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EVOLUTIONARILY RELATED INSERTION PATHWAYS OF BACTERIAL, MITOCHONDRIAL, AND THYLAKOID MEMBRANE PROTEINS

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■ **Abstract** The inner membranes of eubacteria and mitochondria, as well as the chloroplast thylakoid membrane, contain essential proteins that function in oxidative phosphorylation and electron transport processes or in photosynthesis. Because most of the organellar proteins are nuclear encoded, they are synthesized in the cytoplasm and subsequently imported into the organelle before they are inserted into the membrane. This review focuses on the pathways of protein insertion into the inner membrane of eubacteria and mitochondria and into the chloroplast thylakoid membrane. In many respects, insertion of proteins into the inner membrane of bacteria is a process similar to that used by proteins of the thylakoid membrane. In both of these systems a signal recognition particle (SRP) and a SecYE-translocase are involved, as in translocation into the endoplasmic reticulum. The pathway of proteins into the mitochondrial membranes appears to be different in that it involves no SecYE-like components. A conservative pathway, recently identified in mitochondria, involves the Oxa1 protein for the insertion of proteins from the matrix. The presence of Oxa1 homologues in eubacteria and chloroplasts suggests that this pathway is evolutionarily conserved.

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INTRODUCTION

Two unit membranes, similar to those of gram-negative bacteria, surround mitochondria and chloroplasts. Chloroplasts and photosynthetic eubacteria contain an additional membrane system, the thylakoid membrane. Both mitochondria and chloroplasts are believed to have evolved from prokaryotic ancestors, and these organelles still exhibit some structural and functional similarities to prokaryotes. For example, the organelles contain genomes that encode some of their proteins, using their own protein synthesizing machinery. However, the protein conducting capacity of mitochondria and chloroplasts is strongly reduced because the genes originally encoded within these organelles were transferred to the nucleus. Now, most mitochondrial and chloroplast proteins are synthesized in the cytoplasm and are post-translationally imported into the organelle. In this review, we discuss how proteins insert into the inner membrane of bacteria and how this process is related to the translocation pathways of the mitochondria and of the chloroplast thylakoid. Recent work shows that there are some similarities between the protein insertion into the bacterial inner membrane and the chloroplast thylakoid membrane. Mitochondria exhibit multiple mechanisms for inserting proteins into the inner membrane that are quite distinct from the bacterial and chloroplast systems. However, the import pathways of proteins from the matrix side to the mitochondrial inner membrane may have been conserved from the bacterial ancestor.

Routing of nuclear-encoded proteins to the chloroplast and mitochondrion is achieved by unique presequences typically located at the N terminus of the protein. Mitochondrial presequences are enriched in basic amino acids and form amphipathic alpha helices that have positively charged amino acids on one side and hydrophobic amino acids on the opposite face of the helix. The sequences that target proteins to the chloroplast are typically rich in serine and threonine residues but also contain basic amino acids. Receptor proteins localized in the

outer membrane recognize the mitochondrial and chloroplast presequences. The presequences are cleaved by the processing peptidase located in the mitochondrial matrix (MPP) or the chloroplast stroma (SPP), respectively. Translocation of the proteins from the cytoplasm into the organelle occurs through channels in the outer membrane that form transient contacts with a translocase localized in the inner membrane. In addition to the matrix targeting presequence, some inner mitochondrial membrane proteins contain a second signal that directs proteins to the inner membrane and is cleaved by an inner membrane peptidase (IMP). Likewise, thylakoid membrane proteins are often synthesized with bipartite presequences consisting of a stroma targeting signal and a signal that directs that protein to the thylakoid membrane. The stroma targeting signal is cleaved by SPP located in the stroma and the thylakoid signal is cleaved by the thylakoid processing peptidase (TPP) in the thylakoid membrane.

CLASSIFICATION OF MEMBRANE PROTEINS

Hundreds of membrane proteins with diverse orientations reside within the inner membrane of eubacteria, mitochondria, and the chloroplast thylakoid membrane. Some of these proteins perform the complex chemistry of oxidative phosphorylation in bacteria and mitochondria, or photosynthesis in the photosynthetic bacteria and plant chloroplast, as well as transport processes and signaling functions. Membrane proteins are classified according to their topology and whether they contain a cleavable presequence (von Heijne 1992). Single-spanning bitopic membrane proteins have their N termini facing the outside (Type I and III; away from the matrix, the bacterial cytoplasm and the stroma, respectively) or the inside surface (Type II) of the membrane. Type I proteins can possess a cleavable presequence that supports the insertion process, whereas type II and III proteins do not. Multispanning membrane proteins are summarized as Type IV proteins. Polytopic membrane proteins in bacteria lack presequences and insert directly from the cytoplasm. For the inner membrane of mitochondria and thylakoid membrane only a few multispanning proteins have been characterized so far in terms of their topology.

TOPOGENIC SEQUENCES

All membrane proteins contain sequence determinants called topogenic sequences that specify for their insertion into the membrane. These topogenic sequences contain a hydrophobic region surrounded by hydrophilic amino acids. There are at least four different topogenic sequences known within membrane proteins: leader sequence, uncleaved signal sequence, stop-transfer sequence, and reverse signal sequence. There are also sequences functioning as an insertion domain. Leader sequences initiate translocation of the C-terminal hydrophilic regions of proteins and are cleaved during translocation. In bacteria, only a few inner

membrane proteins are known to possess a cleavable leader sequence. Examples include the coat proteins of the M13 bacteriophage, which are made in precursor forms. Uncleaved signal sequences, like cleavable leader sequences, initiate C-terminal translocation of the polypeptide chain but remain as membrane anchor regions. A well-characterized protein with such a topogenic sequence is the *Escherichia coli* leader peptidase (Dalbey & Wickner 1987, Dalbey et al 1987). Both cleaved and uncleaved signal sequences are oriented within the membrane with their N termini residing in the cytoplasm and their C termini at the *trans* side of the membrane. In contrast, stop-transfer domains are oriented within the membrane with the opposite orientation (N_{trans}, C_{cyto}) and function to hold the translocating polypeptide chain in the membrane. Reverse signals initiate translocation of the polypeptide chain in the N-terminal direction and remain as transmembrane anchors (Kuhn et al 1990a). Insertion domains contain two hydrophobic segments separated by a polar region and may insert spontaneously across the lipid bilayer.

In mitochondria and chloroplasts, the nuclear-encoded proteins destined to the inner membrane or thylakoid membrane, respectively, often contain bipartite presequences. The first part of these presequences target the protein to the lumen of the organelle, whereas the second part mediates membrane integration.

How might topogenic sequences operate? Over two decades ago Blobel (1980), in his signal hypothesis, proposed that membrane proteins contain signal and stop-transfer sequences that initiate and stop translocation of a polypeptide chain, respectively. Signal and stop-transfer sequences act independently and in an N-terminal to C-terminal order. According to this hypothesis, a translocation machinery catalyzes the transfer of hydrophilic domains of membrane proteins across the lipid bilayer and allows the hydrophobic regions of the membrane proteins to integrate into the lipid bilayer. The translocation of a number of membrane proteins has been shown to confirm the predictions of the signal hypothesis.

In contrast to what was predicted by the signal hypothesis, some proteins can be inserted by a loop mechanism. Such proteins often contain an insertion domain containing two hydrophobic regions separated by a small intervening hydrophilic region (Engelman & Steitz 1981, Kuhn 1987). By this mechanism, both hydrophobic domains act in concert to initiate translocation of the central polar domain. No protein channel is needed to facilitate translocation by this loop mechanism, as the protein is believed to cross by interaction with the membrane lipids. This mechanism may be used by some multispanning membrane proteins for translocating small domains to the *trans* side.

TRANSLOCATION OF PROTEINS ACROSS MEMBRANES

In mitochondria and chloroplasts, nuclear-encoded proteins must translocate across the two membranes that form their boundaries. Translocation into the matrix of mitochondria and the stroma of chloroplasts is mediated by translocases and is ATP driven. Membrane translocation most likely occurs by a molecular motor

or molecular ratchet mechanism (Schatz & Dobberstein 1996). In contrast to the SecA-driven bacterial system where proteins are pushed across the membrane (Wickner 1994), chloroplast and mitochondrial imported proteins are either pulled across the membrane by the motor or translocated by Brownian motion. The imported proteins are then physically trapped on the *trans* side of the membrane by binding to chaperones. The trapping prevents the polypeptide chain from traveling back across the membrane.

