kDa on SDS-gels; the molecular weight of the functional enzyme is not known. The isoelectric point as determined by chromatofocusing is 6.9. With procoat as a substrate, the pH optimum is between 8.0 and 9.0 and the enzyme appears to be inhibited by NaCl (>160 mM) and Mg^{++} (>1.0 mM). The enzyme is not inhibited by TPCK, TLCK, PMSF, EDTA, *o*-phenanthroline, or 2,6-pyridinedicarboxylic acid. A number of precursor proteins have been shown to be effective substrates (Wolfe *et al.*, 1983). The processing of procoat is not inhibited by a wide variety of commercially available peptides (Wolfe *et al.*, 1983).

Signal Peptidase II or Prolipoprotein Signal Peptidase

Bacteria have a unique group of membrane-associated proteins that contain covalently linked lipids (Wu and Tokunaga, 1986). These proteins contain a glyceride moiety linked through a thioester bond to a cysteine residue at the amino-terminus of the mature protein; these proteins have been designated lipoproteins and exist in both Gram-negative and Gram-positive bacteria (Wu and Tokunaga, 1986). These lipoproteins are synthesized as precursor proteins with a cleavable signal sequence.

Biochemical and genetic data have provided definitive evidence for a distinct signal peptidase for lipoproteins in *E. coli*. Signal peptidase II (SPase II) is coded for the *lsp* gene; the gene for this enzyme has been cloned and sequenced (Yamagata *et al.*, 1983a, b; Regue *et al.*, 1984; Yu *et al.*, 1984). The *lsp* gene maps at 0.5 min on the *E. coli* chromosome and is part of a distinct operon containing five genes (*x-ileS-lep-orf149-orf316*) whose transcription is dependent upon a promoter(s) located upstream of or within gene *x* (Miller *et al.*, 1987; Miller and Wu, 1987; Innis *et al.*, 1984). The *lsp* gene encodes a protein containing 164 amino acids and has a molecular weight of 18 kDa. The essential nature of SPase II has been demonstrated by the isolation of a conditionally lethal mutation and by the cell's sensitivity to globomycin (Hussain *et al.*, 1980; Ichihara *et al.*, 1982; Yamagata *et al.*, 1983a).

SPase II in *E. coli* is an integral inner membrane protein with four hydrophobic regions that share common features with transmembrane segments of many other integral membrane proteins (Tokunaga *et al.*, 1985; Innis *et al.*, 1984). SPase II does not share sequence homology at the DNA level with other known signal peptidases and does not contain a cleavable amino-terminal signal sequence (Tokunaga *et al.*, 1985; Innis *et al.*, 1984; Shelness *et al.*, 1988). How this protein is assembled into the inner membrane and whether its assembly requires the electrochemical potential or functional SecY and SecA proteins has not been determined.

The nature of prolipoprotein signal peptidase in orgnisms other than *E. coli* is not known. When the penicillinase gene from *Bacillus licheniformis* was cloned into *E. coli* and *B. subtilis*, the protein product was modified by lipid, processed and localized correctly (Hayashi and Wu, 1983; Hayashi *et al.*, 1985). Thus it appears that the synthesis of this lipoprotein may involve similar modification and processing enzymes even though there were major differences in the rate of maturation of prepenicillinase and its sensitivity to globomycin when expressed in *B. subtilis* or *E. coli*.

Substrate Specificity

The amino acids surrounding the cleavage site of the known prolipoproteins (Wu and Tokunaga, 1986) are conserved but not unique (Leu-X-Y-Cys). The important component of these sequences is the cysteine residue, which must be fully modified (Tokunaga *et al.*, 1982b, 1984) before the precursor of Braun's prolipoprotein can be processed (Tokunaga *et al.*, 1982a; Dev and Ray, 1984). However, the glyceride modification of the cysteine residue by itself is probably not sufficient for processing. This was indicated by the characterization of a precursor lipoprotein that contained a substitution of threonine for glycine at the -1 position (Pollitt *et al.*, 1986). This specific mutant precursor, which contained a conservative substitution at the processing site, was modified but not processed to its mature form (Pollit, *et al.*, 1986). Assuming that the precursor was translocated and therefore accessible to the enzyme, the lack of cleavage must have resulted from an alteration in the enzyme–substrate interaction (an *in vitro* study with a threonine substituted at -1 for alanine in procoat was a poor substrate for SPase I). In addition, the substitution of a serine or alanine for glycine at the -1 position had no effect on the modification or processing reactions.

