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0014-4754/92/020117-02$1.50 + 0.20/0

Proteolysis in protein import and export: Signal peptide processing in eu- and prokaryotes

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Abstract. Numerous proteins in pro- and eukaryotes must cross cellular membranes in order to reach their site of function. Many of these proteins carry signal sequences that are removed by specific signal peptidases during, or shortly after, membrane transport. Signal peptidases have been identified in the rough endoplasmic reticulum, the matrix and inner membrane of mitochondria, the stroma and thylakoid membrane of chloroplasts, the bacterial plasma membrane and the thylakoid membrane of cyanobacteria. The composition of these peptidases varies between one and several subunits. No site-specific inhibitors are known for the majority of these enzymes. Accordingly, signal peptidases recognize structural motifs rather than linear amino acid sequences. Such motifs have become evident by employing extensive site-directed mutagenesis to investigate the anatomy of signal sequences. Analysis of the reaction specificities and the primary sequences of several signal peptidases suggests that the enzymes of the endoplasmic reticulum, the inner mitochondrial membrane and the thylakoid membrane of chloroplasts all have evolved from bacterial progenitors.
Key words. Signal peptidase; signal sequence; limited proteolysis; protein traffic; endosymbiont theory; membrane proteins.

Introduction

Numerous proteins in both prokaryotes and eukaryotes have to cross biomembranes in order to reach their destinations within the cell. It is generally accepted that these proteins are distinguished, and consequently sorted, from the bulk of cytoplasmic proteins by virtue of discrete sequence sections, termed signal sequences. A signal sequence is defined as a sequence which contains the information necessary and sufficient to guide a protein

bearing that sequence to a distinct cellular membrane, and initiate its transmembrane transport (which will be referred to as translocation in this paper). A functional signal sequence is characterized by (1) its selective occurrence in non-cytoplasmic proteins; (2) its failure to direct membrane transport after certain structural modifications; (3) its ability to function as a transport signal when artificially fused to a normally cytoplasmic protein, whose structure is permissive of transmembrane transport. Signal sequences are in many instances NH_2-terminally located and are cleaved during or shortly after the translocation process. The enzymes involved in the cleavage of signal peptides are the topic of this article. The properties and functions of signal sequences are reflected by the existence of several synonyms for them: presequence, transit sequence, targeting sequence, leader sequence (see also Varshavsky[105]).

Survey of the cellular protein traffic involving removal of signal sequences

The cell envelope of gram-negative bacteria such as *Escherichia coli* is composed of an inner (plasma) membrane confining the cell body, an outer membrane, and an intermediate periplasmic space. After their synthesis in the cytoplasm many proteins are exported to one of the three envelope layers. The periplasmic and outer membrane proteins are proteolytically processed by the removal of their NH_2-terminal signal sequences, which is catalyzed by plasma membrane-located signal peptidases.

In eukaryotes, signal sequences not only have to target to membranes but also to distinguish between various organelles. Hence differently structured signal sequences occur. One group of proteins containing cleavable signal sequences encompasses secretory proteins, lysosomal proteins, house-keeping enzymes of the endoplasmic reticulum (ER) and the Golgi apparatus, and a few membrane proteins. These proteins are initially targeted to the ER membrane by hydrophobic signal sequences, which are cleaved upon entry into the ER cisternae by signal peptidases located on the ER membrane. The ER-targeting signals are functionally interchangeable with those of the periplasmic and outer membrane proteins of gram-negative bacteria.

A different class of cleavable signal sequences, mostly hydrophilic in nature, is required to target nuclear-encoded proteins to mitochondria and chloroplasts and to initiate their import into these organelles. The hydrophilic signal sequences have to be sufficiently specific to avoid mis-sorting between mitochondria and chloroplasts. Hydrophilic signal sequences guide proteins imported into mitochondria to the matrix of the organelle and are removed by a matrix-located processing enzyme. Inner membrane and intermembrane space proteins are then sorted from the pool of matrix-destined proteins by means of the information contained in additional signals. Intermembrane space proteins might undergo a second proteolytic event, which is catalyzed by a signal peptidase of the inner membrane. An even more complex situation is encountered in chloroplasts. There, following import into the stroma of the organelle and removal of the signal sequence by the stromal processing peptidase, proteins have to be redirected to the envelope layers in a similar way to that outlined for mitochondria, but in addition to the membrane and the lumen of the thylakoid. The latter process, again, involves additional signal sequences which are cleaved off by a thylakoidal signal peptidase.

The reader is referred to recent detailed reviews on membrane transport and sorting mechanisms, for bacteria[6,67,90], the ER membrane[5,86,90], mitochondria[37,79,107], and chloroplasts[56,98]. Reviews on signal peptidases have also recently been published elsewhere[16,64].

Signal peptidases of various organelles

Table 1 is a summary of the most extensively studied signal peptidases, including some of their known characteristics.