The Sec translocase facilitates the transfer of most proteins across the membrane in bacteria (for review see Duong et al 1997c, Wickner & Leonard 1996). The translocase is composed of six polypeptides: SecA (Oliver & Beckwith 1981), SecY (Ito et al 1983), SecE (Riggs et al 1988), SecG (Douville et al 1994), SecD and SecF (Gardel et al 1987, 1990), and YajC (Duong & Wickner 1997a). SecB delivers most exported proteins to the translocase (Kumamoto & Beckwith 1983) by interacting with the membrane-bound receptor SecA (Hartl et al 1990), which is part of the translocase. SecA is an ATPase (Lill et al 1989, 1990) found both in the cytoplasm or associated to the membrane (Cabelli et al 1991, Oliver & Beckwith 1982). SecA drives translocation of hydrophilic regions across the membrane (Economou 1998) by undergoing cycles of membrane insertion and deinsertion, which occurs upon ATP binding and hydrolysis, respectively. This SecA cycle is coupled to preprotein binding and release, which promotes the stepwise translocation of a preprotein across the membrane (Economou & Wickner 1994, Economou et al 1995). The proton motive force stimulates protein translocation by promoting membrane deinsertion of SecA (Nishiyama et al 1999). The integral membrane component SecYE is believed to function as a protein channel through which the exported proteins move (Hartl et al 1990). The membrane proteins SecG and YajC, as well as SecD and SecF, which have large periplasmic domains, improve the efficiency of translocation by regulating insertion-deinsertion cycles (Nishiyama et al 1996, Duong & Wickner 1997b, Matsumoto et al 1998, Suzuki et al 1998). How SecG, YajC, SecD, and SecF enhance the SecA cycle is not known.

INSERTION OF BACTERIAL MEMBRANE PROTEINS

Research on how proteins insert into the bacterial membrane began in earnest in the late 1970s and early 1980s. The research was confined primarily to two proteins, the coliphage f1 gene III protein and the M13 major coat protein. These proteins are both synthesized with a cleavable signal peptide. Each spans the membrane once with its N terminus located in the periplasm and the C terminus located in the cytoplasm after cleavage by leader peptidase has occurred.

As predicted by the signal hypothesis, Model and coworkers (Boeke & Model 1982) reported that the f1 gene III protein contains two topogenic sequences: signal and stop-transfer. They showed that the hydrophobic region in the mature part acts as a stop-transfer domain (Davis & Model 1985). This domain plays no role in the translocation of the large hydrophilic domain but functions to stop the translocation

of the polypeptide chain. The expectation was that a proteinaceous machinery would facilitate the translocation of the large hydrophilic domain. However, the signal/stop-transfer concept did not apply to all proteins. Wickner and coworkers (Kuhn et al 1986b) showed that the membrane anchor region in the coat region was just as important for insertion as was the signal peptide. They suggested that the M13 procoat protein contains an insertion domain with two hydrophobic domains that pair up and then insert directly into the membrane, independent of *secA* and *secY* (Kuhn et al 1987).

The first membrane protein shown to require the Sec machinery was leader peptidase, which spans the membrane twice. It has a 28-kDa periplasmic domain that requires both *secA* and *secY* (Wolfe et al 1985) in addition to the membrane electrochemical potential (Wolfe & Wickner 1984) for export. Other membrane proteins that require the SecYE translocase are the polytopic membrane proteins Mal F (Traxler & Murphy 1996), Tsr (Gebert et al 1988), SecY (Werner et al 1992), and MtlA (Beck et al 2000).

It became apparent in early studies that no single mechanism explains the insertion of proteins into the bacterial inner membrane. Today we know that there are at least two widely used pathways for insertion (Figure 1, see color insert). One involves the signal recognition particle (SRP) for targeting the protein to the membrane; the Sec-translocase is required for the translocation of the hydrophilic domains of the membrane protein across the membrane. The other pathway appears to be a Sec-independent mechanism, which in some cases, may not require any protein component for membrane insertion.

SRP-Sec Pathways

The role of SRP in the membrane insertion of bacterial proteins was determined rather late after seminal studies were done in the eukaryotic systems where it was shown that SRP is required for translocation of exported proteins across the mammalian ER membrane. In mammalian cells, SRP is a ribonucleoprotein with six distinct protein subunits and a 7S RNA (Walter & Blobel 1980, 1982). SRP binds the signal peptide of the nascent polypeptide chain as it emerges from the large subunit of the ribosome. Synthesis of the polypeptide is then halted by this binding and the nascent chain-ribosome complex is targeted to the membrane by the interaction of SRP with the membrane bound SRP receptor. Soon after binding to the membrane, SRP dissociates from the SRP receptor, protein synthesis resumes, and the polypeptide chain translocates across the membrane through the Sec61 channel.

In 1989 and 1990, *E. coli* was discovered to have a homologue of the SRP 54 subunit, namely the 48-kDa protein Ffh. In addition, a 4.5S RNA (Bernstein et al 1989, Poritz et al 1990) and the SRP receptor-like protein FtsY were found (Romisch et al 1989). The *E. coli ffh* (Phillips & Silhavy 1992) and *ftsY* (Luirink et al 1994) genes were discovered to be necessary for cell viability (Phillips & Silhavy 1992). Although the exact role of SRP and FtsY in *E. coli* was initially

controversial (Bassford et al 1991), they now appear to play an important role in the targeting of membrane proteins to the cytoplasmic membrane. Crosslinking studies with disuccinimidyl-suberate showed that Ffh is associated with nascent membrane proteins such as FtsQ and leader peptidase (de Gier et al 1996, Valent et al 1998). As expected for a targeting role, depletion of either Ffh or 4.5S RNA had an adverse effect on the membrane insertion of leader peptidase (de Gier et al 1996). Inhibition of the SRP pathway using a synthetic lethality approach blocked the membrane insertion of several polytopic membrane proteins (Ulbrandt et al 1997), but some integral membrane proteins, such as MalF or Tsr, were not affected. Moreover, depletion of FtsY in the cell leads to a marked effect on the insertion of Lac permease and SecY (Seluanov & Bibi 1997). In addition, functional integration of Lac permease (MacFarlane & Mueller 1995, Jensen & Pedersen 1994) was reduced when the 4.5S RNA synthesis was inhibited.

The SRP membrane protein targeting system and the SecB protein targeting system are thought to converge at the translocase (Valent et al 1998). Mannitol permease (MtlA) and the SecY protein, which are targeted by the SRP pathway, require the Sec translocase. Their membrane insertion is inhibited when the SecY translocase components are depleted in vitro. Surprisingly, the translocation of SecY itself is independent of SecA (Koch et al 1999), whereas the translocation of the C-terminal domain of leader peptidase requires SecA and SecY (Wolfe et al 1985). However, not all SRP-targeted proteins require the translocase: A truncated ProW molecule consisting of only the N-terminal and the first transmembrane segment did not require SecE but did need the SRP components for targeting (Cristobal et al 1999). Presumably, alternative translocase complexes exist that support translocation of large N-terminal hydrophilic domains.

For membrane proteins, an important question is when does the hydrophobic signal sequence or membrane anchor region leave the translocase and integrate into the bilayer. If a stop-transfer sequence is present downstream from the translocated domain of a membrane protein, translocation must halt, and the protein must be released laterally from the translocase complex to integrate into the lipid bilayer. How does this occur? Recently, Wickner and colleagues showed that translocation arrest by a stop-transfer domain is controlled by the SecYEG translocase. The lateral release and membrane integration of the exported protein OmpA with a synthetic stop-transfer sequence was directed by the hydrophobicity of the segment (Duong & Wickner 1998). Such studies should also be done with bona fide membrane proteins.

Direct Membrane Insertion Pathway

The paradigm for the Sec-independent insertion pathway is the M13 coat protein; it is synthesized in a precursor form, termed procoat, with a cleavable signal sequence. Studies show that its membrane biogenesis can be dissected into four steps: targeting, hydrophobic partitioning, translocation of the polar region, and leader peptidase cleavage. (*a*) Targeting the protein to the membrane requires

the basic residues in the N- and C-terminal regions (Gallusser & Kuhn 1990). This requirement most likely reflects the electrostatic binding of procoat to the acidic phospholipid head groups. (*b*) The initial partitioning of procoat into the lipid bilayer is driven by hydrophobic forces (Soekarjo et al 1996). In this step, the hydrophobicity of the signal sequence and the membrane anchor sequence is critical (Kuhn et al 1986a). Both hydrophobic regions synergistically contribute to drive the protein into the membrane. (*c*). The translocation of the acidic loop across the membrane is stimulated by the proton motive force. The transmembrane electrical component is most likely important here, with the positively charged periplasmic side of the membrane electrophoretically pulling the acidic protein region across the membrane (Kuhn et al 1990b, Cao et al 1995). In the last step, (*d*) leader peptidase cleaves procoat thus generating the mature coat protein and the leader peptide (Shen et al 1991). M13 procoat was thought to insert by a spontaneous mechanism because it does not require SecA or SecY (Wolfe et al 1985) and because it inserts in vitro into protein-free liposomes (Geller & Wickner 1985, Soekarjo et al 1996).

In this pathway, membrane insertion is driven by two forces: hydrophobicity and the proton motive force (pmf). Hydrophobic force (Kuhn et al 1986a, Cao et al 1995) seems to play the crucial role in membrane translocation. The proton motive force plays a secondary role and only stimulates the translocation. It is required for translocation of negatively charged residues across the membrane when the hydrophobicity of the leader sequence or the membrane anchor region is decreased by deletions (TA Schuenemann et al 1999). The proton motive force only promotes translocation when the region to be translocated contains acidic residues (Kuhn et al 1990b, Cao et al 1995). The primary role of the hydrophobic force for membrane insertion was also shown with the purified procoat protein added to preformed liposomes containing diphenylhexatriene-modified phospholipids. The protein insertion showed a standard free energy gain of ΔG^0 of -10.4 kcal/mol, underscoring the thermodynamically favored membrane insertion of the M13 procoat protein into lipid bilayers (Soekarjo et al 1996).