There have been many studies on mutations and alterations in the signal sequence of prolipoprotein (Duffaud *et al.*, 1985; Wu and Tokunaga, 1986), but only a few have yielded information on the processing site. In an attempt to elucidate the role of the effect of substitutions around the cleavage site ($-6/+2$), Inouye and co-workers (Duffaud *et al.*, 1985; Pollitt *et al.*, 1986; Inouye *et al.*, 1986) have constructed many different mutations. However, many of these mutations eliminate the modification of the precursor, and since the modification is a prerequisite for processing, the specific effect of these mutations on processing cannot be assessed. Clarification of the substrate requirements will probably require an *in vitro* translocation, modification, and processing system that has recently been reported (Krishnabhakdi and Müller, 1988; Tian *et al.*, 1989).

Properties of SPase II

SPase II is localized in the inner membrane and requires a nonionic detergent for activity but does not require added phospholipids (Dev and Ray, 1984; Tokunaga *et al.*, 1984). The enzyme has been purified to near homogeneity (Dev and Ray, 1984). The purified enzyme is not inhibited by a number of protease inhibitors nor is it dependent on divalent cations for activity. The enzyme is inhibited by globomycin in a noncompetitive manner with a K_i of 36 nM (Dev *et al.*, 1985). Utilizing prolipoprotein with a measured specific activity, we determined the K_m of SPase II to be 6 μM.

Attempts to make antibody to the cloned purified enzyme have not yet been successful.

Mammalian Signal Peptidase(s)

Jackson and Blobel (1977) initiated studies on the isolation and purification of the mammalian signal peptidase. Utilizing the substrates bovine preprolactin and pre-growth hormone, they demonstrated that a signal peptidase activity could be solubilized from rough microsome but not from smooth microsomes. Purification of processing activities from various sources indicated that these enzymes were similar in many respects (Mollay *et al.*, 1982; Jackson and White, 1981; Lively and Walsh, 1983; and Fujimoto *et al.*, 1984).

Two eukaryotic signal peptidases have been purified. The canine microsomal signal peptidase has been purified as a complex by Evans *et al.* (1986). This complex contains five polypeptides with apparent molecular weights of 12, 18, 21, (22/23), and 25 kDa. The complex remained associated after solubilization with detergent, ion exchange chromatography, sieving, ultracentrifugation, and affinity chromatography. The polypeptides designated SPC (signal peptidase complex) 22/23 were found to be differentially glycosylated forms of the same 19-kDa protein. Due to the glycoprotein subunit, the entire complex could be purified using ConA-Sepharose affinity chromatography. SPC proteins 22 and 23 eluted differently from the ConA-Sepharose column and for this reason are thought not to exist in the same complex.

The signal peptidase complex from hen oviduct has been purified by Baker and Lively (1987). After isolation of the hen oviduct rough microsomes, the membranes were extracted with cold sodium carbonate at pH 11.5, to remove the majority of membrane-associated proteins, followed by solubilization of the membrane with 2.5% NP-40. After ion-exchange and ConA-Sepharose affinity chromatography, only four polypeptides, with relative molecular weights of 19, 22, 23, and 24 kDa, were associated with the signal peptidase activity. The 22–24–kDa proteins were shown to be differentially glycosylated forms of the same 19.5-kDa polypeptide. The complex for the hen oviduct signal peptidase is composed of only two proteins, a 19-kDa and a 19.5-kDa protein whose tryptic maps are different. The stoichiometry of the functional enzyme has not been determined.

Recently, Shelness *et al.* (1988) cloned and sequenced the gene that encodes the SPC polypeptide (22/23) and showed that it shares homology with the tryptic peptides derived from the hen oviduct 19.5-kDa glycoprotein. The sequence indicates that the SPC (22/23) polypeptide is synthesized

without a cleavable signal peptide and that it contains a single site for N-linked glycosylation. Utilizing the Kyte–Doolittle hydrophobicity scale and the position of the glycosylation site in the protein, they gave a possible topography of the protein. The SPC 22/23 protein would span the membrane once through a hydrophobic domain at the amino-terminus (residues 11–32), and the remainder of the protein would be oriented on the lumenal side of the membrane.

