13.4 Targeting of Proteins to Mitochondria and Chloroplasts

In the remainder of this chapter, we examine how proteins synthesized on cytosolic ribosomes are sorted to discrete organelles: mitochondria, chloroplasts, peroxisomes, and the nucleus (see Figure 13-1). Mitochondria and chloroplasts are closely related organelles that contain an internal lumen — called the *matrix* in mitochondria and the *stroma* in chloroplasts — that is surrounded by a double membrane. Within the stroma, chloroplasts contain a third membrane known as the *thylakoid*, which is where the light-harvesting reactions of photosynthesis take place. In contrast to mitochondria and chloroplasts, peroxisomes are bounded by a single membrane and have a single luminal matrix compartment. The mechanism of protein transport into and out of the nucleus, which differs in many respects from sorting to other organelles, is discussed in the last section of the chapter.

In addition to being bounded by two membranes, mitochondria and chloroplasts share similar types of electron-transporting proteins and use an F-class ATPase to synthesize ATP (see Figure 12-26). Remarkably, these characteristics are shared by gram-negative bacteria. Also like bacterial cells, mitochondria and chloroplasts contain their own DNA, which encodes organelle rRNAs, tRNAs, and some proteins (see Chapter 8). Moreover, growth and division of mitochondria and chloroplasts are

not coupled to nuclear division. Rather, these organelles grow by the incorporation of cellular proteins and lipids, and new organelles form by division of preexisting organelles. These numerous similarities to free-living bacterial cells have led to the understanding that mitochondria and chloroplasts arose when bacteria were incorporated into ancestral eukaryotic cells, forming endosymbiotic organelles (see Figure 12-7). The sequence similarity of the many membrane translocation proteins shared by mitochondria, chloroplasts, and bacteria provides the most striking evidence for this ancient evolutionary relationship. In this section, we examine these membrane translocation proteins in detail.

Proteins encoded by mitochondrial DNA or chloroplast DNA are synthesized on ribosomes within the organelles and directed to the correct subcompartment within the parent organelle immediately after synthesis. However, the majority of proteins located in mitochondria and chloroplasts are not encoded by organelle DNA — they are encoded by genes in the nucleus and are imported into the organelles after their synthesis in the cytosol. Apparently, as eukaryotic cells evolved over a billion years, much of the genetic information from the ancestral bacterial DNA in these endosymbiotic organelles moved, by an unknown mechanism, to the nucleus. Precursor proteins synthesized in the cytosol that are destined for the matrix of mitochondria or the equivalent stroma in chloroplasts usually contain specific N-terminal targeting sequences that specify binding to receptor proteins on the organelle surface. Generally these sequences are cleaved once the protein reaches the matrix or stroma. Clearly these targeting sequences are similar in their location and general function to the signal sequences that direct nascent proteins to

the ER lumen. Although the three types of signals share some sequence features, their specific sequences differ considerably, as summarized in Table 13-1.

In both mitochondria and chloroplasts, protein import requires energy and occurs at points where the outer and inner organelle membranes are in direct contact. Because mitochondria and chloroplasts contain multiple membranes and membrane-limited spaces, the sorting of many proteins to their correct locations requires the sequential action of two targeting sequences and two membrane-bound translocation systems: one to direct the protein into the organelle and the other to direct it into the correct organelle subcompartment or membrane. As we will see, the mechanisms for sorting various proteins to mitochondria and chloroplasts are related to SRP-dependent recognition of a signal sequence as discussed previously.

Amphipathic N-Terminal Targeting Sequences Direct Proteins to the Mitochondrial Matrix

All proteins that travel from the cytosol to the same mitochondrial destination have targeting signals that share common motifs, although the signal sequences are not identical. Thus the receptors that recognize such signals are able to bind to a number of different but related sequences. The most extensively studied sequences for localizing proteins to mitochondria are the *matrix-targeting sequences*. These sequences, located at the N-terminus, are usually 20–50 amino acids in length. They are rich in

hydrophobic amino acids, positively charged basic amino acids (arginine and lysine), and hydroxylated ones (serine and threonine) but tend to lack negatively charged acidic residues (aspartate and glutamate).

Mitochondrial matrix–targeting sequences are thought to assume an α-helical conformation in which positively charged amino acids predominate on one side of the helix and hydrophobic amino acids on the other. Sequences such as these that contain both hydrophobic and hydrophilic regions are said to be amphipathic. Mutations that disrupt the amphipathic character of these sequences usually disrupt targeting to the matrix, although many other amino acid substitutions do not. These findings indicate that the amphipathicity of matrix-targeting sequences is critical to their function.

The cell-free assay outlined in Figure 13-23 has been widely used to define the biochemical steps in the import of mitochondrial precursor proteins. Respiring (energized) mitochondria extracted from cells can incorporate mitochondrial precursor proteins that have been synthesized in the absence of mitochondria if they are carrying appropriate targeting sequences. Successful incorporation of the precursor into the organelle can be assayed by resistance to digestion by an added protease such as trypsin. In other assays, successful import of a precursor protein can be shown by the proper cleavage of the N-terminal targeting sequences by specific mitochondrial proteases. The uptake of completely presynthesized mitochondrial precursor proteins by the organelle in this system contrasts with the cell-free cotranslational translocation of secretory proteins into

the ER, which generally occurs only when microsomal (ER-derived) membranes are present during synthesis (see Figure 13-4).