The major coat protein of the *Pseudomonas aeruginosa* phage Pf3 is similar to the M13 coat protein in that it also inserts into the bacterial membrane in a Sec-independent fashion. In contrast to the M13 coat protein, Pf3 coat is synthesized without a cleavable leader sequence. Basically, the two coat proteins share the same topology: The Pf3 coat protein has an 18-residue periplasmic domain at the N terminus, an 18-residue transmembrane segment, and an 8-residue C-terminal tail. The Pf3 coat absolutely requires the electrochemical membrane potential for insertion (Rohrer & Kuhn 1990). Its membrane translocation is not affected by the temperature-sensitive mutations in the SecY or SecA protein, nor by azide, which inactivates SecA (Oliver et al 1990). Notably, the translocation of the N-terminal tail into the periplasm requires at least one acidic residue in that region, which suggests that a negative charge plays an active role in translocation (Kiefer et al 1997). More recent studies show that it is primarily the transmembrane electrical component of the pmf that drives insertion. Interestingly, the

pmf is not only required to translocate the N terminus across the membrane but also to maintain this domain in the periplasm (Kiefer & Kuhn 1999). The Pf3 coat protein was efficiently translocated in vitro into trypsin-treated membrane vesicles where Sec-dependent proteins were blocked for translocation (Kiefer & Kuhn 1999). Therefore, Pf3 coat inserts directly into the lipid bilayer or requires a protein component that is protease resistant.

A Sec-independent mechanism is also proposed for the translocation of the N terminus of leader peptidase, even though the Sec-pathway is needed for translocation of the large C-terminal hydrophilic domain (Lee et al 1992). In contrast to the Pf3 coat protein, translocation of the N terminus of leader peptidase to the periplasm does not require the pmf (Lee et al 1992) unless the hydrophobicity of the first hydrophobic region is decreased (Delgado-Partin & Dalbey 1998).

How prevalent is this Sec-independent insertion mechanism? Many believe it is quite common. This is, however, a contentious point in the membrane field. A number of investigators believe that the Sec-independent mechanism is quite rare and applies only to viral coat proteins. These scientists believe that almost all membrane proteins require the SecYE protein conducting pore for translocation of hydrophilic domains of membrane proteins. Indeed, recent studies call into question whether Sec-independent insertion really means that insertion is truly not facilitated by the Sec machinery. Traxler & Murphy (1996) showed that small amounts of the Sec-components are sufficient for MalF insertion, indicating that the so-called Sec-independent MalF protein really is Sec-dependent. Likewise, the Sec-independent inverted Lep (von Heijne 1989) appears to insert in a Sec-dependent manner (de Gier et al 1998). In these latter studies, a conditionally lethal strain was used where SecE was efficiently depleted. In the previous studies temperature-sensitive *secA* or *secY* mutants were used.

Extending the periplasmic loop of the M13 procoat protein changes the membrane insertion from a Sec-independent to a Sec*A*- and Sec*Y*-dependent mechanism (Kuhn 1988). Mutants of leader peptidase behave similarly in their Sec dependence, which corresponds to the length of the hydrophilic portion that translocates across the membrane (Andersson & von Heijne 1993). Each translocated region may require different components of the translocase.

YidC—A New Component Involved in Membrane Protein Insertion

Another protein that might play an important role in the insertion of proteins into the inner membrane of *E. coli*, is the six-spanning membrane protein YidC. YidC is the bacterial homologue of Oxa-l in mitochondia, which participates in a novel pathway for insertion of polytopic membrane proteins into the mitochondrial inner membrane (Hell et al 1997). YidC was recently found to be associated with the Sec translocase complex and to interact with the transmembrane segment of the membrane protein FtsQ during its insertion into the membrane (Scotti et al 2000). When cells are depleted of YidC the membrane integration of the Sec-dependent

proteins such as leader peptidase and ProW is impaired (Samuelson et al 2000). Even the insertion of the Sec-independent M13 procoat protein, which was thought to insert into the membrane by a spontaneous mechanism, is strongly inhibited in the YidC-deficient *E. coli* cells. The preproteins that are exported into the periplasm or outer membrane are not affected under these conditions (Samuelson et al 2000). This suggests that YidC is a new translocation component specific for membrane proteins (Figure 1, see color insert). It may also function to catalyze the lateral exit of transmembrane segments from the Sec translocase into the lipid bilayer. This idea requires further investigation.

INSERTION OF THYLAKOID MEMBRANE PROTEINS

The protein import systems in the thylakoidal membranes in chloroplasts appear to be analogous to the phylogenetically ancient insertion systems of cyanobacteria. These systems could date back to the early eubacteria, which developed the specialized membranes of thylakoids. There are few comparative investigations between chloroplasts, cyanobacteria, and purple bacteria (Heins & Soll 1998, Drews 1996, Reumann et al 1999). Certainly, the study of these systems will elucidate the evolution of the thylakoid system.

Most of the nuclear-encoded thylakoidal proteins in plants are synthesized in a precursor form with an aminoterminal bipartite chloroplast presequence and are imported from the cytoplasm into the stroma. These proteins bind to receptor proteins at the outer envelope membrane of the chloroplast and then translocate across both the outer and inner envelope membrane [for recent reviews see Chen & Schnell (1999) and May & Soll (1999)]. The translocase complexes in the outer and inner envelope membrane of chloroplasts are referred to as TOC and TIC, respectively (Figure 2, see color insert). Toc160 and Toc34 are the receptor proteins that mediate binding to the preproteins (Hirsch et al 1994, Kouranov & Schnell 1997). Toc160 was originally identified as Toc86 since it was detected as a proteolytic fragment. The receptor function might be regulated by GTP binding of Toc86 and Toc34 (Kessler et al 1994). Toc75 is a protein-conducting channel through which the preprotein is translocated (Hinnah et al 1997). In the inner envelope, or associated with the intermembrane surface of the inner envelope, are three membrane proteins, Tic110, Tic 55, Tic22, and a peripheral protein, Tic20. The Tic proteins are associated with the TOC complex to form a TOC-TIC supercomplex (Nielsen et al 1997, Kouranov et al 1998). Tic22 is believed to function by recognizing the preprotein moving through TOC, based on crosslinking of preproteins trapped during translocation (Wu et al 1994). The exact function of Tic20 is not known, but it is proposed to be involved in protein conductance (Kouranov et al 1998). Tic55, co-immunoprecipitated with Tic110, contains a Rieske-type iron-sulfur center and a mononuclear iron site. Presently, it is not clear which protein makes up the protein-conducting channel of the inner envelope. Tic110 provides the docking site for the Hsp100 homologue ClpC in the

intermembrane space (Nielsen et al 1997), which facilitates translocation across the membrane. ATP is the only energy source required for the chloroplast import process (Theg et al 1989). Its hydrolysis by stromal chaperones might pull the preprotein into the chloroplast. Concomitantly, some proteins are folded by the GroEL homologue Cpn60 in the stroma (Kessler & Blobel 1996), which is also found close to TOC-TIC. After translocation, the presequence is removed by the stroma processing peptidase (SPP).

Cyanobacterial homologues of Toc75, Toc34, Tic20, Tic22, Tic55, and Hsp110 were identified in *Synechocystis* (Reumann et al 1999, Soll & Tien 1998). This corroborates the view that an early protein import machinery already existed in cyanobacteria and that both chloroplast envelope membranes have evolved from a cyanobacterial ancestor. The insertion pathways of membrane proteins into the thylakoid membrane could be quite similar to those used in the inner membrane of eubacteria. In the simplest pathway, proteins are believed to insert into the thylakoid membrane in the absence of both soluble factors and proteinaceous membrane components. This is similar to the direct insertion mechanism used by some bacterial membrane proteins. The other assisted pathways require SecA, SRP homologues, GTP hydrolysis, or ΔpH (Robinson & Mant 1997, Settles & Martienssen 1998, Dalbey & Robinson 1999).

In vitro studies with isolated thylakoid membranes are useful in deciphering membrane insertion pathways. In these studies, the radiolabeled preproteins are incubated with thylakoid membranes and with (or without) stromal fractions, either in the presence or absence of apyrase, which hydrolyzes ATP. The assisted pathway is blocked when the stromal fraction is not added or when it is added together with apyrase because the SRP/SecA pathways depend on nucleoside triphosphates. Moreover, the assisted pathways are blocked when the membrane-bound translocase (presumably a Sec-like protein channel) is inactivated by mild pretreatment of the thylakoids with proteases.