Signal peptidases of the endoplasmic reticulum (ER)

Most secreted, lysosomal, and some integral membrane proteins, are targeted to the ER via NH_2-terminal signal sequences which are cleaved off upon entry into the ER by a signal peptidase. Two of these signal peptidases have been purified to homogeneity from mammalian tissue; canine pancreas signal peptidase complex (SPC)[22] and hen oviduct signal peptidase (HOSP)[2]. Recently, the yeast enzyme has also been purified to near homogeneity[126]. All three enzymes purify as complexes composed of several subunits (see 'Composition of signal peptidases', below). Three subunits of the dog pancreas enzyme[35,96,97], one of the hen oviduct peptidase[64], and one of the yeast enzyme[9] have been sequenced, and none of them was found to be similar to any of the sequences of other known proteinases. One of the subunits of yeast signal peptidase is identical to SEC11[126]. The *SEC11* locus had previously been identified by a ts-mutation which leads to an accumulation of unprocessed secretory protein precursors and ultimately to cell death at the non-permissive temperature[9]. Mammalian signal peptidases have also been solubilized and partially purified from rat liver[55,68] and porcine pancreas[26].

Signal peptidases of the prokaryotic plasma membrane

Signal peptidases of the prokaryotic plasma membrane proteolytically process proteins destined for the periplasmic space and the outer membrane, in the case of gram-negative bacteria (e.g. *Escherichia coli*), and for the extracellular milieu, in the case of gram-positive bacteria (e.g. *Bacillus subtilis*). Essentially all of our present knowledge about bacterial signal peptidases stems from studies with

Table 1. Signal peptidases

Organelle	Organism	Nomenclature	Membrane anchored	Subunits	Molecular mass (kDa)[a]	Inhibitors	Carbohydrate	Cleavable signal sequence	Solubilized	Purified	Sequenced
Endoplasmic reticulum	Dog pancreas	(Canine) signal peptidase complex (SPC)	+	5	12 18 21 22/23[c] 25	–[b]	– – – + –	– – – –	(49)	(22)	(96) (35) (97)
	Hen oviduct	Hen oviduct signal peptidase (HOSP)	+	2	19 22–24[c]	–[b]	– +		(66)	(2)	(64)
	Yeast	Yeast signal peptidase (ySP)	+	3–4	(13)[d] 18 = SEC11 20 25	high salt	– – – +		(125)	(126)	(9)
Mitochondria											
Matrix	*Neurospora crassa*	MPP[e] + PEP[e]	soluble[f]	2	57 (MPP) 52 (PEP)	high salt and Me-chelators		+		(39) (39)	(93) (39)
	Yeast	MAS1[e] + MAS2	soluble	2	51 (MAS2) 48 (MAS1)	Me-chelators		+ +		(130) (130)	(50, 82) (119)
	Rat liver	Matrix processing protease	soluble	2	55 52	Me-chelators		+		(59, 77) (59, 77)	(59)
Inner membrane	Yeast	Inner membrane protease I[g]	+		21.4[h, i]	EDTA		+	(94)		(4)
Chloroplasts											
Stroma	Pea	Stromal processing peptidase	soluble		~180	Me-chleators				partially (87)	
Thylakoidal membrane	Pea	Thylakoidal processing peptidase	+			–				partially (57)	
Bacterial plasma membrane	*E. coli*	Signal peptidase I (SPase I; leader peptidase)	+	1	37	high salt				(121)	122)
		Signal peptidase II (SPase II)	+	1	18	globomycin				(15)	(131)

a) determined by SDS-PAGE; b) none of the classical proteinase inhibitors has been found to inactivate this peptidase; c) differently glycosylated forms of one polypeptide; d) the association of the 13 kDa-protein with the enzymatic activity is tentative[126]; e) MPP, matrix processing peptidase; PEP, processing enhancing protein; MAS, mitochondrial assembly; f) PEP is partly found associated with the inner mitochondrial membrane[39]; g) a mutation of this proteinase interferes with the second processing step of only a subgroup of the intermembrane space proteins analyzed. Hence other proteinases of the same location and function might exist[4]; h) a gene encoding a 21.4 kDa-protein complements the phenotype of a yeast mutant which accumulates incompletely processed, intermembrane space proteins of mitochondria[4]; i) overproduction of the 21.4 kDa-protein does not enhance peptidase activity, which might point to the existence of an additional subunit[94].

the two *E. coli* enzymes (table 1). Signal peptidase I (SPase I), also termed leader peptidase, has been purified[121] and its gene (*lep*) cloned and sequenced[122]. SPase I was found to consist of a single polypeptide chain of 37 kDa molecular mass. It is essential for cell growth[14]. The enzyme is anchored within the plasma membrane and its assembly is dependent on SecA and SecY[120], proteins which are required in general for protein export in *E. coli* (recently summarized in Saier et al.[90]).

Little is known about signal peptidases of other bacteria. The *lep*-gene of *Salmonella typhimurium* has also been cloned and sequenced and shown to be 92% identical to the *E. coli* enzyme at the amino acid level[103]. A peptidase correctly cleaving the signal sequence of the in vitro synthesized preprosubtilisin has been identified in Triton X-100-disrupted membrane vesicles prepared from *B. subtilis*[123]. Recently a SPase I-activity could be solubilized also from intracytoplasmic membranes of the facultatively phototrophic, gram-negative bacterium *Rhodobacter capsulatus* (B. Wieseler and M. Müller, submitted).