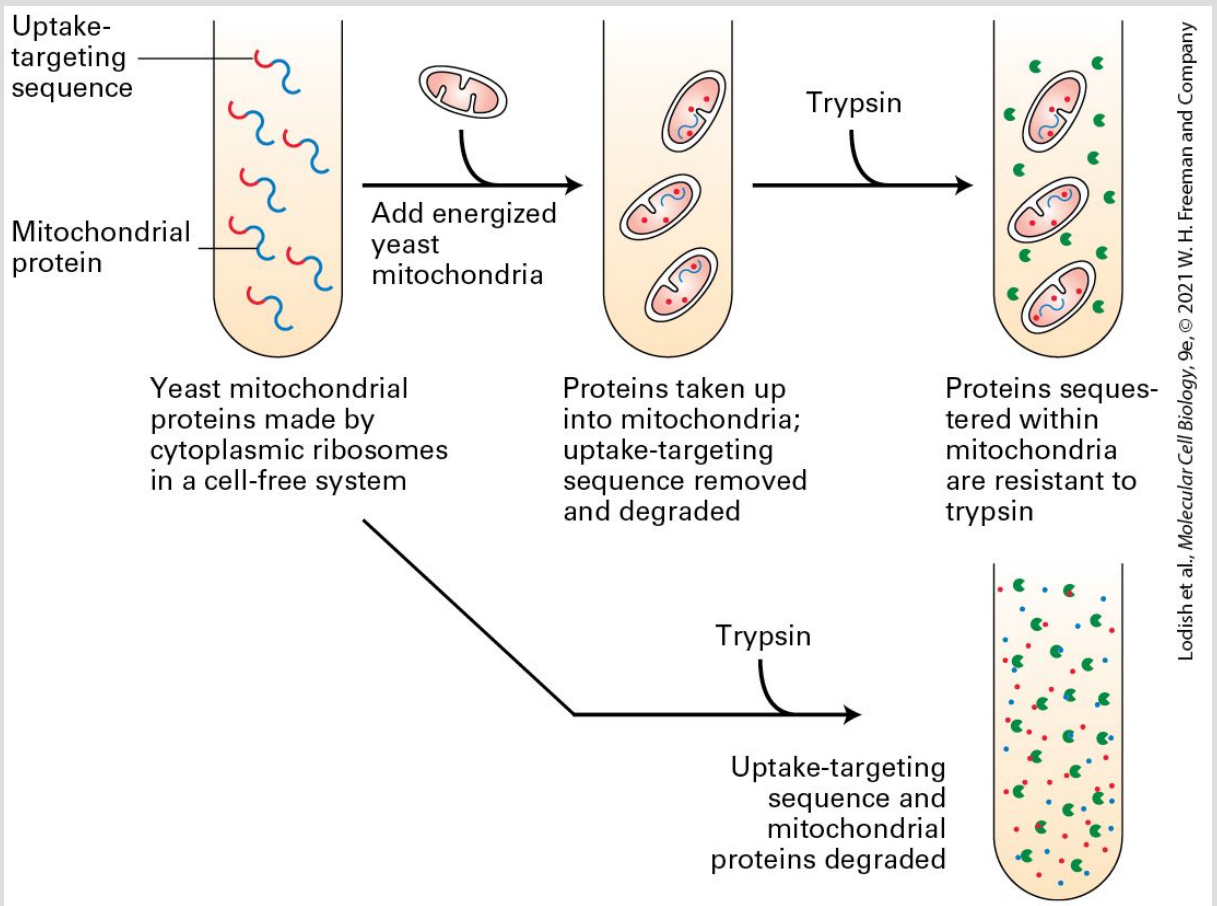


FIGURE 13-23 Import of mitochondrial precursor proteins is assayed in a cell-free system. Mitochondrial precursor proteins with attached uptake-targeting signals can be synthesized on ribosomes in a cell-free reaction. When respiring mitochondria are added to these synthesized mitochondrial precursor proteins (*top*), the proteins are taken up by mitochondria. Inside mitochondria, the proteins are protected from the action of proteases such as trypsin. When no mitochondria are present (*bottom*), the mitochondrial proteins are degraded by added protease. Only energized (respiring) mitochondria, which have a proton electrochemical gradient (proton-motive force) across the inner membrane, take up these proteins. Furthermore, the imported proteins must contain an appropriate uptake-targeting sequence. Uptake also requires ATP and a cytosolic extract containing chaperone proteins that maintain the precursor proteins in an unfolded conformation. This assay has been used to study targeting sequences and other features of the translocation process.

Description
The first illustration shows a test tube having Yeast mitochondrial proteins made by cytoplasmic ribosomes in a cell-free system. The mitochondrial proteins are tagged by Uptake-targeting sequence. Next, energized yeast mitochondria is added resulting the mitochondria to take up proteins; uptake-targeting sequence are removed and degraded. Next, trypsin is added and shows the mitochondria unaffected by trypsin activity. Proteins sequestered within mitochondria are resistant to trypsin. However, when trypsin is added to the initial test tube, uptake-targeting sequence and mitochondrial proteins are degraded.

Mitochondrial Protein Import Requires Outer-Membrane Receptors and Translocons in Both Membranes

Figure 13-24 presents an overview of protein import from the cytosol into the mitochondrial matrix, the route into the mitochondrion followed by most imported proteins. Here we discuss in detail each step of protein transport into the matrix, then consider how some proteins are subsequently targeted to other compartments of the mitochondrion.