Direct Membrane Insertion Pathway

A growing number of proteins are found to insert into the thylakoid membrane from the stroma by a direct or "unassisted" pathway (Table 1). These include the single-spanning membrane proteins CF_oII, PsbX, and PsbW, as well as the polytopic membrane proteins Elip2 and PsbS (Michl et al 1994; Kim et al 1998, 1999). Strikingly, these proteins are able to insert into the thylakoid membrane in the absence of SRP, nucleotide triphosphates, SecA, a ΔpH, and a functional SecYE complex. Since these proteins also insert into thylakoid membranes that have been pretreated with protease, they might sponanteously translocate. A model was proposed by Robinson & Mant (1997) for the membrane insertion of the ATP synthase subunit CF_oII, and the PsbX and PsbW protein of the photosystem II. In this spontaneous model the membrane proteins insert by a similar pathway originally proposed for the M13 procoat protein. Remarkably, these thylakoidal proteins do not require a transmembrane potential for insertion. This might correlate

TABLE 1 Thylakoid membrane proteins

Direct pathway	
	PsbS
	PsbW
	PsbX
	PsbZ
	Elip2
	CFoI
	CFoII
SRP interacting proteins	
	CytF
	LHCP
	PsbA (D1)
Sec-dependent proteins	
	Cyt f
Tat-pathway	
	OE 16 kD
	OE 23 kD
	Psa N
	Psb T

to the fact that thylakoid proteins possess higher hydrophobic forces that drive membrane insertion. The membrane anchor regions of the thylakoidal proteins are also much longer than the one found in the M13 procoat protein. Because the M13 procoat protein requires YidC for membrane translocation (Samuelson et al 2000), these thylakoidal proteins might use the Albino3 protein, which is the YidC homologue in chloroplasts (Sundberg et al 1997).

The thylakoidal proteins CF_oII, PsbZ, and PsbW are synthesized in the cytoplasm with a bipartite presequence, containing an envelope transit signal and a thylakoid signal sequence. The latter is reminiscent of a bacterial N-terminal hydrophobic leader sequence. In addition, the mature regions of these proteins possess a hydrophobic anchor domain. After the proteins translocate across both envelope membranes, the stromal peptidase SPP removes the envelope transit signal peptide. Targeting and binding of the preproteins to the thylakoid membrane are believed to occur by electrostatic interactions with the positively charged N-terminal signal peptide region and the negatively charged membrane surface. The hydrophobic domains then insert into the membrane via a transmembrane loop. This loop structure promotes the translocation of a hydrophilic domain of 20 or fewer residues across the thylakoid membrane (Thompson et al 1998). Cleavage by the thylakoid processing peptidase TPP generates the mature protein in the transmembrane form.

Interestingly, CF_oI, a protein closely related to CF_oII, is encoded by the plastidal chromosome (Herrmann et al 1993). It is synthesized without a bipartite presequence and has a presequence of 17 residues (Bird et al 1985). Michl et al (1999) found that even this short presequence is not required for the translocation of the 8-residue long N-terminal tail of CF_oI. In contrast, the N-terminal tail of CF_oII, which has additional charged residues, requires a thylakoidal signal sequence. This is reminiscent of the Sec-independent insertion of Pf3 and M13 coat proteins that translocate without a bacterial leader sequence when only few charged residues are in the N-terminal region (Rohrer & Kuhn 1990).

SRP and Sec Pathways

Some thylakoid membrane proteins require the chloroplast homologue of the bacterial Ffh for targeting (Franklin & Hoffman 1993), as well as a FtsY homologue (Kogata et al 1999) and GTP hydrolysis (Hoffman & Franklin 1994). Despite the presence of these targeting homologues, no RNA component appears to be required for the chloroplast SRP. Rather, there is a novel SRP43 protein, an as yet not well characterized 43-kDa polypeptide in the stroma (D Schuenemann et al 1998).

The best studied chloroplast thylakoid membrane protein in this pathway is the light-harvesting chlorophyll a/b-binding protein (LHCP) of the photosystem II. LHCP is synthesized with only an envelope transit sequence, which is cleaved by SPP. The nucleotide GTP is sufficient for integration of LHCP into the thylakoid membrane, and nonhydrolyzable analogs of GTP are inhibitory (Hoffman & Franklin 1994). Recently, Hoffman and colleagues showed that in the stroma LHCP is bound to a complex containing cpSRP54 and SRP43 (D Schuenemann et al 1999). Both of these SRP proteins are necessary to form a transient complex with LHCP, and they are, along with additional soluble factors, sufficient for the post-translational targeting of LHCP to the thylakoid membrane.

SRP preferentially recognizes hydrophobic precursors such as integral membrane proteins. In photocrosslinking experiments, efficient crosslinking was observed for only those proteins that had hydrophobic signals (High et al 1997). The SRP efficiently crosslinked to the D1 thylakoidal protein, as well as to LHCP at its third transmembrane region (Nilsson et al 1999). Currently, the membrane translocation components of this pathway are not characterized. It is assumed that for some of these SRP-dependent membrane proteins, homologues of the SecYE translocase will be required. The SRP and SecA-dependent pathways converge at SecYE in bacteria (Valent et al 1998). Homologues of SecY and SecE were found in *Synechococcus* PCC7942, *Pisum sativum*, and *Arabidopsis thaliana* (Nakai et al 1992, Laidler et al 1995) and are components of a 180-kDa complex in *Arabidopsis* (D Schuenemann et al 1999). Interestingly, antibodies to SecY, which inhibit Sec pathway substrates, do not inhibit the insertion of LHCP into the thylakoid membrane (Mori et al 1999). This suggests that insertion, at least for the SRP-dependent LHCP, does not require the SecYE translocase.

The only integral membrane protein known to require SecA in thylakoids is cytochrome *f*. The addition of azide, which inhibits the SecA activity, slows the insertion of cytochrome *f* across the membrane (Mould et al 1997). Furthermore, the insertion of cytochrome *f* was inhibited in a $SecA^-$ mutant in maize (Voelker et al 1997). Cytochrome *f* might require SecA because it has a large lumenal domain preceding the C-terminal transmembrane segment. The peripheral 33-kDa membrane protein of the PSII oxygen-evolving complex in the thylakoidal lumen requires SecA and SecY for correct import (Haward et al 1997, D Schuenemann et al 1999).

Twin Arginine Pathway

The nuclear-encoded 16 and 23-kD proteins of the oxygen-evolving complex use the twin arginine or ΔpH pathway for import into the thylakoidal lumen (Creighton et al 1995, Chaddock et al 1995). Strikingly, they do not require stromal factors or ATP for translocation. Similarly, the subunits N and T of photosystem I and II, respectively, insert using this pathway. All these proteins are synthesized with a bipartite presequence, which is processed by SPP first. The thylakoid signal sequence has two arginine residues between the N and H region, similar to some eubacterial proteins. Functional investigations showed that the import of these proteins is sensitive to nigericin and that the twin arginines in the signal sequence prevent the interaction with the components of the Sec-dependent pathway (Chaddock et al 1995).

In *E. coli*, periplasmic redox proteins were found that also contain a twin-arginine motif in their leader sequence (Berks et al 2000). The *tat* locus in *E. coli*, containing four different genes, is required for twin-arginine protein translocation (Sargent et al 1998); one of which showed homology to a maize gene involved in the ΔpH-dependent pathway (Settles et al 1997). Other homologues of this gene exist in *Archaeoglobus fulgidus* and *Pseudomonas stutzeri* (Sargent et al 1998, Glockner & Zumft 1996). The ΔpH pathway probably developed specifically for the translocation of cofactor-binding proteins early in evolution. The mechanism by which proteins are translocated by this pathway is unknown. It appears that tightly folded proteins are transported by the central component TatC (Bogsch et al 1998) and that the bacterial and thylakoidal systems are interchangeable (Wexler et al 1998).

INSERTION OF MITOCHONDRIAL INNER MEMBRANE PROTEINS

Most inner membrane proteins of mitochondria are nuclear encoded and synthesized in the cytoplasm. In yeast, for example, only eight proteins are encoded by the mitochondrial DNA and synthesized in the mitochondrial matrix. The majority of proteins directed from the cytosol to the mitochondrial inner membrane are

made with bipartite presequences. These bipartite signals include an N-terminal presequence followed by an intra-mitochondrial sorting sequence. However, some mitochondrial inner membrane proteins are made with only an N-terminal presequence or contain an internal targeting sequence that is not removed during export (Stuart & Neupert 1996).

Current evidence demonstrates that more than one mechanism accounts for the integration of inner mitochondrial membrane proteins. Of the varieties of mechanisms, the stop-transfer and conservative mechanisms are the most prevalent. Before the mechanisms are discussed in detail, we describe the protein import pathway from the cytoplasm into the matrix because some nuclear-encoded proteins must first cross both the outer and inner mitochondrial membrane. Protein import into the matrix involves five steps (Figure 3, see color insert): targeting to the mitochondria, translocation across the outer membrane, translocation across the inner membrane, removal of the matrix signal peptide, and folding in the matrix (Hartl & Neupert 1990). In the cytoplasm, the mitochondrial-stimulating factor (MSF) (Hachiya et al 1993) and Hsp70 (Endo et al 1996) promote export of a number of mitochondrial proteins by binding to mitochondrial exported proteins. MSF binds selectively to certain preproteins, whereas Hsp70 binds to a wider range of preproteins. Translocase outer membrane (TOM) and translocase inner membrane (TIM) complexes mediate translocation across the outer and inner membrane. Proteins are targeted in an ATP-dependent manner (Pfanner et al 1997, Hachiya et al 1993) to the outer membrane by interacting with membrane-bound receptors. The receptor Tom20-Tom22 binds to preproteins containing N-terminal presequences (Schneider et al 1991, Kiebler et al 1993, Brix et al 1997), whereas Tom70 preferentially binds preproteins containing internal targeting signals (Schlossmann et al 1994, Brix et al 1997). After targeting, the preproteins interact with the TOM-TIM complex.