Another component of the SPC complex, the 21-kDa subunit, has recently been cloned and sequenced by Greenburg *et al.*, (1989), and the deduced amino acid sequence (192 amino acids with a calculated molecular weight of 21 kDa) shows that this polypeptide is 47% homologous with the recently sequenced *Saccharomyces cerevisiae* protein SEC11 (Böhni *et al.*, 1988) that is required for signal peptide cleavage, normal secretion, and cell survival. From the deduced amino acid sequence of the 21-kDa protein, the authors noted no cleavable leader sequence but that there was a large amino terminal hydrophobic domain (residues 29–69) that could serve as a membrane-spanning segment and that this region was followed by a large hydrophilic domain that could lie within the lumen of the endoplasmic reticulum. There is a small hydrophobic domain in the carboxyl-terminus of the protein (residyes 157–168), but this region is too small to span the lipid bilayer. The authors point out that this structure resembles SPase I in that the bacterial protein contains a small hydrophobic segment in the periplasmic domain (which corresponds to the lumen) and that this structual motif might be involved in the cleavage reaction. There is no sequence homology with the two bacterial SPases, perhaps indicating that another protein of the SPC contains the signal peptidase activity and that the 21-kDa and 22/23-kDa proteins function in an ancillary way.

Substrate Specificity

From *in vivo* studies the mammalian SPase cleaves a wide variety of substrates including bacterial precursors (Talmadge *et al.*, 1980; Roggenkamp *et al.*, 1985; Müller *et al.*, 1982; Jackson and Blobel, 1977; Mollay *et al.*, 1982; Lively and Walsh, 1983). From the signal sequences of several hundred secreted proteins, von Heijne (1985, 1986) has defined features of the polar carboxyl-terminus segment that defines the signal peptidase cleavage site. *In vitro* studies with the purified HOSP or SPC concerned with the specificity of the cleavage site have just begun. Recently, Cioffe *et al.* (1989) have, by site-directed mutagenesis, modified the length of the hydrophobic core in the signal peptide of the bovine parathyroid hormone. Their results indicated that deletions in the amino-terminus of the hydrophobic core were inhibitory for processing activity; however, deletions in the carboxyl-terminus of the

hydrophobic core had no effect or increased processing when assayed either by translocation experiments or post-translationally with the addition of purified HOSP. These data suggest that the proteins within the HOSP complex might be part of the membrane proteins involved with translocation. Since the HOSP complex is composed of only two proteins, the authors present a model whereby one of the subunits binds to the carboxy-terminal region of the signal peptide and is responsible for the catalytic activity but does not influence translocation. The other subunit could interact with the amino-terminus of the hydrophobic core such that when it interacted with a signal peptide it would activate the catalytic part of the complex. This model is supported by the recent studies of Caulfield *et al.* (1989) that showed, with a synthetic cleavable peptide, that polar substitutions in the hydrophobic core (12 residues from the cleavage site) inhibited processing.

Properties of the Mammalian Signal Peptidases

The mammalian signal peptidases are integral membrane proteins (Lively and Walsh, 1983) that require added phospholipid, principally phosphatidylcholine, for activity (Jackson and White, 1981). Depletion of the SPC for phospholipid resulted in a nonreversible loss of activity (Evans *et al.*, 1986); HOSP also requires phosphatidylcholine for maximal activity (Baker *et al.*, 1986). It is interesting that the substrates utilized for measuring the activity of the purified enzymes are not completely hydrolyzed under any conditions tested (Baker and Lively, 1987; Evans *et al.*, 1986; Caulfield *et al.*, 1989). These enzymes are not metallo-enzymes and are not inhibited by a wide range of protease inhibitors (Jackson and Blobel, 1980; Strauss *et al.*, 1979; Fujimoto *et al.*, 1984; Lively and Walsh, 1983). There is no evidence indicating the parameters of the enzyme's active site. The kinetic properties of these complex enzyme systems remain to be defined. Utilizing a synthetic substrate of 31 amino acids that was processed by HOSP, Caulfield *et al.* (1989) calculated a K_m of 3.2 μM and a V_{max} of 70 nM/min. The K_m is in the same range as that reported for SPase II (Dev and Ray, 1984).