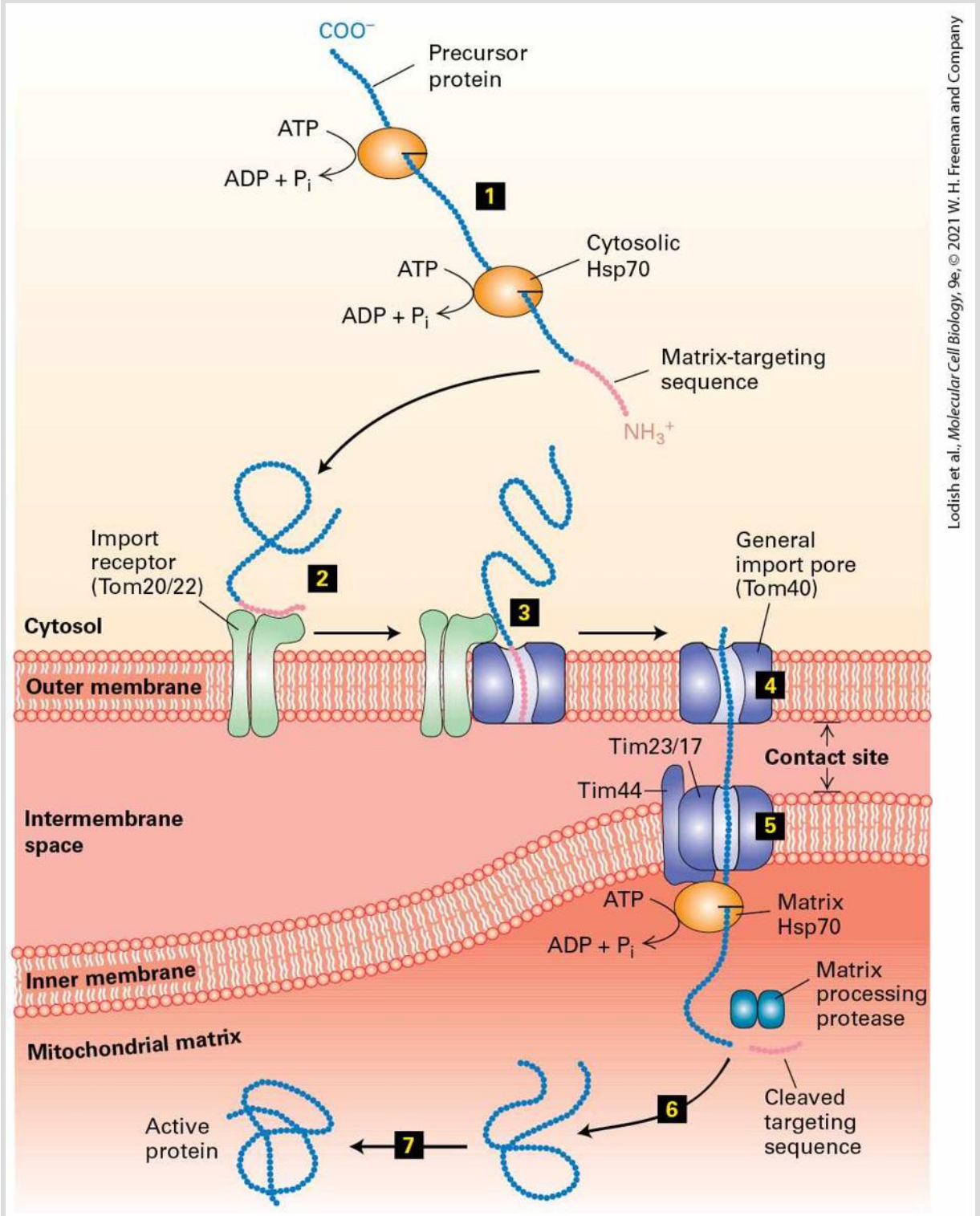


FIGURE 13-24 Protein import into the mitochondrial matrix. Precursor proteins synthesized on cytosolic ribosomes are maintained in an unfolded or partially folded state by bound chaperones, such as cytosolic Hsp70 (step 1). After a precursor protein binds to

an import receptor near a site of contact with the inner membrane (step 2), it is transferred into the general import pore (step 3). The translocating protein then moves through this channel and an adjacent channel in the inner membrane (steps 4–5). Note that translocation occurs at rare "contact sites" at which the inner and outer membranes appear to touch. Binding of the translocating protein by matrix Hsp70 and subsequent ATP hydrolysis by Hsp70 helps drive import into the matrix. Once the targeting sequence is removed by a matrix protease and Hsp70 is released from the newly imported protein (step 6), the protein folds into its mature, active conformation within the matrix (step 7). Folding of some proteins depends on matrix chaperonins. See G. Schatz, 1996, *J. Biol. Chem.* **271**:31763; and N. Pfanner et al., 1997, *Annu. Rev. Cell Dev. Biol.* **13**:25.

Description

The steps are as follows: Step 1. A precursor protein in the cytosol is bound by cytosolic H s p 70 and has a matrix targeting sequences at the N-terminal. The A T P of cytosolic H s p 70 hydrolysis into A D P and P i. Step 2. The cytosolic H s p 70 detaches and the matrix targeting sequence binds to an import receptor, TOM 20 slash 22, embedded in the outer membrane of the mitochondrion. Step 3. The matrix targeting sequence is transferred to a general import pore TOM 40 lying adjacent to import receptor. Step 4. The protein is translocated through TOM 40 into intermembrane space. Step 5. The protein is transferred through a second pore (Tim 23 slash 17) embedded in the inner membrane at a site where the outer and inner membranes are in close contact. A protein complex, Tim 44, attached to Tim 23 slash 17 attaches a matrix H s p 70 to the entering protein chain as A T P is hydrolyzed to A D P and P i. A matrix processing protease cleaves the targeting sequence. Steps 6 and 7. The protein folds resulting into an active protein.

After synthesis in the cytosol, the soluble precursors of mitochondrial proteins (including hydrophobic integral membrane proteins) can interact directly with the mitochondrial membrane. Import of an unfolded mitochondrial precursor protein is initiated by the binding of a mitochondrial targeting sequence to an *import receptor* in the outer

mitochondrial membrane. These import receptors were first identified by experiments in which antibodies to specific proteins of the outer mitochondrial membrane were shown to inhibit protein import into isolated mitochondria. Subsequent genetic experiments in which the genes for specific mitochondrial outer-membrane proteins were mutated showed that specific receptor proteins were responsible for the import of different classes of mitochondrial proteins. For example, N-terminal matrix-targeting sequences are recognized by a complex of Tom20 and Tom22 (proteins in the outer mitochondrial membrane involved in targeting and import, designated *Tom* proteins for *t*ranslocon of the *o*uter *m*embrane).

Many proteins can be imported into the mitochondrion only in an unfolded state. Chaperone proteins such as cytosolic Hsp70 and Hsp90 use energy derived from ATP hydrolysis to keep nascent and newly made proteins in a disaggregated state so that they are available to be taken up by mitochondria. For some mitochondrial precursor proteins, the mitochondrial outer-membrane protein Tom70 serves as an import receptor by binding to both Hsp90 and portions of the unfolded precursor protein.

The import receptors subsequently transfer the precursor protein to an import channel in the outer membrane. This channel, composed mainly of the Tom40 protein, is known as the **general import pore** because all known mitochondrial precursor proteins gain access to the interior compartments of the mitochondrion through it. When Tom40 is purified and incorporated into liposomes, it forms a transmembrane channel with a pore wide enough to accommodate an unfolded polypeptide chain. The