Translocation across the outer membrane occurs through the Tom40 channel (Pfanner et al 1997). Electron micrographs of purified and reconstituted Tom40 show an internal channel of about 2 nm (Kunkele et al 1998) through which proteins can move. Tom40 forms a stable complex with Tom22. Translocation into the matrix requires additional machinery within the inner membrane of mitochondria called TIM. TIM and TOM likely form a supercomplex during preprotein import into the matrix (Schulke et al 1999). To date, two different TIM complexes, TIM22 and TIM23, have been characterized that are used by preproteins recognized by Tom20 and Tom70, respectively. Preproteins with an N-terminal presequence are translocated by the TIM23 complex that contains Tim17, Tim23, and Tim44 (Berthold et al 1995, Blom et al 1995). Proteins with internal topogenic sequences use the TIM22 complex made up of Tim22 and Tim54 and the associated proteins Tim9, 10, and 12 (Koehler et al 1998, Adam et al 1999). Tim23 and Tim17 are most likely components of a preprotein-conducting channel because a direct contact with a preprotein was detected (Ryan & Jensen 1993, Kubrich et al 1994). Initial translocation of the presequence through TIM23 could be driven by an electrophoretic mechanism because the translocation depends strictly on

$\Delta\psi$ (Martin et al 1991). Tim44 catalyzes the vectorial movement of preproteins across the TIM23 complex. This occurs in conjunction with mitochondrial Hsp70 (Kronidou et al 1994, Rassow et al 1994, Schneider et al 1994). The interaction of Tim44 with mt-Hsp70 in the matrix occurs in cycles that depend on ATP hydrolysis. Mge1 is also involved in import and folding of proteins in the matrix (Bolliger et al 1994, Laloraya et al 1994). The bacterial homologue of Mge1 is GrpE, which is a nucleotide exchange factor that promotes the release of nucleotides from the chaperone DnaK (Schneider et al 1996). Therefore, Mge1 most likely promotes import by interacting with mt-Hsp70.

After translocation across the inner membrane, the matrix-processing peptidase (MPP) removes the targeting sequence (Kalousek et al 1993). The final step involves folding of the imported protein in the matrix. This requires additional chaperones such as Mdj1 (Westermann et al 1996), Hsp60 (Cheng et al 1989, Ostermann et al 1989), and Hsp10 (Hohfeld & Hartl 1994).

Stop-Transfer Insertion

The best example of an inner membrane protein that uses a stop-transfer mechanism is the single-spanning cytochrome *c* oxidase subunit Va (CoxVa; Figure 3, see color insert; see Table 2). Prior to assembling into the inner membrane, CoxVa is synthesized in a precursor form with a matrix targeting signal and is imported across the outer membrane via the TOM complex (Gartner et al 1995). A strong dependence on the matrix Hsp70 for assembly into the inner membrane was found (Gartner et al 1995), suggesting that the mtHsp70 catalyzes the translocation of

TABLE 2 Mitochondrial inner membrane proteins

Stop-transfer (TIM2)	
	Cox Va
	LD
TIM22 pathway	
	AAC
	PiC
	DiC
	OgC
Conservative pathway	
	ATP synthase subunit 9
	CoxII
	Oxa1
Mitochondrial-encoded proteins	
	Cox II (mt)
	ATPase Su9
	Cox III

the short N-terminal region into the matrix (Cyr et al 1994). The transmembrane segment is involved in halting translocation, most likely within Tim17-23. Consistent with the transmembrane segment exerting a stop-transfer activity, a deletion of this hydrophobic region leads to mislocalization of the protein to the matrix (Glaser et al 1990).

Recently, the properties of a transmembrane segment that are required for stop-transfer activity were investigated with D-lactate dehydrogenase (LD) (Rojo et al 1998). This protein, which is synthesized with a cleavable matrix signal, is located in the inner membrane of mitochondria. It spans the membrane with an N_{matrix} and C_{ims} orientation, with most of the protein exposed to the intermembrane space region. Import into the mitochondria requires a transmembrane electrochemical potential. The stop-transfer activity of the inner membrane signal depends on both the cluster of charges on the C-terminal side of the transmembrane segment and on the hydrophobicity (Rojo et al 1998). Deletion of the hydrophobic transmembrane segment within a DHFR-LD fusion protein led to the import of the DHFR moiety into the matrix. Increasing the hydrophobicity of the transmembrane region by 8-leucine residues led to the assembly of the protein into the outer membrane of mitochondria. The mechanism by which a topogenic sequence acts as a stop-transfer is not known. One possibility is that the cluster of charged residues may slow translocation and allow inner membrane proteins to leave TIM23 laterally into the lipid bilayer. Another possibility is that the charge cluster is recognized by a protein component that disengages from the translocase and allows the protein to integrate into the lipid bilayer.

TIM22 Pathway

A number of membrane proteins travel across the mitochondrial intermembrane space in order to insert into the mitochondrial inner membrane (Koehler et al 1999, Tokatlidis & Schatz 1999). These membrane proteins include the multispanning mitochondrial inner membrane proteins, such as the ADP/ATP carrier (AAC), the phosphate carrier (PiC), the dicarboxylate carrier (DiC), and the 2-oxoglutarate carrier (OgC; see Table 2). The members of the mitochondrial carrier protein family have six membrane spans and about 50-residue-long hydrophilic regions within the matrix. The well-studied AAC protein binds to the Tom70 receptor first and then translocates across the outer membrane (see Figure 3). AAC then binds to a soluble intermembrane 70-kDa complex composed of three small proteins, Tim9, 10, and 12, prior to interacting with Tim22 (Koehler et al 1998). Tim22 mediates the membrane potential–dependent insertion of the AAC protein into the inner membrane. If the membrane potential is dissipated, the AAC protein only partially translocates across the outer membrane and does not insert into the inner membrane. For the AAC protein it was suggested that the intermembrane intermediate protein is inserted as three modules, each having two hydrophobic regions and a connecting loop region (Endres et al 1999). Whereas the first two loops translocate across the outer membrane, only the third loop shows a

$\Delta\psi$-dependent response to direct the insertion into the inner membrane. Another protein that uses the TIM22 pathway is Tim23. However, it binds to a different soluble complex in the intermembrane space made up of Tim8, 9, and 13. The soluble complex escorts Tim23 to the TIM22 complex (Leuenberger et al 1999) where it then inserts into the membrane bilayer in a potential-dependent manner.

Conservative Mechanism

Many membrane proteins are routed to the inner membrane by the so-called conservative mechanism, including subunit 9 of F_o-ATPase (Su9) (Figure 3) (Rojo et al 1995), subunit II of cytochrome oxidase (CoxII) (Hermann et al 1995), the Rieske FeS protein (van Loon & Schatz 1987), and the Oxa1 protein (Herrmann et al 1997). In this pathway, proteins are first imported into the matrix, processed, and then inserted into the inner membrane. The transmembrane region(s) acts as a membrane insertion sequence (directed from matrix), but not as a stop-transfer sequence. It is called the conservative mechanism because assembly from the matrix into the inner mitochondrial membrane is reminiscent of bacterial membrane insertion from the cytoplasm. Hence, this mitochondrial pathway may have evolved from a bacterial pathway.

Su9, which spans the membrane twice with both its N and C termini located in the intermembrane space, typifies a protein that inserts by the conservative pathway (Figure 3). It is synthesized in a precursor form with a bipartite signal sequence and is cleaved twice during assembly into the membrane. Using dihydrofolate reductase fusions, Rojo et al (1995) showed that the first transmembrane segment of Su9 is completely translocated into the matrix. After the removal of the matrix targeting signal by MPP, the first transmembrane segment initiates the membrane insertion and the export of the N-terminal region. Export of Su9 out of the matrix into the inner membrane requires a pH gradient and ATP hydrolysis.

The precise mechanism by which inner membrane proteins are inserted from the matrix is unknown. Possibly, there is a novel translocase that promotes membrane potential-dependent insertion, one of the components being the Oxa1 protein. Recent studies (Hell et al 1998) using a temperature-sensitive yeast mutant defective in *Oxa1* showed that Su9, Oxa1, and CoxII accumulate in the matrix at the nonpermissive temperature. Moreover, Oxa1 was shown to interact directly with the importing pCoxII polypeptide chains synthesized in vitro. The translocation of the subunit II of cytochrome oxidase (CoxII) from the matrix into the intermembrane space requires the electrical component of the membrane potential (Herrmann et al 1995). A membrane potential is also required for insertion of the nuclear encoded Oxa1 of *Saccharomyces cerevisiae*, which spans the membrane five times with the N terminus located in the intermembrane space. Oxa1 is made in a precursor form with a matrix targeting peptide. After cleavage of the imported Oxa1 by the MPP, the membrane potential promotes insertion of the first transmembrane segment and translocation of the negatively charged 90-residue tail. Subsequently,

the other transmembrane segments insert into the membrane to generate the final topology in the inner membrane (Herrmann et al 1997).