Prokaryotic plasma membranes contain yet another signal peptidase activity, designated signal peptidase II (SPase II), whose substrate specificities are different from SPase I. SPase II is specific for preproteins that undergo a distinct lipid modification, such as Braun's lipoprotein of *E. coli*. The precursor of this lipoprotein is first modified by the attachment of a diglyceride to the prospective NH_2-terminal Cys of the mature protein. Only the diglyceride-bearing precursor is accepted as substrate by the lipoprotein-specific SPase II, which removes the signal peptide immediately upstream of the modified Cys. Braun's lipoprotein is a prototype of many glyceride-modified proteins in gram-negative and gram-positive bacteria (reviewed in Wu and Tokunaga[124]). SPase II has been purified[15] and its gene (*lsp*) cloned and sequenced[131]. It does not share any sequence homology with the *lep*-gene or genes encoding other known signal peptidases. Like SPase I, SPase II is essential for cell growth[128]. Recently, the *lsp*-genes of *Enterobacter aerogenes*[48] and *Pseudomonas fluorescens*[47] have also been identified and sequenced.

Mitochondrial signal peptidases

The majority of constituent mitochondrial proteins are synthesized in the cytoplasm and therefore have to be imported into the organelle. This process is driven by mitochondria-specific, hydrophilic signal sequences. Import occurs in most cases at contact sites of the inner and outer membranes and involves cleavage of the hydrophilic presequence by a matrix-located processing peptidase. The enzyme consists of two subunits (table 1)

termed MPP (matrix processing peptidase) and PEP (processing enhancing protein) in *Neurospora crassa*, and MAS1 and MAS2 (MAS stands for *m*itochondrial *as*sembly) in *Saccharomyces cerevisiae*. Both enzymes have been obtained in pure form[39,130] and the nucleotide sequences of all four subunits have been determined[39,50,82,93,119], revealing that the subunits themselves are synthesized as precursors with hydrophilic signal sequences typical for matrix-directed import proteins. The peptidases are soluble within the matrix, with PEP being found partially associated with the inner membrane[39]. Mutants in either of the two subunits have been isolated from *S. cerevisiae*[82,127], which are temperature-sensitive for growth under conditions in which cells depend on mitochondrial metabolism. An equivalent matrix processing peptidase has also been purified from rat liver[59,77] and an additional enzyme has also been reported[52].

Some of the imported proteins, once delivered to the matrix, have to be redirected to either the inner mitochondrial membrane or the intermembrane space. This routing requires individual hydrophobic signal sequences which, in the case of translocation across the inner membrane into the intermembrane space, are cleaved off by a distinct membrane-located signal peptidase. Thus, nuclear-encoded intermembrane space proteins are synthesized with a bipartite NH_2-terminal signal sequence, the NH_2-terminal part specifying import into the matrix, the COOH-terminal part being an intermembrane space-targeting signal. An alternative view suggests that the processing-intermediates of intermembrane space proteins, resulting from cleavage by the matrix processing peptidase, have not crossed the inner mitochondrial membrane completely[53,104], but rather remain anchored to it via the stop transfer information of the COOH-terminal, hydrophobic part of the bipartite signal sequence[54], which is subsequently cleaved by the membrane-located signal peptidase.

The enzyme which cleaves the second part of the bipartite signal sequence, and which is different from the matrix peptidase, also processes mitochondria-encoded proteins destined for the inner mitochondrial membrane or the intermembrane space. This is inferred from the finding that the *pet ts2858* mutant accumulates an incompletely processed precursor of an imported, intermembrane space protein (cytochrome b_2) as well as a mitochondrially encoded precursor of the inner membrane protein cytochrome oxidase subunit II[83]. The protein affected in this mutant was shown to be the signal peptidase of the inner mitochondrial membrane of *S. cerevisiae*, designated inner mitochondrial protease (IMP 1)[94]. Recently, a gene was cloned by complementation of the *pet ts2858* mutant[4], and the enzyme was shown to contain a membrane-embedded 21.4 kDa-subunit[4,94]. However, since an overproduction of the 21.4 kDa-polypeptide does not increase the peptidase activity of mitochondria, active IMP 1 is presumably composed of yet another subunit. The existence of an additional inner membrane peptidase is suggested by the finding that two other intermembrane space proteins encoded by the nucleus and equipped with a bipartite signal sequence (cytochrome c_1 and cytochrome c peroxidase) are correctly processed in the IMP 1-mutant[4].

Signal peptidases of the chloroplast and the thylakoid of cyanobacteria

The compartment structure of the chloroplast resembles that of mitochondria with the exception of the additional thylakoidal membrane system. Accordingly, the chloroplast requires processing peptidase activities similar to those of mitochondria, but, in addition, a thylakoidal enzyme. Chloroplasts contain a processing peptidase of about 180 kDa, soluble in the stroma of the organelle, which has been partially purified[87]. Little is known about the mechanism by which proteins of the chloroplast envelope (outer and inner membrane) are sorted. However, it was demonstrated that proteins destined for the thylakoid contain additional targeting information[56,98]. In the case of thylakoid lumen proteins the additional information resides in a second signal sequence immediately downstream of the one required for transport into the stroma. Hence, this class of proteins also contains a bipartite signal sequence, which is cleaved in two successive steps. After the NH_2-terminal signal specifying import into the stroma has been removed by the stromal processing peptidase, an integral thylakoid membrane peptidase cleaves the thylakoid-specific part of the signal sequence upon transport into the lumen of the thylakoid. This peptidase has also been partially purified[57]. Presumably this enzyme also processes the precursor of the chloroplast-encoded thylakoid membrane protein cytochrome f[117].