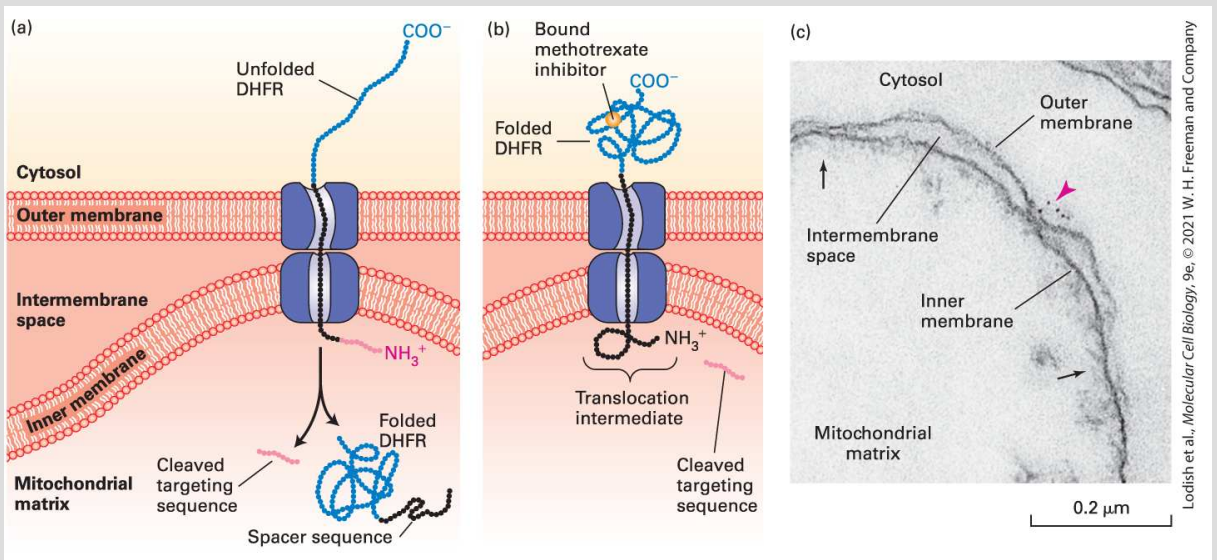
general import pore forms a largely passive channel through the outer mitochondrial membrane; the driving force for unidirectional transport comes from within the mitochondrion, as we will see shortly. In the case of precursors destined for the mitochondrial matrix, transfer through the outer membrane occurs simultaneously with transfer through an inner-membrane channel composed of the Tim23 and Tim17 proteins. (*Tim* stands for *t*ranslocon of the *i*nner *m*embrane.) Translocation into the matrix thus occurs at contact sites where the outer and inner membranes are in close proximity.

Soon after the N-terminal matrix-targeting sequence of a protein enters the mitochondrial matrix, it is removed by a protease that resides within the matrix. The emerging protein is also bound by matrix Hsp70, a chaperone that is localized to the translocation channels in the inner mitochondrial membrane by interaction with transmembrane protein Tim44. Together, Tim44 and Hsp70 are thought to power translocation of proteins into the matrix.

Some imported proteins can fold into their final, active conformation without further assistance. Final folding of many matrix proteins, however, requires chaperonins. As discussed in Chapter 3, chaperonin proteins actively facilitate protein folding by a process that depends on ATP. Yeast mutants defective in Hsc60, a chaperonin in the mitochondrial matrix, can import matrix proteins and cleave their targeting sequences normally, but the imported polypeptides fail to fold and assemble into their native tertiary and quaternary structures.

Studies with Chimeric Proteins Demonstrate Important Features of Mitochondrial Protein Import

Dramatic evidence for the ability of mitochondrial matrix–targeting sequences to direct the import of any protein to the matrix was shown by adding the matrix-targeting sequence of alcohol dehydrogenase to the enzyme dihydrofolate reductase (DHFR), which normally resides in the cytosol. Cell-free translocation assays show that in the presence of chaperones, which prevent the DHFR segment from folding in the cytosol, the chimeric protein is transported into the matrix (Figure 13-25a). However, the DHFR inhibitor methotrexate, which binds tightly to the active site of DHFR and greatly stabilizes its folded conformation, renders the chimeric protein resistant to unfolding by cytosolic chaperones and prevents the chimeric protein from completely entering the translocon of the matrix. This finding demonstrates that a precursor must be unfolded in order to traverse the import pores in both mitochondrial membranes.



EXPERIMENTAL FIGURE 13-25 Experiments with chimeric proteins elucidate mitochondrial protein import processes. These experiments show that a matrix-targeting sequence alone directs proteins to the mitochondrial matrix and that only unfolded proteins are translocated across both mitochondrial membranes. The chimeric protein in these experiments contained a matrix-targeting signal at its N-terminus (red), followed by a spacer sequence of no particular function (black), and then by dihydrofolate reductase (DHFR), an enzyme normally present only in the cytosol. (a) When the DHFR segment is unfolded, the chimeric protein moves across both membranes to the matrix of an energized mitochondrion, and the matrix-targeting signal is then removed. (b) When the C-terminus of the chimeric protein is locked in the folded state by binding of methotrexate, translocation is blocked. If the spacer sequence is long enough to extend across both transport channels, a stable translocation intermediate with the targeting sequence cleaved off is generated in the presence of methotrexate, as shown here. (c) The C-terminus of the translocation intermediate in (b) can be detected by incubating the mitochondria with antibodies that bind to the DHFR segment, followed by gold particles coated with bacterial protein A, which binds nonspecifically to antibody molecules (see Figure 4-33). An electron micrograph of a sectioned sample reveals gold particles (red arrowhead) bound to the translocation intermediate at a contact site between the inner and outer membranes. Other contact sites (black arrows) are also evident. See J. Rassow et al., 1990, *FEBS Lett.* **275**:190.

[Part (c) M. Schwaiger, V. Herzog, and W. Neupert, 1987, *J. Cell Biol.* **105**:235–246; https://doi.org/10.1083/jcb.105.1.235.]

Description

The illustration (a) shows an unfolded D H F R protein entering mitochondrial matrix from cytosol through pores in outer and inner membrane at the contact site. The C-terminal lies in cytosol, the N-terminal having target sequence lies in matrix, while the spacer sequence is present in the pores. Next, targeting sequence is cleaved and the D H F R having spacer sequence is folded.

The illustration (b) shows a folded D H F R protein with a methotrexate inhibitor bound to it entering mitochondrial matrix from cytosol through pores in outer and inner membrane at the contact site. The C-terminal lies in cytosol and the N-terminal having spacer sequence lies in matrix. A cleaved targeting sequence is present in the mitochondrial matrix.

The black-and-white micrograph (c) shows an outer and inner membrane of mitochondria with mitochondrial matrix inside and cytosol outside. A pink arrow points to a contact site where protein translocation occurs and two black arrows point to two contact sites.

Additional studies revealed that if a sufficiently long spacer sequence separates the N-terminal matrix-targeting sequence and the DHFR portion of the chimeric protein, then, in the presence of methotrexate, a translocation intermediate that spans both membranes can be trapped (Figure 13-25b). Microscopy studies of these stable trapped intermediates show that they accumulate at sites where the inner and outer mitochondrial membranes are close together (Figure 13-25c). Since roughly a thousand stuck chimeric proteins can be observed at these *contact sites* in a typical yeast mitochondrion, it is thought that mitochondria have approximately a thousand general import pores for the uptake of mitochondrial proteins.