One of the issues in the mitochondrial field not studied extensively is how proteins that are encoded by the mitochondrial genome assemble into the membrane. One possibility is that a direct mechanism is used, similar to the bacterial coat proteins (Clarkson & Poyton 1989). For more complex proteins, it was first believed that they insert into the membrane from the matrix via a mechanism like that of the Sec-dependent proteins in bacteria. This is unlikely because no SecA or SecY homologues have been discovered in mitochondria. However, an intriguing possibility suggests that that an additional translocase is involved that includes the Oxa1 protein (Hell et al 1997). The homologue of Oxa1 in *E. coli* plays an important role in membrane protein insertion in bacteria (Samuelson et al 2000). In addition, the homologue of Oxa1 in *Arabidopsis thaliana* was capable of complementing a yeast mutant in *Oxa1*, which suggests the existence of an evolutionarily conserved protein translocation pathway (Hamel et al 1997).

MEMBRANE PROTEIN ORIENTATION

Since 1986 it has been clear that there is a correlation in bacteria between positively charged amino acids flanking transmembrane segments and the membrane topology (von Heijne 1986, Dalbey 1990). By statistical analysis it was found that basic amino acids were four times more likely to be within cytoplasmic loops than periplasmic loops, but less correlation was found with acidic residues. Later, a charge bias was also found for membrane proteins in the endoplasmic reticulum, chloroplasts, and mitochondria, suggesting that the positive-inside rule is quite general in nature (von Heijne 1992, Wallin & von Heijne 1998).

The idea that positively charged residues were determinants of the membrane protein topology was supported by site-directed mutagenesis studies (Laws & Dalbey 1989, von Heijne 1989, Nilsson & von Heijne 1990) and PhoA fusion studies (Boyd & Beckwith 1989). The topology of leader peptidase could be inverted by altering the basic residues flanking a transmembrane segment (von Heijne 1989). Whereas arginines have slightly higher determinant power than lysines, no effect was seen with histidine residues (Andersson et al 1992).

What is the physical basis of the positive-inside rule? Examination of membrane properties raises several possibilities. The surface charge of a typical membrane is negative due to the phospholipid headgroups. The basic residues on membrane proteins may bind electrostatically to the acidic phospholipid head groups and thereby be retained in the cytoplasm (Gallusser & Kuhn 1990). Likewise, the topology of leader peptidase was affected by manipulating the amount of acidic phospholipids within *E. coli* (van Klompenburg et al 1997). Another possible determinant is the pmf wherein the periplasmic face of the membrane is positively charged and the cytosolic face of the membrane is negatively charged. Evidence for this comes from studies in which a topology reversal of leader peptidase occurred

upon dissipation of the transmembrane potential (Andersson & von Heijne 1994). Although the effects of the pmf on the translocation of positively charged residues were not shown directly in this study, recent work definitively shows that the pmf hinders translocation of basic residues and is thereby a determinant of the positive-inside rule of membrane proteins (TA Schuenemann et al 1999). A third possible way positively charged residues could be retained in the cytoplasm and act as topological determinants may be the transmembrane dipole potential. This dipole potential (positive inside the bilayer) is such that positively charged residues would be impeded from entering the membrane (Flewelling & Hubbell 1986).

An interesting investigation was recently reported using membrane proteins of *Sulfolobus acidocaldarius*. This archaeal organism lives in a highly acidic environment (pH 0.5–2.5) and has a positive-inside electrical potential that maintains an internal neutral pH and a $\Delta\psi$ of -200 eV (Moll & Schafer 1988). Analysis of SecY and subunit 1 of cytochrome *c* oxidase showed that the charge bias of the hydrophilic loops of these proteins is also positive inside (van de Vossenberg et al 1998). This implies that the positive-inside rule is not related to the membrane potential and rather that the electrostatic interactions may be the functional basis.

The positive-inside rule also applies to thylakoid membrane proteins and to mitochondrial-encoded inner membrane proteins (Gavel et al 1991, Gavel & von Heijne 1992). In these proteins, the positively charged residues in the hydrophilic loops are located in the chloroplast stroma and mitochondrial matrix. Interestingly, most of the inner membrane proteins that are nuclear encoded do not show this positive-inside bias. Rather, they show a bias for glutamic acid residues in the hydrophilic loops that face the mitochondrial inner membrane space. Recently, Rojo et al (1999) studied a derivative of preSu9 of *Neurospora crassa* and showed that this protein adheres to a negative-outside paradigm. The model protein preSu9-DHFR spans the inner membrane once with its N-terminal tail in the intermembrane space and the DHFR region in the matrix. The N-terminal tail located in the intermembrane space has a net negative charge with two glutamic acid residues and one lysine residue, whereas the C-terminal domain bordering the transmembrane segment is positively charged. Rojo et al (1999) showed that the N-terminal tail must contain a net negative charge for export. Reversing the net charge flanking the hydrophobic segment resulted in an inverted protein.

The orientation of the single-spanning Pf3 coat protein is also determined by negatively charged residues in the periplasmic region (Kiefer et al 1997). The Pf3 coat protein has two negatively charged residues in the periplasmic tail and two basic residues flanking the single transmembrane segment on the cytoplasmic side. All positively charged residues can be removed from the Pf3 coat protein, and the protein still inserts into the membrane with the same defined topology as long as one negatively charged residue is present in the periplasmic domain and an electrical potential across the membrane exists (Kiefer & Kuhn 1999). This finding is consistent with the idea that the electrical gradient $\Delta\psi$ across the inner membrane acts directly on the negatively charged residue within the protein.

ASSEMBLY OF MEMBRANE PROTEINS

Once polytopic membrane proteins insert into the membrane, the next step in the folding pathway is the formation of the transmembrane domain structure by the interaction of several transmembrane helical regions. Many membrane proteins are organized as complexes consisting of a number of subunits. Each subunit must recognize the partner protein(s) and interact. Recent research has shed some light on these interesting processes. Another related problem for future research is the ordered disassembly of membrane complexes. Mechanisms must exist to replace malfunctioning subunits in an assembled complex.

Membrane Anchoring and Folding of Transmembrane Helices

Membrane-spanning regions within integral membrane proteins function to hold the protein in the lipid bilayer. Typically, these regions consist of 20 or more non-polar amino acids (Kuroiwa et al 1991). Sixteen non-polar residues are sufficient for anchoring a protein. However, it can be as short as 11 residues if the region has a high degree of overall hydrophobicity (Davis & Model 1985, Davis et al 1985, Chen & Kendall 1995). For some proteins, flanking polar residues are involved in the anchoring (Kuroiwa et al 1991, Falcone et al 1999). In membrane anchoring, hydrophobicity is an important property. The hydrophobicity is measured as the sum of partition coeffients of the individual amino acyl residues within a hydrophobic phase and can be calculated as the Gibbs free energy G^o (Engelman et al 1986). In addition, the free energy of each peptide bond in the membrane must be considered in the context of H-bonding interactions (White & Wimley 1998). The conformational state of the protein is, therefore, important in thermodynamic considerations. If the conformation of a transmembrane protein is α-helical, the cost of free energy is much lower than if it is in an unordered state.

The structure of integral membrane proteins are of two basic types. One is the α-helical transmembrane segment type and the other is the β-sheet type. The β sheets forming barrel structures are predominantly found in porins, which are located in the outer membrane of bacteria, mitochondria, and chloroplasts and are not discussed here. All organellar and bacterial inner membrane proteins known so far are of the α-helical type. The size of the α-helical transmembrane segments are inferred from structures of membrane proteins crystallized in detergents and are an average of 26.4 residues (Bowie 1997). The transbilayer distance of a membrane is about 6 nm, which includes a hydrocarbon core of 3 nm and a interface region of 1.5 nm including the phospholipid head groups (Gally et al 1979, 1980). Significantly, an α-helix of about 20 residues matches with the thickness of the hydrocarbon core of a membrane. In the interface region of the membrane, certain amino acid residues of membrane proteins are preferentially positioned in proteins. Phenylalanine and tryptophan are often found at the ends of membrane-spanning regions where they interact with the phospholipid head groups (Wallin et al 1997, Spruijt et al 1996).