Cyanobacteria contain a thylakoid membrane peptidase involved in proteolytic processing of thylakoid-imported proteins, which has similar reaction specificities to the plant thylakoid peptidase (see section below). A thylakoid peptidase has now been solubilized and partially characterized from the cyanobacterium *Phormidium laminosum*[114].

Structural and functional similarities between signal peptidases of different origin

Substantial homologies exist between the various organelle-specific signal peptidases which have been prepared from different organisms (table 2). Remarkably, this applies also to enzymes of the phylogenetically distinct organisms mammals and yeast (ER-signal peptidases and matrix processing peptidases of mitochondria, respectively). This conservation of structure underlines the vital function signal peptidases fulfill in all living organisms.

Structural and functional homologies are observed even between signal peptidases of different membrane sys-

Table 2. Sequence homologies between signal peptidase proteins

		refs
Canine signal peptidase subunit 18	Yeast *SEC11* (subunit 18 of yeast signal peptidase)	(9, 96)
Canine signal peptidase subunit 21	Yeast *SEC11* (subunit 18 of yeast signal peptidase)	(9, 35)
Canine signal peptidase subunit 21	Hen oviduct signal peptidase subunit 19	(64)
Canine signal peptidase subunit 22/23	Hen oviduct signal peptidase subunit 22–24	(64, 97)
Neurospora crassa PEP	Yeast *MAS1*	(82)
Neurospora crassa MPP	Yeast *MAS2*, 55 kDa-subunit of matrix processing protease from rat liver	(93) (59)
Yeast mitochondrial inner membrane protease 1	*E. coli* signal peptidase I	(4)

tems. Thus, structural relatedness has been found for an inner mitochondrial membrane peptidase and signal peptidase I of the *E. coli* plasma membrane[4]. This is consistent with the finding that the COOH-terminal part of the bipartite signal sequence of mitochondrial intermembrane space proteins is very similar in structure to the signal sequence of bacterial export proteins. For example, *R. capsulatus*, which contains a cytochrome bc_1 complex highly homologous with that found in mitochondria, synthesizes cytochrome c_1 with a hydrophobic signal sequence which shows considerable similarity to the COOH-terminal part of the presequence of yeast cytochrome c_1[37, 79]. These findings are consistent with the endosymbiont theory proposing the origin of mitochondria from prokaryotic ancestors. This idea implies the conservation of the mechanisms of bacterial protein export in mitochondria. These mechanisms are now functioning to direct proteins imported from the cytoplasm and proteins synthesized in the mitochondrial matrix to the inner mitochondrial membrane and the intermembrane space, respectively[37].

A similar conservation of prokaryotic protein transport mechanisms involving signal peptidases and the respective signal sequences appears to prevail in the chloroplasts of a plant cell. Chloroplasts are thought to have arisen from cyanobacterial progenitors after endocytosis by an ancestral plant cell[34]. Cyanobacteria contain a thylakoidal membrane system, and transport of proteins into the lumen of this organelle involves cleavage of a hydrophobic signal sequence[42]. This process has been conserved in the case of chloroplast-encoded proteins destined for the lumen of the thylakoid, as well as in the case of nuclear-encoded proteins directed into the thylakoid after they have been imported into the stroma of the chloroplast. The similarity of these processes is again suggested by the fact that the same type of hydrophobic signal sequence is found on thylakoid proteins of cyanobacteria and chloroplasts[98, 114].

No sequence data are as yet available on the thylakoidal signal peptidases of chloroplasts and cyanobacteria. Therefore the degree to which these peptidases are homologous with bacterial plasma membrane signal peptidases has not yet been determined. However, the reaction specificities of the thylakoidal enzyme of chloroplasts and *E. coli* SPase I are identical, and those of thylakoidal peptidases from chloroplasts and cyanobacteria are similar. Thus, SPase I correctly and efficiently processes a precursor of a chloroplast-thylakoid lumen protein, and the thylakoidal processing peptidase cleaves a precursor with a bacterial signal peptide[36]. Although the precursor of a plant thylakoid lumen protein is processed correctly by the thylakoid processing peptidase prepared from both plants and cyanobacteria, the two enzymes cleave the cyanobacterial precursor differently[114]. It should be noted, however, that the failure of solubilized signal peptidase preparations to cleave a precursor protein does not necessarily reflect a lack of reaction specificity, but has often been found to be due to an unfavorable conformation of the precursor. For example, inner mitochondrial membrane protease 1 processes the intermediate-sized precursor of cytochrome b_2 accumulating in *pet ts2858*-mitochondria, but not if it is synthesized in vitro[94]. Similarly, yeast prepro-α factor requires SDS in order to be cleaved by its cognate signal peptidase[125] whereas it is cleaved by bacterial SPase I and thylakoidal processing peptidase only when the enzymes are added during cell-free synthesis of the precursor[36, 57]. Processing of preplacental lactogen by mammalian signal peptidase is stimulated if the precursor is unfolded by an anti-precursor antibody[65]. Therefore, lack of reaction specificity cannot be concluded from the fact that a given signal peptidase is inactive with a particular precursor protein. On the other hand, the positive demonstration of reactivity of signal peptidases towards non-cognate precursors does in fact indicate relatedness of substrate and enzyme. Taken together, the findings described clearly support the idea of conserved protein transport mechanisms operating in bacteria and chloroplasts.