Three Energy Inputs Are Needed to Import Proteins into Mitochondria

As noted previously and as indicated in Figure 13-24, ATP hydrolysis by Hsp70 chaperone proteins in both the cytosol and the mitochondrial matrix is required for the import of mitochondrial proteins. Cytosolic Hsp70 expends energy to maintain bound precursor proteins in an unfolded state so that they can be translocated into the matrix. The importance of ATP to this function was demonstrated in studies in which a mitochondrial precursor protein was purified and then denatured (unfolded) by urea. When tested in the cell-free mitochondrial translocation system, the denatured protein was incorporated into the matrix in the absence of ATP. In contrast, the same precursor protein in its native, undenatured state was not imported in the absence of ATP, even in the presence of cytosolic chaperones.

The sequential binding to and ATP-driven release of multiple matrix Hsp70 molecules from a translocating protein may simply trap the unfolded protein in the matrix. In this case, the functions of matrix Hsp70 and Tim44 would be analogous to those of the chaperone BiP and Sec63 complex, respectively, in post-translational translocation into the ER lumen (see Figure 13-9).

The third energy input required for mitochondrial protein import is an H^+ electrochemical gradient, or *proton-motive force*, across the inner membrane. Recall from Chapter 12 that protons are pumped from the

matrix into the intermembrane space during electron transport, creating an electric potential across the inner membrane. In general, only mitochondria that are actively undergoing respiration, and therefore have generated a proton-motive force across the inner membrane, are able to translocate precursor proteins from the cytosol into the mitochondrial matrix. Treatment of mitochondria with inhibitors or uncouplers of oxidative phosphorylation, such as cyanide or dinitrophenol, dissipates this proton-motive force. Although precursor proteins can still bind tightly to receptors on such poisoned mitochondria, the proteins cannot be imported, either in intact cells or in cell-free systems, even in the presence of ATP and chaperone proteins. Once a protein is partially inserted into the inner membrane, it is subjected to a membrane potential of 200 mV (matrix negative). This seemingly small potential is established across the very narrow hydrophobic core of the lipid bilayer, which gives an enormous electrochemical gradient, equivalent to about 400,000 $\mathrm{V/cm}$. The positive charges in the amphipathic matrix-targeting sequence may simply be pulled into the matrix by the inside-negative membrane potential.

Multiple Signals and Pathways Target Proteins to Submitochondrial Compartments

Unlike targeting to the matrix, targeting of proteins to the intermembrane space, inner membrane, or outer membrane of mitochondria generally requires more than one targeting sequence and occurs via one of several

pathways. Figure 13-26 summarizes the organization of targeting sequences in proteins sorted to different mitochondrial locations.

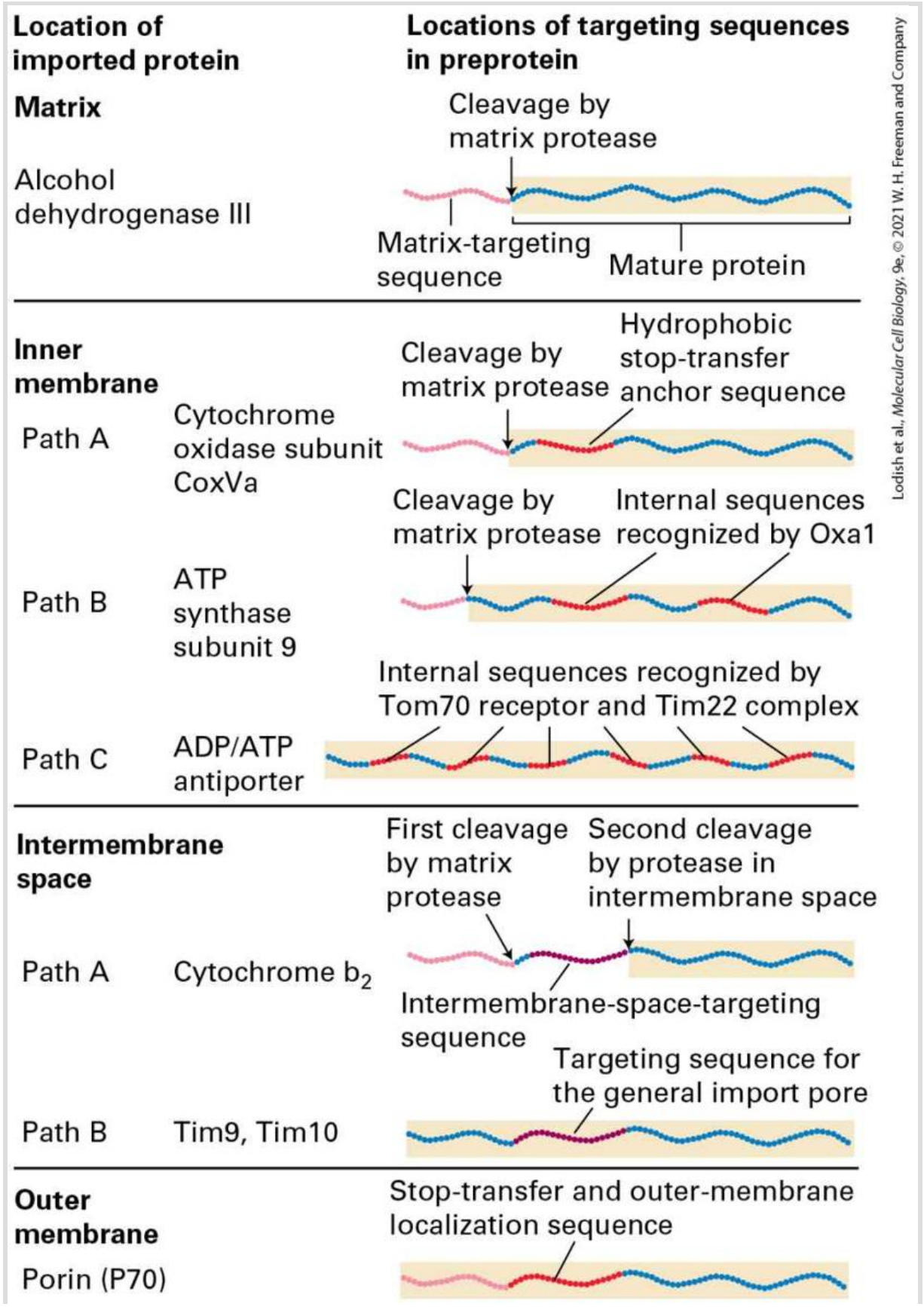

Location of imported protein
Locations of targeting sequences in preprotein
Matrix
Alcohol dehydrogenase III
Cleavage by matrix protease
Matrix-targeting sequence
Mature protein
Inner membrane
Path A
Cytochrome oxidase subunit CoxVa
Cleavage by matrix protease
Hydrophobic stop-transfer anchor sequence
Path B
ATP synthase subunit 9
Cleavage by matrix protease
Internal sequences recognized by Oxa1
Path C
ADP/ATP antiporter
Internal sequences recognized by Tom70 receptor and Tim22 complex
Intermembrane space
Path A
Cytochrome b2
First cleavage by matrix protease
Second cleavage by protease in intermembrane space
Intermembrane-space-targeting sequence
Path B
Tim9, Tim10
Targeting sequence for the general import pore
Outer membrane
Porin (P70)
Stop-transfer and outer-membrane localization sequence
Lodish et al., Molecular Cell Biology, 9e, © 2021 W. H. Freeman and Company