Packing of Transmembrane Helices

How do transmembrane regions interact? This question was investigated for glycophorin A (Lemmon et al 1992), the M13 phage coat protein (Haigh & Webster 1998), and the transmembrane region of phospholamban (Arkin et al 1997), a Ca^{2+} ATPase in the cardiac sarcoplasmic reticulum. We concentrate on what is known about the transmembrane interactions for glycophorin, a protein that spans the membrane with a stretch of 23-amino acid residues. The single transmembrane segment of glycophorin can form stable dimers even in SDS micelles (Lemmon et al 1992). Mutagenesis experiments (Fleming et al 1997) identified the critical residues for dimerization, which were along one face of the transmembrane α-helix. The dimerization domain motif is L*xx*GV*xx*GV*xx*T, with *x*s standing for amino acids that can be exchanged with little effect on dimerization, whereas the other amino acids play an important role in the dimerization process. Using this information, computational methods were used to build a three-dimensional model of the right-hand dimer where the helix faces, which are critical for packing, are formed by the residues that are very sensitive to mutagenesis. The NMR structure of a glycophorin A dimer in dodecylphosphocholine (MacKenzie et al 1997) proved the model valid. In the case of phospholamban, which forms a pentamer via its transmembrane helical regions, the critical residues are L37, I40, I44, and I47. These results and others suggest that the intolerant residues cluster on one face of the helix when projected at 3.5 residues per turn. The M13 major coat protein also forms a dimer via the transmembrane helical regions prior to the assembly onto phage particles (Haigh & Webster 1998). This was shown by disulfide bond formation of cysteine residues placed along the helix.

In polytopic membrane proteins, many transmembrane helices interact and contribute to the specific three-dimensional arrangement. In fact, some helices can find their appropriate partners even if the membrane protein is made as fragments. This was nicely demonstrated with Lac permease, which spans the membrane 12 times. Ehring and coworkers (Wrubel et al 1990) showed that Lac permease still functions when it is synthesized as two split proteins. Bibi & Kaback (1990) made a split Lac permease with an N-terminal fragment spanning the membrane 6 times, and a C-terminal fragment spanning the membrane 6 times. The two fragments inserted into the membrane independently and, amazingly, found each other to form a functional permease. Different combinations of Lac permease fragments were combined and showed similar reconstitution capability (Wrubel et al 1994). Within Lac permease the packing of the helices was determined, despite the lack of a three-dimensional X-ray crystal structure, using site-directed spin labeling and crosslinking studies (Wu & Kaback 1996, Wolin & Kaback 1999). This protein is also a good example of a membrane protein with intramembrane salt bridges (Sahin-Toth et al 1992). Helices VII and X are connected by D240 and K319, and helices VII and XI are connected by D237 and K358. Similarly, precise contacts between helices VIII and X, and between IX and X, were shown by excimer fluorescence (Jung et al 1993). Helix-helix contacts

of bacteriorhodopsin were determined by electron diffraction studies (Grigorieff et al 1996), by structural analysis with X-ray crystallography (Pebay-Peyroula et al 1997, Luecke et al 1998), and via site-directed spin labeling (Altenbach et al 1990).

Transient interactions between two hydrophobic regions may also occur during membrane insertion. This interaction was proposed for the insertion of the M13 procoat protein, which requires the hydrophobic signal sequence and the hydrophobic membrane anchor region. These two hydrophobic domains enter the membrane and insert as a helical hairpin (Kuhn 1987). Alternatively, the tight packing of membrane proteins may occur as a late step. Popot & Engelman (1990) proposed a two-stage mechanism. In the first stage, the protein helices are surrounded by lipid molecules and are distant from each other. In the second stage they interact with their interfaces and the final folded structure forms.

Oligomeric Assembly of Membrane Complexes

One of the most challenging future problems in membrane biology is characterizing the mechanism of assembly of complexes in the membrane. Following the membrane integration of the individual proteins, a defined order must assure that the correct complexes are assembled in a sequential process. This process may be very complex, as in the case of the mitochondrial cytochrome *c* oxidase, which is composed of 13 subunits. Many of the steps in the formation of membrane complexes—membrane insertion, cofactor binding, folding, and quarternary assembly—are poorly understood.

Although daunting, studies of the assembly of many membrane complexes possessing multiple membrane-spanning subunits, organic cofactors, and metal clusters are underway. The photosynthetic reaction center of *Rhodopseudomonas viridis* and the ATP synthase and cytochrome *c* oxidase of *E. coli* are attractive models for these studies because their structures are solved at high resolution (Deisenhofer & Michel 1991, Michel et al 1998, Rastogi & Girvin 1999). The cytochrome *c* oxidase assembly pathway has been addressed in yeast and eubacteria. Cytochrome *c* oxidase is the terminal enzyme in the mitochondrial and bacterial respiratory chains. The mitochondrial enzyme contains 13 subunits and the bacterial enzyme contains 4 subunits. In mitochondria, the deletion of subunit IV, VI, VII, and VIIa leads to the loss of cytochrome *c* oxidase activity. Most likely, these nuclear-encoded subunits are important for either the folding or the stability of the mitochondrial encoded subunits I, II, and III in the holoenzyme (Poyton & McEwen 1996). The assembly of cytochrome *c* oxidase also depends upon a number of proteins that are not part of the oxidase complex. For instance, Cox10 and Cox11 genes encode proteins that are involved in the heme A biosynthetic pathway. Heme A is necessary for assembly or stability of protein subunits within the enzyme complex. Also, *Cox17*, a gene encoding a protein important for copper delivery to mitochondria, is necessary for the formation of a functional cytochrome *c* oxidase molecule (Glerum et al 1996). The results with cytochrome *c* oxidase

are typical of the assembly of other membrane complexes. That is, membrane complexes are often unstable when one of the subunits is not expressed (Baba et al 1994, Omote et al 1998). The addition of a metal ion or cofactor is also necessary for correct folding or in vivo stability.

Another example where the assembly of membrane complexes is studied is the ATP synthase of mitochondria and *E. coli* (Straffon et al 1998, Deckers-Hebestreit & Altendorf 1996). Here, the formation of a dimer between two b subunits was found to be a prerequisite if assembly of the F1 part is to occur (Sorgen et al 1998).

Membrane complexes are detected by extraction methods using mild detergents. For example, the Sec-translocase complex was extracted with β-octylglucoside (Brundage et al 1990). When digitonin was used for extraction, the loosely bound components SecD, SecF, and YajC remained bound to the complex (Duong & Wickner 1997b). Also, blue native gel electrophoresis traps membrane complexes simultaneously, thus allowing estimation of the molecular weight of the complexes (Dekker et al 1997). An attractive approach for studying the assembly of membrane complexes is the reconstitution into liposomes, which was studied with the photosystem I of cyanobacteria (Kruip et al 1999). However, the details of the assembly process remain incomplete.

CONCLUDING REMARKS

Protein insertion into membranes in bacteria and the chloroplast thylakoids are conserved and quite similar. These systems use both the Sec-pathway and Sec-independent pathway. The only difference is that in chloroplasts the proteins must first be imported into the stroma, prior to using these conserved pathways. In bacteria and chloroplasts, the key components of the Sec translocase, namely SecA, SecE, and SecY, and the components of the targeting pathway, SRP and the SRP receptor FtsY, are all present. The Sec-independent pathways seem to apply to those proteins that have short hydrophilic regions to translocate across the membrane. The twin-arginine translocation pathway (or ΔpH pathway) is used for insertion of some peripheral membrane proteins as well.

The mechanism in mitochondria is different and a little more complicated. A conservative pathway is present but is used for only certain inner membrane proteins. In the stop-transfer pathway, translocation of the inner membrane protein is halted as it passes through the TIM23 channel. In the conservative mechanism, nuclear-encoded mitochondrial inner membrane proteins are imported first into the matrix and then inserted into the inner membrane. The insertion of proteins from the matrix is analogous to the bacterial pathway. Some inner membrane proteins require Oxa1 for insertion, of which homologues exist in bacteria and chloroplasts. Interestingly, YidC, the bacterial homologue of Oxa1, is important for membrane protein insertion in *E. coli*. Whether mitochondrial-encoded inner membrane proteins also use a translocase, such as Oxa1, is an open question. A Sec translocase clearly is not used; mitochondria do not have SecA, SecY, SecE,

SRP, or FtsY homologues. Finally, there are at least two pathways (one involves Tim9/10 and the other Tim 8/13) that direct mitochondrial inner membrane proteins through the intermembrane space.

Both bacterial and organellar systems require translocases to pass proteins across the membrane. With this commonality, it is puzzling that the major translocases SecYEG, TOM-TIM, and TOC-TIC are not evolutionarily related. This might be due to the direction of the transport, i.e. proteins are exported in bacteria but imported into chloroplasts and mitochondria, having lost most of their genome. These organelles had to invent new protein machineries to import nuclear-encoded proteins from the cytoplasm. The bacterial Sec-system was limited to export function only and could not convert into an import machine. The mitochondrial TIM was derived most likely from a bacterial permease, since sequence homology was found between Tim 17, 22, and 23 with LivH of *E. coli* (Rassow et al 1999). In contrast to the mitochondria, the chloroplasts kept the bacterial-derived Sec-system, along with the newly developed TOC-TIC complex. Protein insertion systems that play an essential role for membrane proteins were evolutionarily conserved among the bacterial and organellar membranes. Homologues of the Oxa1 protein are found in mitochondria, chloroplasts, and bacteria. In addition, homologues that are not directly functionally related have also been detected. Toc36 of the outer envelope membrane shows homology to the bacterial SecA (Pang et al 1997).