Translocation of preproteins across the membrane of the mammalian endoplasmic reticulum and the prokaryotic plasma membrane involves cleavage of signal sequences which have a highly conserved secondary structure (see 'Hydrophobic signal sequences', below). The compatibility of both transport machineries in recognizing and processing the foreign substrate has also frequently been demonstrated both in vitro and in vivo[90]. In addition, purified bacterial SPase I correctly processes a variety of eukaryotic precursor proteins. However, no sequence

similarities between SPase I and signal peptidases from mammalian sources have been described. Since the catalytically active polypeptide has not yet been identified among the various subunits copurifying with mammalian signal peptidase activity, it remains to be seen whether one of the, as yet, unidentified subunits turns out to be similar to the single polypeptide signal peptidase of *E. coli*.

Composition of signal peptidases

Signal peptidases of the endoplasmic reticulum and the mitochondrial matrix were found to consist of more than one subunit (cf. table 1). This is shown by the copurification of the various subunits with the catalytic activity, and by the lack of a pool of monomers[96]. The activity of the mitochondrial matrix processing peptidase is lost if the two subunits reconstituting the enzyme are separated[39,130]. A polymeric structure, however, has not been found for the two *E. coli* signal peptidases, SPase I and SPase II, both of which contain a single polypeptide. Interestingly, in those cases in which sequence data are already available, it has become evident that different subunits of the same signal peptidase complex might show substantial sequence homology: this is true of the subunits 18 and 21 of canine signal peptidase[96] and the two subunits of the mitochondrial matrix processing peptidases from yeast and *Neurospora crassa*[50,82,93]. These similarities suggest that the homologous subunits have evolved from a common ancestor.

Why have some of the known signal peptidases a multimeric structure? The isolated subunit MPP of the mitochondrial matrix enzyme of *N. crassa* (MAS2 in yeast) shows low activity compared to the MPP-PEP complex[30,39]. This finding suggests that MPP is the actual catalytic subunit whereas PEP, which is inactive when isolated, has an enhancing function. It is conceivable that the catalytic activity of all signal peptidases resides in a single polypeptide, as is the case in the monomeric signal peptidases of prokaryotes. Ancillary subunits like PEP would then be activators of the catalytic subunit. This view is supported by the finding that PEP, which is about 15-fold more abundant in mitochondria than MPP[39], is either identical (*Neurospora crassa*) or highly homologous (yeast) with the subunit I of the cytochrome bc_1 complex of the inner mitochondrial membrane[95]. Subunit I does not participate in electron transfer but is required for the reductase activity and assembly of cytochrome bc_1 (for refs see Schulte et al.[95]). Therefore, PEP may have a stabilizing or even assembly-mediating function. If this conclusion is extended to the function of PEP in signal peptide cleavage, PEP and related subunits could be envisioned as precursor-binding proteins presenting the cleavage site to the catalytic subunit[79]. A precedent was described for the N-glycosylation of secretory nascent chains within the ER. It was suggested[29] that the translocating chain interacts first with the so-called glycosylation site binding protein (GSBP), before the acceptor sequence Asn-X-Ser/Thr is recognized by oligosaccharyl transferase. GSBP has now been found to be a multifunctional protein of the ER-lumen, involved in recognizing polypeptide domains and participating in modification reactions of newly synthesized proteins[61]. Certainly these interpretations are tentative (GSBP has recently been found not to be essential for N-glycosylation[72]), and other mechanisms by which ancillary subunits of signal peptidases might function are possible. For example, binding of a mitochondrial matrix targeting sequence has recently been found by cross-linking studies to occur at the MAS2 subunit of the yeast matrix processing peptidase[129]. This subunit corresponds to MPP of *N. crassa*.

Translocation of preproteins into the ER and subsequent processing occur predominantly in a cotranslational manner[8]. Therefore, the signal peptidase of the ER should be intimately associated with the putative pore through which nascent polypeptide chains probably traverse the membrane. The oligomeric structure of ER-signal peptidase may simply reflect an association of the catalytic protein with other subunits potentially involved in the translocation process. At the present time, however, this is still speculation.