FIGURE 13-26 Targeting sequences in imported mitochondrial proteins. Most mitochondrial proteins have an N-terminal matrix-targeting sequence (pink) that is similar, though not identical, in different proteins. Proteins destined for the inner membrane, the intermembrane space, or the outer membrane have one or more additional targeting sequences that function to direct the proteins to these locations by several different pathways. The lettered pathways correspond to those illustrated in Figures 13-27 and 13-28. See W. Neupert, 1997, *Annu. Rev. Biochem.* **66**:863.

Description

Each part highlights proteins, location of imported protein, and locations of targeting sequences in preprotein. The first part for location of imported protein in matrix has protein, alcohol dehydrogenase 3, and the location of targeting sequences shows cleavage by matrix protease between matrix-targeting sequence and mature protein sequence.

The second part shows three paths for location of imported protein in inner membrane. Path A: The protein is Cytochrome oxidase subunit Cox V a and the location of targeting sequences shows a cleavage by matrix protease between matrix-targeting sequence and hydrophobic stop-transfer anchor sequence which is attached to a mature protein sequence. Path C: The protein is A T P synthase subunit 9 and the location of targeting sequences shows a cleavage by matrix protease between matrix-targeting sequence and mature protein sequence. The mature protein sequence has two internal sequences recognized by Oxa 1. Path C: The protein is A D P slash A T P antiporter and the location of targeting sequences shows a mature protein having six internal sequences recognized by Tom 70 receptor and Tim 22 complex.

The third part shows two paths for location of imported protein in intermembrane space. Path A: The protein is Cytochrome b 2 and the location of targeting sequences shows a first cleavage by matrix protease between matrix-targeting sequence and intermembrane-space-targeting sequence followed by a second cleavage by protease in intermembrane space between intermembrane-space-targeting sequence and mature protein sequence. Path B: The protein is Tim 9, Tim 10 and the location of targeting sequences shows a targeting sequence for the general import pore between two mature protein sequences.

The fourth part for location of imported protein in outer membrane has protein, Porin P 70, and the location of targeting sequences shows a stop-transfer and outer-membrane localization sequence between matrix-targeting sequence and mature protein sequence.

Inner-Membrane Proteins

Three separate pathways are known to target proteins to the inner mitochondrial membrane. One pathway makes use of the same machinery that is used for the targeting of matrix proteins (Figure 13-27, path A). A cytochrome oxidase subunit called *CoxVa* is one protein transported by this pathway. The precursor form of CoxVa, which contains an N-terminal matrix-targeting sequence recognized by the $\mathrm{Tom20/22}$ import receptor, is transferred through the Tom40 general import pore of the outer membrane and the inner-membrane $\mathrm{Tim23/17}$ translocation complex. In addition to the matrix-targeting sequence, which is cleaved during import, CoxVa contains a hydrophobic stop-transfer anchor sequence. As the protein passes through the $\mathrm{Tim23/17}$ channel, the stop-transfer anchor sequence blocks translocation of the C-terminus across the inner membrane. The membrane-anchored intermediate is then transferred laterally into the bilayer of the inner membrane, much like type I integral membrane proteins are incorporated into the ER membrane (see Figure 13-11).