In essence, it was the evolutionary change after endosymbiosis that led to the development of new translocases that use similar mechanisms. In mitochondria, the translocase catalyzes translocation and lateral integration of hydrophobic segments into the bilayer by a stop-transfer mechanism. The translocases work in conjunction with the protein folding and secondary translocation systems that descended from a bacterial ancestor. The evolution of these pathways in this manner explains why some mitochondrial proteins are first transported into the matrix and are then inserted into the inner membrane by a bacterial-type conserved pathway.

Our knowledge of the similarities of the export pathways used to insert proteins into membrane provides the impetus for the elucidation of the underlying molecular mechanism. More emphasis must be directed toward unraveling the structure/function details of the Tim, Tic, and Oxa1 proteins so that the mechanism of how proteins are inserted into the membranes may be understood.

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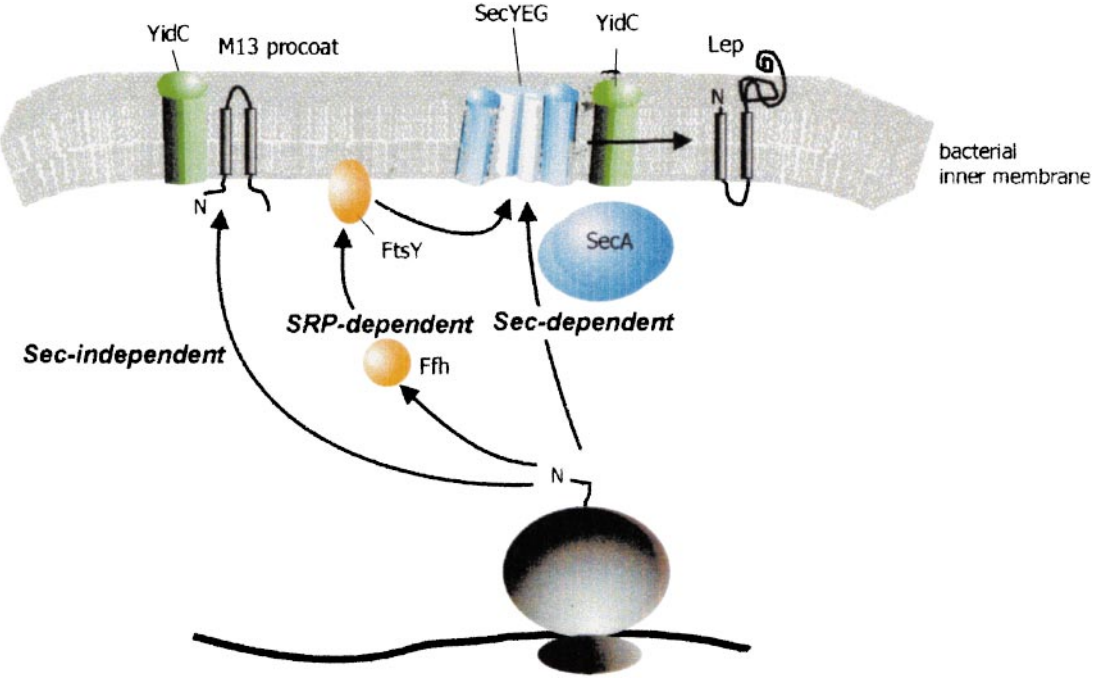

Figure 1 Membrane protein insertion in *E. coli*. Proteins synthesized in the cytoplasm are inserted into the membrane by two pathways. By the first pathway, the proteins are directly inserted into the lipid bilayer or may be assisted by YidC. Examples are Pf3 coat and M13 procoat (shown here as the uncleaved protein). The second pathway requires Ffh for targeting the protein to its receptor FtsY. Translocation across the membrane occurs through SecYEG. Examples are the leader peptidase and the mannitol permease MtlA. In addition, SecA is required for translocation of large periplasmic domains, as seen for the leader peptidase protein.

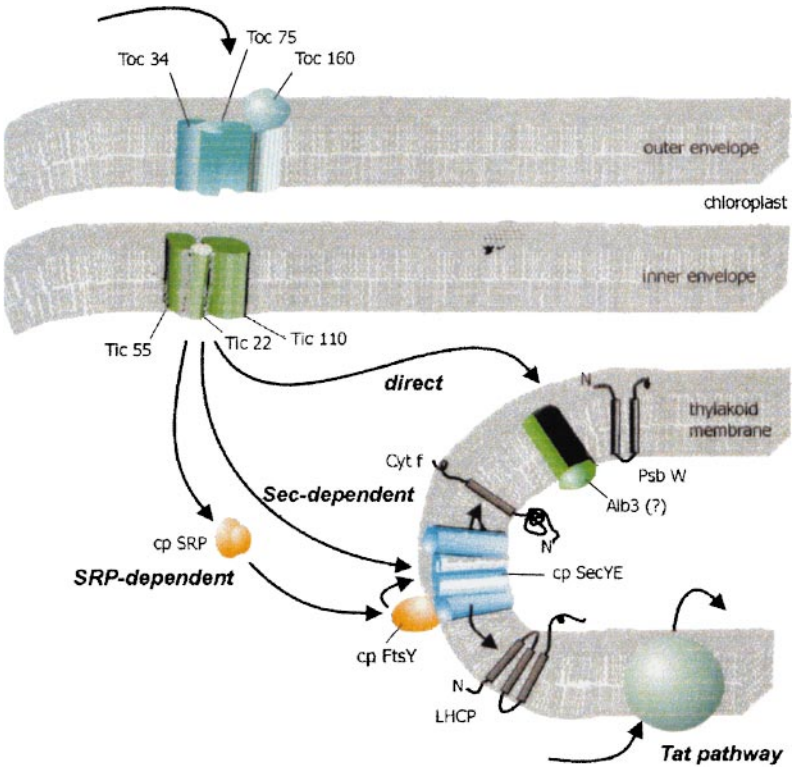

Figure 2 Membrane insertion into thylakoidal membrane. Nuclear-encoded proteins are synthesized in a precursor form in the cytoplasm. Chaperones interact with the preprotein to prevent misfolding. The protein is translocated across both the inner and outer envelope membrane through the TOC-TIC complex driven by ATP hydrolysis. A processing peptidase located in the stroma cleaves off the presequence. A direct pathway not involving the Sec proteins is used to insert both bitopic and polytopic membrane proteins and might be assisted by the Alb3 protein. As an example, the PsbW protein is shown in its uncleaved state. The SRP pathway requires GTP hydrolysis and cpFtsY for membrane integration of proteins. The Sec-dependent pathway is believed to use SecYE for membrane insertion and SecA for translocation of large lumenal domains of membrane proteins. The twin-arginine pathway (TAT or DpH) is used to transport folded proteins into the thylakoid lumen.

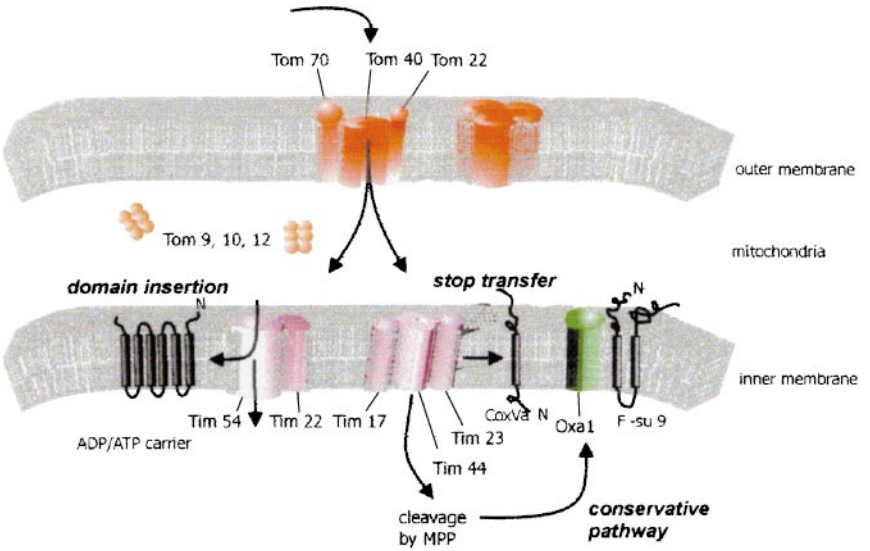

Figure 3 Insertion of proteins into the mitochondrial inner membrane. Nuclear-encoded proteins are typically made with a presequence. The protein is targeted to the TOM complex and translocated across the outer membrane. In the stop-transfer pathway, translocation across the inner membrane through the TIM23 complex is interrupted after its initiation. The model protein for this pathway is Cox Va. The carrier proteins are exported by the TIM22 pathway or domain insertion pathway where they enter the intermembrane space prior to the insertion into the inner membrane. The carrier proteins (such as ADP/ATP carrier) interact with a 70-kDa complex (Tim9, 10, 12) in the intermembrane space and then insert into the inner membrane by domain insertion. In the conservative pathway, the preprotein is imported into the matrix through the TOM-TIM complex. The matrix presequence is removed by the matrix processing peptidase and the protein is then inserted into the inner membrane by an Oxal-dependent mechanism. The ATP synthase subunit 9 uses the conservative pathway. The mitochondrial type 1 signal peptidase (Imp1/Imp2) cleaves off the sorting sequence when present on the inner membrane proteins.