Topography of signal peptidases

Signal peptidases of the ER, the bacterial plasma membrane and related enzymes of the mitochondrial inner membrane and the thylakoidal membrane of chloroplasts and cyanobacteria, are all integral membrane proteins requiring detergents for solubilization (summarized in table 1). The 18- and 21 kDa-subunits of canine pancreas signal peptidase were each found to be resistant to alkaline extraction, and the 22/23 kDa-subunit contains a potential NH_2-terminal anchor sequence[97], suggesting that each of these subunits is embedded in the lipid bilayer of the ER-membrane. In contrast, processing peptidases of the mitochondrial matrix and the chloroplast stroma are soluble, with the exception of the PEP-subunit of the mitochondrial peptidase from *N. crassa*. This subunit has a dual function in signal peptide cleavage and assembly of cytochrome bc_1, which explains why it is a peripheral membrane protein[95].

A body of direct and circumstantial evidence has been accumulated showing that the integral membrane peptidases are located at the *trans*-side of the membrane with respect to the transport direction of the precursor proteins. This has been investigated most extensively for the *E. coli* signal peptidase I. This protein has a large (about two-thirds of the molecule) COOH-terminal domain facing the periplasm, while the NH_2-terminal part anchors SPase I within the plasma membrane[69,91]. The anchor consists of an internal, i.e. uncleaved, signal sequence and a positively charged cytoplasmic domain[62,113].

Similarly, the major part of SPase II is thought to protrude into the periplasm. Four significantly hydrophobic domains are potential transmembrane segments, giving rise to a topography of SPase II such that 2 long hydrophilic loops extend into the periplasm while the NH_2-terminus, the COOH-terminus, and a small loop between the second and third transmembrane domain are located at the cytoplasmic side of the membrane[47].

Inner membrane protease 1 of yeast mitochondria has been found through protease accessibility studies to be largely exposed to the mitochondrial intermembrane space[94], and the thylakoid processing peptidase of chloroplasts faces the lumen of the organelle[58].

Several pieces of evidence indicate that the ER-signal peptidases are also oriented towards the lumen: (1) the fact that some of the subunits are glycosylated (cf. table 1) indicates that at least parts of these polypeptides must have been translocated across the ER membrane in order to be accessible to the oligosaccharyl transferase; (2) hydropathy profiles of all subunits of mammalian signal peptidases sequenced so far suggest NH_2-terminal anchor sequences with the remainder of the polypeptide chains extending into the lumen of the ER[9,35,96,97]; (3) trypsin treatment of right-side out microsomal vesicles does not abolish signal peptidase activity[115].

Substrate specificities of signal peptidases

Hydrophobic signal sequences

Extensive computer analyses of numerous pro- and eukaryotic, hydrophobic, NH_2-terminal signal sequences have unravelled the anatomy of a canonical signal peptide[109]. Thus a typical hydrophobic signal sequence is 15–25 amino acids long. It has a tripartite structure consisting of a positively charged NH_2-terminal region (n-region, 1–5 amino acids), a central hydrophobic core (h-region, 7–15 amino acids) probably arranged in an α-helix, and, separated by an α-helix-breaking Pro or Gly, the more polar COOH-terminal part (c-region, 3–7 amino acids), representing half of the cleavage site. No sequence conservation exists between the many signal sequences whose primary structure has been determined. It is even possible to synthesize idealized surrogates, e.g. Met-Lys-Asn-Ser-Thr-$(Leu)_{10}$-$(Ala)_6$[60], that are fully functional. A general organization into the domain structure outlined above holds true for prokaryotic and eukaryotic signal sequences, although distinct differences are detected if large enough samples of sequences are compared[27,110].

Removal of (part of) the c-terminal domain results in a complete loss of processing[73,80], indicating that this part of the signal sequence is involved in recognition by the peptidases. A detailed structure comparison of the c-region revealed the almost exclusive occurrence of Ala, Gly, Ser, Thr (amino acids with short, neutral side chains) at positions -1 and -3, with $-1/+1$ representing the cleavage site. This is known as the "$(-3,-1)$-rule"[78,108,109]. It has frequently been confirmed in investigations employing site-directed mutagenesis of the $(-3,-1)$-amino acids of signal sequences[23,25,60,81].

An additional characteristic feature of the c-region of a hydrophobic signal sequence appears to be the acquisition of a β-pleated conformation and a β-turn around the cleavage site[108]. The latter is consistent with the hairpin loop structure proposed for the signal sequence[46] after it has integrated into the lipid bilayer: the NH_2-terminal part of the signal sequence remains in the cytoplasm, while the α-helical core and parts of the β-sheet span the membrane. The β-turn would then expose the cleavage site to the signal peptidase on the *trans*-side of the membrane, with the remainder of the polypeptide chain running back through the bilayer. The existence of such a precursor conformation has recently been verified experimentally for the bacterial plasma membrane by demonstrating the occurrence of a translocation-intermediate which has already been proteolytically processed by SPase I but which is still accessible to external proteinases from the *cis*-side of the membrane[31,100,102]. On the other hand, experimental data on the requirement of β-structures for cleavage are controversial[19,60,74].

Less information is available on how the amino acid sequence downstream of the cleavage site influences proteolytic processing. Introduction of a Pro at $+1$ of the precursor of maltose-binding protein results in a competitive inhibition of *E. coli* signal peptidase I (G. Gallagher and P. J. Bassford Jr., unpublished), and a deletion of 6 residues downstream of the cleavage site of preproparathyroid hormone results in inefficient processing[118].