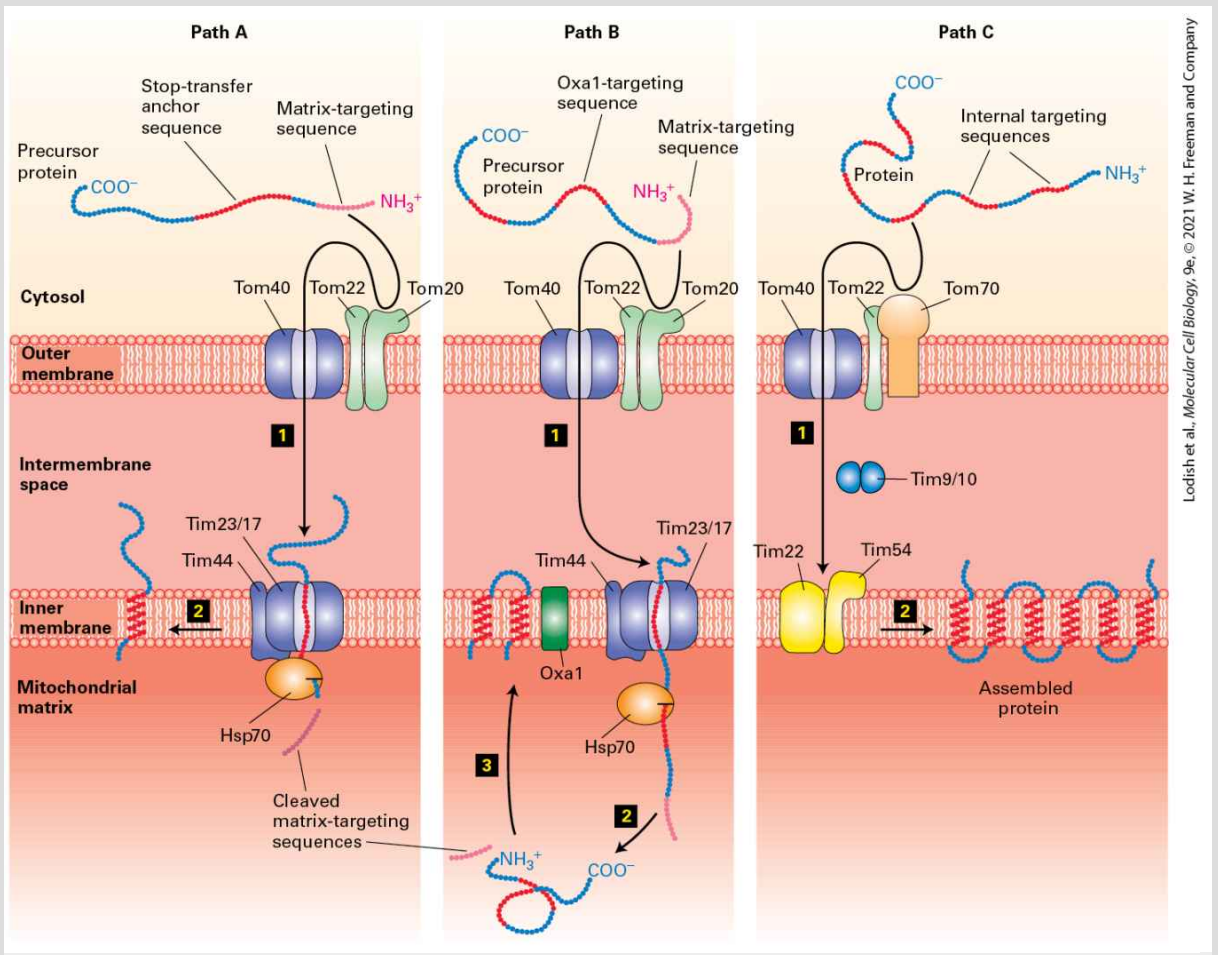


FIGURE 13-27 Three pathways to the inner mitochondrial membrane from the cytosol. Proteins with different targeting sequences are directed to the inner membrane via different pathways. In all three pathways, proteins cross the outer membrane via the Tom40 general import pore. Proteins delivered by pathways A and B contain an N-terminal matrix-targeting sequence that is recognized by the **Tom20/22** import receptor in the outer membrane. Although both these pathways use the **Tim23/17** inner-membrane channel, they differ in that the entire precursor protein enters the matrix and is then redirected to the inner membrane in pathway B. Matrix Hsp70 plays a role similar to its role in the import of soluble matrix proteins (see Figure 13-23). Proteins delivered by pathway C contain internal sequences that are recognized by the **Tom70/Tom22** import receptor; a different inner-membrane translocation channel **(Tim22/54)** is used in this pathway. Two intermembrane proteins (Tim9 and Tim10) facilitate transfer between the outer and inner channels. See the text for discussion. See R. E. Dalbey and A. Kuhn, 2000, *Annu. Rev. Cell Dev. Biol.* **16**:51; and N. Pfanner and A. Geissler, 2001, *Nat. Rev. Mol. Cell Biol.* **2**:339.

Description
The illustration shows from top to bottom: cytosol, outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and mitochondrial matrix. Path A shows a precursor protein chain with a matrix-targeting sequence at N-terminal followed by a stop-transfer anchor sequence. The outer membrane has a Tom 40 channel next to a Tom 20, Tom 22 receptor; and the inner membrane has Tim 23 slash 17 channel attached to a Tim 44 receptor. Step 1: The precursor protein enters mitochondrial matrix via Tom 40, intermembrane space, and Tim 23 slash 17 where stop-transfer anchor sequence attaches to internal of Tim 23 slash 17 channel. H s p 70 in matrix cleaves the matrix-targeting sequence. Step 2 shows the protein embedded in the inner membrane with C-terminal in intermembrane space, stop-transfer anchor sequence in inner membrane, and N-terminal in matrix.

Path B shows a precursor protein chain with a matrix-targeting sequence at N-terminal followed by two Oxa 1-targeting sequences. Step 1: The precursor protein enters mitochondrial matrix where the second Oxa 1-targeting sequence attaches to internal of Tim 23 slash 17 channel. H s p 70 in matrix cleaves after first Oxa 1-targeting sequence. Step 2 shows a protein chain in matrix with two Oxa 1-targeting sequences and a cleaved matrix-targeting sequence. Step 3: The protein gets embedded in the inner membrane next to an Oxa 1 embedded in the inner membrane. The protein embedded has both C-terminal and N-terminal in matrix, both Oxa 1-targeting sequence in inner membrane, and middle protein sequence in intermembrane space.

Path C shows a protein chain with several internal targeting sequences. The outer membrane has a Tom 40 channel next to a Tom 22, Tom 70 receptor; and inner membrane has Tim 22 attached to a Tim 54 embedded in it. Step 1: The precursor protein enters intermembrane space via Tom 40, binds to Tim 9 slash 10 and enters Tim, Tim 54 of inner membrane. Step 2 shows the protein embedded in the inner membrane with both C-terminal and N-terminal in intermembrane space, internal targeting sequences in inner membrane, and middle protein sequence in matrix.

A second pathway to the inner membrane is used by proteins such as ATP synthase subunit 9, whose precursors contain both a matrix-targeting sequence and internal hydrophobic domains recognized by an inner-

membrane protein termed *Oxa1*. This pathway is thought to involve translocation of at least a portion of the precursor into the matrix via the Tom40 and Tim23/17 channels. After cleavage of the matrix-targeting sequence, the protein is inserted into the inner membrane by a process that requires interaction with Oxa1 and perhaps other inner-membrane proteins (see Figure 13-27, path B). Oxa1 is related to a protein involved in inserting some plasma-membrane proteins in bacteria. This relatedness suggests that Oxa1 may have descended from the translocation machinery in the endosymbiotic bacterium that eventually became the mitochondrion. However, the proteins that form the inner-membrane channels in mitochondria are not related to the proteins in bacterial translocons. Oxa1 also participates in the inner-membrane insertion of certain proteins, such as subunit II of cytochrome oxidase, that are encoded by mitochondrial DNA and synthesized in the matrix by mitochondrial ribosomes.

The final pathway for insertion in the inner mitochondrial membrane is followed by multipass proteins that contain six membrane-spanning domains, such as the ATP/ADP antiporter. These proteins, which lack the usual N-terminal matrix-targeting sequence, contain multiple internal mitochondrial targeting sequences. After the internal sequences are recognized by a second import receptor composed of outer-membrane proteins Tom70 and Tom22, the imported protein passes through the outer membrane via the general import pore (see Figure 13-27, path C). The protein then is transferred to a second translocation complex in the inner membrane composed of the Tim22, Tim18, and Tim54 proteins. Transfer to the Tim22/18/54 complex depends on a multimeric complex of two

small proteins, Tim9 and Tim10, which reside in the intermembrane space. These small Tim proteins are thought to act as chaperones, guiding imported protein precursors from the general import pore to the $\mathrm{Tim22/18/54}$ complex in the inner membrane by binding to their hydrophobic regions, preventing them from forming insoluble aggregates in the aqueous environment of the intermembrane space. Ultimately the $\mathrm{Tim22/18/54}$ complex is responsible for incorporating the multiple hydrophobic segments of the imported protein into the inner membrane.