Microsomal Signal Peptidase of *Saccharomyces cerevisiae*

YaDeau and Blobel (1989) have solubilized a signal peptidase that cleaves prepro-alpha-factor to its mature form from the microsomal membranes of *S. cerevisiae*. The membrane association of yeast signal peptidase is resistant to carbonate extraction, indicating that it is an integral membrane protein. The enzyme displays a broad, alkaline pH optimum, retaining activity at pH 12. Moderately high temperatures (35°C) excess detergent

(> 0.5% Nikkol), or high salt (> 300 mM KOAc) will inactivate the enzyme. Phosphatidylcholine is necessary for optimal activity.

Among the collection of temperature-sensitive secretion-defective mutants of *S. cerevisiae* (Scheckman, 1985), the characteristics of one mutant, designated *SEC11*, were found to fit the predictions of a signal peptidase lesion (Böhni *et al.*, 1988). *SEC11* was required for signal peptide cleavage, normal rate of secretion, and cell survival in *S. cerevisiae,* A DNA fragment that complements the *SEC11* mutation has been cloned and sequenced. The sequence predicts a basic protein (estimated pI of 9.5) of 167 amino acids including an amino-terminal hydrophobic region that may function as a signal and/or membrane anchor. One potential N-glycosylation site was found in the 19-kDa predicted protein. The deduced amino acid sequence of *SEC11* was found to be 47% homologous with the 21-kDa subunit of the pentameric complex of canine signal peptidase (Greenburg *et al.*, 1989). Whether these two proteins are the catalytic subunit of the signal peptidase complex remains to be determined.

Mitochondrial Signal Peptidases

Hawlitschek *et al.* (1988) have shown that mitochondrial signal peptidase of *Neurospora crassa* is a dimeric enzyme. The fully active enzyme consists of two proteins, the matrix processing peptidase (MPP, 57 kDa) and a processing enhancing protein (PEP, 52 kDa). The MPP component appears to be the catalytic component, which is stimulated by PEP. The two proteins are present in unequal amounts in mitochondria, PEP being 15-fold more abundant. The MPP polypeptide is localized in the soluble matrix space, while PEP is partly associated with the inner membrane. A full-length *c*-DNA clone for PEP was isolated and sequenced. The coding region corresponds to a precursor protein of 476 amino acid residues having a molecular mass of 52 kDa.

Yaffe and Schatz (1984) isolated temperature-sensitive mutants of *S. cerevisiae* that accumulate uncleaved mitochondrial precursor proteins under restrictive conditions. Two complementation groups designated *MAS1* and *MAS2* (for mitochondrial assembly) were identified. The *MAS1* mutants lacked the matrix-localized processing activity *in vitro* (Yaffe *et al.*, 1985). The *MAS1* (*mif1*) gene was cloned, sequenced, and shown to encode a precursor protein of 462 amino acids with a molecular weight of 51 kDa. (Witte *et al.*, 1988). The precursor protein encoded by *MAS1* gene was cleaved *in vivo* by the purified enzyme to a 48-kDa protein. The overall amino acid sequence of the *MAS1* gene product is that of a typical soluble protein that lacks any obvious membrane-spanning domains. The deduced amino

acid sequence of *MAS1* was found to be 70% identical to the 52-kDa (PEP) subunit of the mitochondrial signal peptidase from *N. crassa* (Hawlitschek *et al.*, 1988). The *MAS2* (*mif2*) gene has been cloned and sequenced by two independent groups (Jenson and Yaffe, 1988; Pollock *et al.* 1988); this gene encodes a protein of 482 amino acids (53.3 kDa). The *MAS2* gene product corresponds to the catalytic subunit (MPP) of the mitochondrial signal peptidase from *N. crassa*. The gene products of both *MAS1* and *MAS2* are localized in the soluble matrix space, share more than 25% homology, and are important for cell viability and the processing of mitochondrial precursor proteins.