A more direct demonstration of the substrate requirements of pro- and eukaryotic signal peptidases was obtained when purified enzymes were assayed with synthetic peptides[17,18]. Thus a pentapeptide comprising the three COOH-terminal residues of the signal sequence and the two NH_2-terminal amino acids of mature maltose-binding protein was found to be a substrate of *E. coli* signal peptidase I, although it was several hundredfold less efficient than the authentic precursor[18]. A decrease in length of the peptide is usually associated with a decrease in substrate efficiency[17,18] although exceptions have been reported[18].

In addition to the structural properties of the cleavage domain, determinants of processing efficiency outside this region have been identified. Additions to, and deletions within, the n-region negatively affect processing[101], most probably by changing the position of the signal cleavage site at the *trans*-side of the membrane[75]. Similarly, mutations within the hydrophobic core of the signal sequence exert an influence on the processing efficiency[1,33]. Using homopolymeric units of Ile, Leu, Val or Ala to replace the natural core segment of the *E. coli* alkaline phosphatase signal peptide, it was demonstrated in vivo that the net hydrophobicity determines the total extent of precursor processing[12]. It is again likely that, in

many of these instances, mutations of the h-region affect processing via a positioning effect on the signal sequence within the membrane. However, evidence for the h-region being directly involved in recognition by the signal peptidase was also provided by experiments using synthetic substrates and isolated peptidases[10,13]. The failure of a set of mutant synthetic peptides in which an apolar residue had been replaced by a charged one, to act as a competitive inhibitor of signal peptidase, suggests that these mutations influence binding to the enzyme[10]. Due to the lack of a unique consensus sequence for the signal sequence cleavage site, alternate processing sites might occur within secretory and membrane proteins as long as the primary structure of these cryptic cleavage sites complies with the (−3, −1)-rule. Such cryptic cleavage sites may become manifest if the authentic sites are rendered inactive by mutation[23,32]. Alternatively, they can be made accessible to the peptidase by changing the upstream membrane anchor sequence of a previously non-cleavable signal sequence[63].

Hydrophilic signal sequences

The targeting sequences of imported mitochondrial proteins are between 20 and 80 amino acids long, lack uninterrupted stretches of hydrophobic residues and are rich in basic and hydroxylated residues. They appear to form amphipathic α-helical structures in a lipid environment with a transition to a β-turn around the cleavage site[88,89,92,106,112]. Although a consensus sequence at the cleavage site has not been identified, Arg is frequently found at the −2 position[38,40,112]. Recently, the purified matrix processing enzyme from yeast was shown to correctly cleave peptides which contain the authentic cleavage site of an imported mitochondrial protein[129]. These peptides, when added in excess, inhibited the enzyme's activity towards a purified precursor protein in a competitive manner and the extent of inhibition decreased as the sequence of the peptide deviated from the authentic sequence[129]. Taken together, these findings strongly support the view that the matrix-processing enzyme recognizes structural motifs of the signal sequence, including the cleavage site. The involvement of regions outside the cleavage site in recognition and/or cleavage by the peptidase is further demonstrated by site-directed mutations inactivating a precursor protein[41,43,71].

There is also a lack of significant sequence homology between the various chloroplast targeting sequences (29–100 amino acids long). A tripartite structure has been elucidated: an uncharged NH_2-terminal domain, a central domain lacking acidic residues and a COOH-terminal domain with a potential amphiphilic β-strand[112]. Structural analysis suggests that chloroplast targeting sequences are essentially flexible peptides devoid of regular secondary or tertiary structure[111]. There appears to be a loosely conserved consensus motif characteristic of the cleavage site[28].

Reaction products

The reaction products of the signal peptidase-catalyzed reactions are the mature protein free of the signal sequence, and the signal sequence. Although proteolytic processing of precursor proteins by membrane preparations containing signal peptidases has been demonstrated in countless experiments, usually the signal peptide is not recognized among the reaction products. Only by the use of purified peptidases[132] or, alternatively, in the presence of protease inhibitors can the signal peptide be detected[45]. Therefore signal peptides normally do not accumulate because they are rapidly degraded. This process has been studied in some detail for the *E. coli* lipoprotein. The signal peptide which accumulated when proteinase inhibitors were added was found to be intact, which indicated that signal peptidase cleaves off the entire signal peptide[99]. In vivo, a membrane-bound endoproteinase, termed protease IV, cuts the signal peptide at various sites, and the resulting oligopeptides are degraded further by cytoplasmic proteinases[76]. The physiological reason for the degradation of signal peptides is presumably to prevent inhibition of signal peptidases by the signal peptides generated, a phenomenon that has been demonstrated with purified enzymes[11,116]. (For a more detailed discussion of the matter, see Dev and Ray[16]).

Mode of catalysis

Signal peptidases are endoproteinases. Little is known about the catalytic properties of these enzymes, mostly because no site-specific inhibitors are known. This, however, does not apply to the soluble processing peptidases of mitochondria and chloroplasts, which are inhibited by metal chelators (table 1).

As discussed above, signal peptidases recognize structural motifs around the cleavage site, including up- and downstream sequences, of the precursor proteins. This is most convincingly shown by the inhibition of signal peptidase activity by isolated signal peptides[11,36,116], indicative of a competition between peptides and authentic precursors for the common substrate binding site.

Signal peptidases reside on the *trans*-side of a membrane across which a certain protein is translocated. This fact, in combination with structural analyses of signal sequences, has led to the hairpin-loop model[46], in which the precursor inserts into the membrane so that the cleavage site becomes exposed to the active center of the signal peptidase. Thus the conformation of the substrate to be cleaved is ultimately dependent on the lipid environment of the membrane. Moreover, non-catalytic components of the known signal peptidase complexes are likely to participate in the maintenance of a cleavage-competent structure of the precursor. In some instances, covalent modification of the precursor is a prerequisite for cleavage (for example, heme-attachment[37]), which is an additional indication that signal peptidases require a distinct

tertiary structure of their substrates if they are to be active. To sum up, signal peptidases recognize structural epitopes of their substrates – as opposed to linear sequence motifs – and the concerted action of several factors helps to form these epitopes.

Recently a first study of the structure-function relationship of the *E. coli* signal peptidase I was performed by analyzing the effects of mutant enzymes on the processing of signal peptides, both in vivo and in vitro. The results obtained suggest that the second transmembrane segment of SPase I and the immediate downstream region protruding into the periplasm are directly involved in catalysis[7]. These results are consistent with the presumed location of the signal sequence cleavage site on the *trans*-face of the membrane.

Physiological function

The overwhelming majority of NH_2-terminal signal peptides that promote translocation of secretory proteins are cleaved off during or shortly after the translocation process. Why does proteolytic processing occur? Clearly, transmembrane translocation and signal sequence cleavage are independent processes. This has been repeatedly documented by different experimental approaches: (1) cell-free synthesis/translocation systems prepared from *E. coli* consistently allow for translocation of a fraction of uncleaved precursor into membrane vesicles[70]; (2) mutations within the c-region of signal peptides have been described which prevent cleavage but do not affect the translocation efficiency of the mutated precursor[24, 73]; (3) depletion of yeast cells of the mitochondrial matrix processing protease results in the expected failure to cleave mitochondrial precursor proteins, but still allows for import[30]; (4) many random sequences were found to function as signals for transmembrane translocation of proteins into the ER and mitochondria[3, 44, 51]. However, these surrogate signals are not proteolytically processed. Therefore, signal peptides are not removed in order to render membrane transport possible.

It has been proposed[79] that the signal sequence could function as a modulator of the folding rate of a precursor molecule. Since experimental data have accumulated which demonstrate that stably folded precursors are translocation-incompetent[20, 84], molecular mechanisms have to be postulated that prevent premature folding of a precursor molecule. To what extent in vivo the signal peptide influences folding kinetics per se, or to what extent it does so by interacting with recognition proteins that primarily or concomitantly function as molecular chaperones[21], has to await a complete elucidation of the protein transport mechanisms operating at cellular membranes. In any event, after its complete translocation across a membrane a polypeptide chain does not have to be kept in an unfolded conformation, so that the signal peptide becomes dispensable in that respect also.

Presumably, the most important aspect of signal sequence cleavage is the release of the translocated polypeptide chain from the membrane with which it otherwise remains associated via the signal sequence. This is inferred from studies in which site-directed mutagenesis has led to cleavage of a normally non-cleavable signal sequence (summarized in Saier et al.[90]), thereby converting an anchor sequence to a classical signal sequence. Furthermore, repression of synthesis of *E. coli* signal peptidase I was shown to result in the membrane-anchorage of periplasmic and outer membrane proteins[14], clearly underlining the function of signal peptidases in completing the translocation process. It is evident that proteins which are destined for subcellular locations different from the compartment into which they have been initially translocated (i.e. outer membrane proteins in gram-negative bacteria, truly secreted proteins, etc.) have to be detached from the membrane across which they are translocated. Mis-sorting of translocated proteins by retaining them at the outer surface of the plasma membrane (ER-membrane) is a lethal event[9, 14] presumably due to blockage of the translocation machinery. Therefore it appears that the major reason for cleavage of signal sequences is to ensure that the translocated protein is liberated in order to reach further destinations, and to unblock the export sites of the membrane.

Note added after completion of the manuscript. Bauerle et al. (J. biol. Chem. *266* (1991) 5876 and 5884) recently described experiments raising doubts about an obligate two-step processing pathway of proteins destined for the chloroplast thylakoid. According to their results a full-length precursor rather than a partially processed, stromal intermediate represents the actual substrate for the thylakoidal signal peptidase.

Acknowledgments. The work from the author's laboratory was supported by the DFG grant Dr 29/31-6C, the Sonderforschungsbereich 206, and the Fonds der Chemischen Industrie. I thank Dr J. MacFarlane and I. Spitznagel for their help with the preparation of the manuscript. I am grateful to Drs P. Bassford Jr, R. Dalbey, M. O. Lively, G. Michaelis, S. Mizushima, W. Neupert, C. Robinson, G. Schatz, P. Weisbeck for communicating results in part prior to publication.

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