Substrate Specificity

So far a consensus sequence at the cleavage site of presequences for the matrix protease has not been identified. However, recent surveys show that the configuration around the cleavage site is often -Arg-X # Y- (Hartl *et al.*, 1989; Hendrick *et al.*, 1989). The lack of a consensus sequence could be due to the following reasons: (1) there may be differences in cleavage specificity among the mitochondria of different organisms, even though heterologous import and processing show a high degree of conservation; (2) the presence of an alternate protease which can cleave the mitochondrial presequences has not been completely ruled out, since Kalousek *et al.* (1988) have identified two different processing enzymes in rat liver mitochondria; (3) the enzyme may recognize some three-dimensional motif rather than a specific amino acid sequence around the cleavage site; (4) many studies have suggested that the specificity of the peptidase is dependent on regions either in the signal peptide or in the mature part of the precursor that are at some distance from the actual cleavage site (Vassaroti *et al.*, 1987; Hurt *et al.*, 1987; Kraus *et al.*, 1988).

Properties of the Matrix Mitochondrial Signal Peptidase

Signal peptidases localized in the soluble matrix space of mitochondria that cleave the matrix-targeting sequences from various precursor proteins were first identified in yeast, rat tissues, and maize (Bohni *et al.* 1980) and have been further characterized from various sources (McAda and Douglas, 1982; Miura *et al.*, 1982; Conboy *et al.*, 1982; Bohni *et al.*, 1983; Schmidt *et al.*, 1984; Kumamoto *et al.*, 1986; Hawlitschek *et al.*, 1988; Yang *et al.*, 1988; Ou *et al.*, 1989). The enzyme isolated from different sources exhibits similar properties. This signal peptidase is a soluble protein that is sensitive to metal chelators, such as EDTA or 1, 10-phenanthroline, and is stimulated by divalent cations, such as Co^{2+}, Mn^{+2}, and Zn^{+2}. The enzyme is insensitive to a number of protease inhibitors including phenylmethylsulfonyl flouride

(PMSF) and has a neutral pH optimum. These proteases are specific for mitochondrial precursors and do not cleave denatured precursors. The specificity for cleavage seems to reside in the three-dimensional motif rather than a specific amino acid sequence around the cleavage site (Bohni *et al.*, 1980; McAda and Douglas, 1982). The dimeric enzymes have been purified to near homogeneity from *N. crassa* (Hawlitschek *et al.*, 1988), *S. cerevisiae* (Yang *et al.*, 1988), and rat liver (Ou *et al.*, 1989). The purified matrix proteases from *N. crassa* and *S. cerevisiae* correctly cleave the presequences of several authentic mitochondrial precursors; however, the rat liver mitochondrial enzyme cleaves the precursor forms of ornithine carbamolytransferase and malate dehydrogenase to intermediate forms. Kalousek *et al.* (1988) have shown that two separate processing activities are required, in sequence, for the correct maturation of these two precursors.

Additional Mitochondrial Signal Peptidases

Most of the imported mitochondrial proteins localized in the soluble intermembrane space or anchored to the inner membrane are proteolytically processed in two steps. These proteins are initially cleaved by the signal peptidase localized in the soluble matrix space. The second cleavage is performed by one of the four enzymes located either at the outer surface on the inner memebrane or at the intermembrane-space surface of the inner membrane. The role of two-step processing in the intramitochondrial sorting of the proteins and the properties of the additional enzymes has recently been reviewed by Hartl *et al.* (1989).

Signal Peptide Hydrolase (Peptidases)

Signal peptides, once removed by signal peptidase, do not accumulate but are rapidly degraded (Habener *et al.* 1979; Hussain *et al.*, 1982; Jackson and Blobel, 1977; Ray *et al.*, 1986; Zwizinski and Wickner, 1980). The reasons for this rapid degadation are not clear. However, the data from various laboratories clearly indicate that the addition of an exogenous signal peptide inhibits translocation (Koren *et al.*, 1983; Austen *et al.*, 1984; Chen *et al.*, 1987). The inhibition by the externally added signal peptide could be due to competition for binding a component located at the membrane surface (e.g., signal sequence receptor). Wickner *et al.*, (1987), however, demonstrated that the signal peptide of procoat could inhibit the processing of procoat and pMBP by purified SPase I. Recently, Chen and Tai (1989) showed that antipain, an inhibitor of a membrane-bound signal peptide hydrolase (see

below), inhibited protein translocation but did not inhibit the processing of the precursors by purified SPase I. These results suggest that the rapid removal and degradation of the cleaved signal peptide is necessary to maintain proper export function and that the signal peptide peptidase is an integral part of the export machinery.

The protein(s) responsible for the degradation of the signal peptide in mammalian cells has not been identified. Three different peptidases capable of cleaving an intact signal peptide from *E. coli* have been identified. Ichihara *et al.* (1984) demonstrated that a membrane-bound enzyme, designated protease IV (Pacaud, 1982), can hydrolyze the prolipoprotein signal peptide. The gene for protease IV (*spp A*) has been cloned (Ichihara *et al.*, 1986) and sequenced. The polypeptide deduced from the DNA sequence contains 618 amino acids and would correspond to a 67-kDa protein. Although several hydrophobic domains were observed in the deduced amino acid sequence, the protein was not as hydrophobic as other integral membrane proteins (Ichihara *et al.*, 1986). Mutants deleted for the *spp* gene grew normally (Suzuki *et al.*, 1987). Digestion of the prolipoprotein signal peptide in isolated cell envelope fractions derived from a deletion mutant suggested that another membrane-bound protease was capable of cleaving the signal peptide, although more slowly. The nature of this enzyme is unknown.

Novak *et al.* (1986) have shown that there are two cytoplasmic enzymes that can degrade a signal peptide *in vitro*. The majority of the cytoplasmic signal peptide hydrolase activity is due to a 68-kDa protein similar to the oligopeptidase A protein of *Salmonella typhimurium* (Vimr *et al.*, 1983) and less than 10% of the activity is due to protease So (Chung and Goldberg, 1983). The role of these peptidases in the degradation of the signal peptide *in vivo* is unknown. The intact signal peptide is not accumulated in *E. coli* mutants lacking protease IV (Suzuki *et al.*, 1987) or in mutants of *S. typhimurium* deficient in oligopeptidase A (I. Dev, unpublished). The data suggest that these two major peptidases individually are not responsible for the cleavage of an intact signal peptide. Protease IV cleaves prolipoprotein signal peptide in the hydrophobic segment only (Novak and Dev, 1988). *In vitro*, the oligopeptidase degrades the signal peptide of prolipoprotein much more rapidly than does protease IV. Neither enzyme, however, cleaves the signal peptide while it is part of the precursor (Novak and Dev, 1988).

Properties of Signal Peptide Hydrolases

Protease IV, a membrane-bound signal peptide hydrolase, was first identified by Pacaud (1982) based on its ability to hydrolyze N-acyl-amino acid-*p*-nitrophenol esters containing val, leu, phe, and ala residues. The

enzyme was solubilized by a nonionic detergent (0.3% emulphogen) and purified by conventional chromatography. Protease IV is inhibited by diisopropyl phosphofluoridate and PMSF. Ichihara *et al.* (198) purified protease IV to near homogeneity from an overproducing strain and estimated the protein to be 67 kDa from SDS gel electrophoresis. Cross-linking studies indicated that the active enzyme is a tetramer of the *sppA* gene product. The substrate specificity and other properties of the cloned enzyme were similar to the enzyme isolated from a wild-type strain (Pacaud, 1982; Hussain *et al.*, 1982; Ichihara *et al.*, 1984), except that the previously reported molecular mass of the latter was 34,000. This discrepancy probably resulted from insufficient purification of Pacaud's enzyme preparation, and the protein with a molecular mass of 34,000 observed by Pacaud was a contaminant.

The major cytoplasmic signal peptide hydrolase activity corresponds to oligopeptidase A (*optA*) that was identified by Vimr *et al.* (1982) in *S. typhimurium* as a peptidase that could hydrolyze N-acetyl-tetra-alanine. Mutants lacking this activity grew normally. The enzyme in crude extracts was stimulated by Co^{2+} and Mn^{2+} and inhibited by EDTA. Novak *et al* (1986) showed that most of the signal peptide-degrading activity in *E. coli* is localized in the cytoplasm and is due to an enzyme similar to oligopeptidase A. They purified the hydrolase to near homogeneity and showed that the major activity is associated with a monomeric protein of 68 kDa. Oligopeptidase A cleaved prolipoprotein signal peptide on either side of a glycine or alanine residue (Novak and Dev, 1988). The enzyme requires a peptide of at least five amino acid residues, with a minimum of two to three residues on either side of the scissile bond (Vimr *et al.*, 1982; Novak and Dev, 1988).

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