Intermembrane-Space Proteins

Two pathways deliver cytosolic proteins to the space between the inner and outer mitochondrial membranes. The major pathway is followed by proteins such as cytochrome b_2, whose precursors carry two different N-terminal targeting sequences, both of which are ultimately cleaved. The most N-terminal of the two sequences is a matrix-targeting sequence, which is removed by the matrix protease. The second targeting sequence is a hydrophobic segment that blocks complete translocation of the protein across the inner membrane (Figure 13-28, path A). After the resulting membrane-embedded intermediate diffuses laterally away from the $\mathrm{Tim23/17}$ translocation channel, a protease in the membrane cleaves the protein near the hydrophobic transmembrane segment, releasing the mature protein in a soluble form into the intermembrane space. Except for the second proteolytic cleavage, this pathway is similar to that of inner-membrane proteins such as CoxVa (see Figure 13-27, path A).

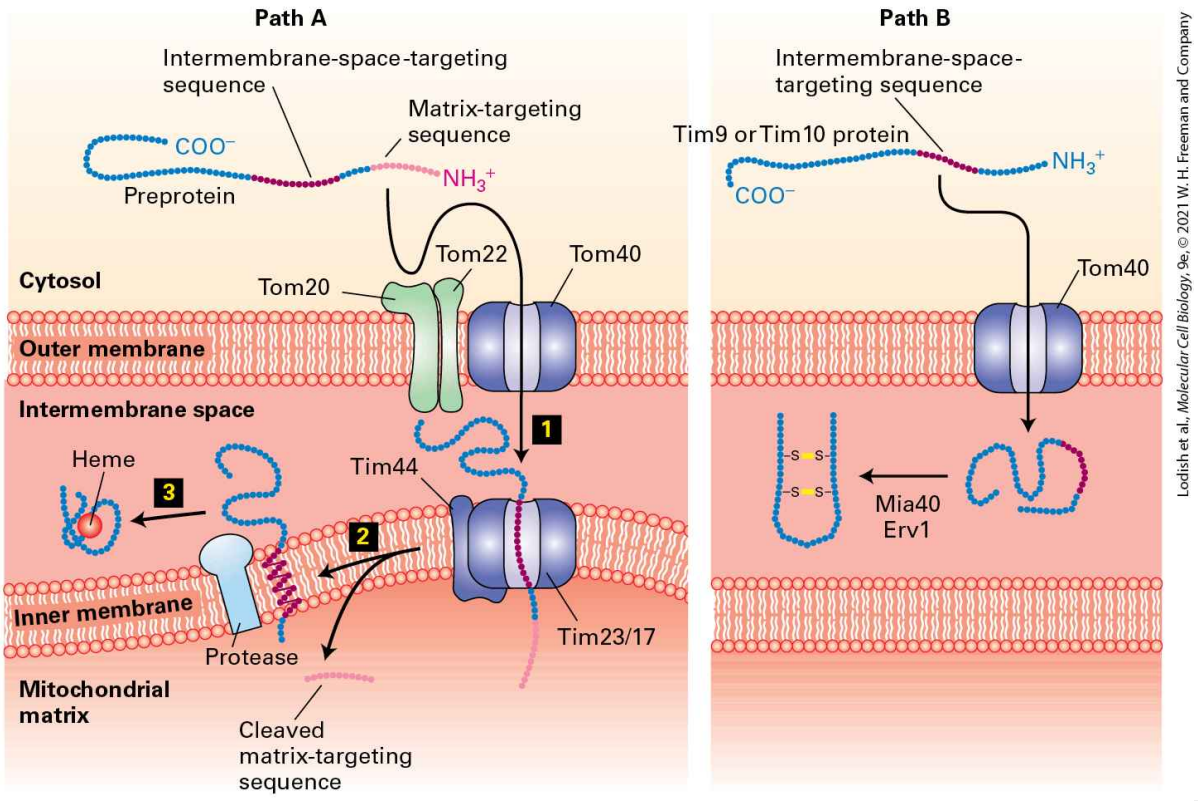


FIGURE 13-28 Two pathways to the mitochondrial intermembrane space. Pathway A, the major one for delivery of proteins from the cytosol to the intermembrane space, is similar to pathway A for delivery to the inner membrane (see Figure 13-26). The major difference is that the internal targeting sequence in these proteins, such as cytochrome b_2, is recognized by an inner-membrane protease, which cleaves the protein on the intermembrane-space side of the membrane. The released protein then folds and binds to its heme cofactor within the intermembrane space. Pathway B is a specialized pathway for delivery of the proteins Tim9 and Tim10 to the intermembrane space. These proteins readily pass through the Tom40 general import pore, and once they are in the intermembrane space, they fold and form disulfide bonds that prevent reverse translocation through Tom40. The disulfide bonds are generated by Erv1 and are transferred to Tim9 and Tim10 by Mia40. See R. E. Dalbey and A. Kuhn, 2000, *Annu. Rev. Cell Dev. Biol.* **16**:51; N. Pfanner and A. Geissler, 2001, *Nat. Rev. Mol. Cell Biol.* **2**:339; and K. Tokatlidis, 2005, *Cell* **121**:965–967.

Description

The illustration shows from top to bottom: cytosol, outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and mitochondrial matrix. Path

A shows a preprotein chain with an intermembrane-space-targeting sequence and matrix-targeting sequence at N-terminal in the cytosol. The outer membrane has a Tom 40 channel next to a Tom 20, Tom 22 receptor; and the inner membrane has Tim 23 slash 17 channel attached to a Tim 44 receptor. Step 1: The preprotein enters mitochondrial matrix via Tom 40, intermembrane space, and Tim 23 slash 17 where intermembrane-space-targeting sequence attaches to internal of Tim 23 slash 17 channel. Step 2: A protease embedded in inner mitochondrial membrane cleaves matrix-targeting sequence leaving behind an embedded preprotein. Step 3: The preprotein chain present in the intermembrane space cleaves and folds by enclosing a heme within.

Path B shows a Tim 9 or Tim 10 protein chain having an intermembrane-space-targeting sequence toward N-terminal in the cytosol. The chain enters intermembrane space via Tom 40 channel where Mia 40 E r v 1 acts on it resulting in a U-shaped protein with two disulfide bonds.

The small Tim9 and Tim10 proteins, which reside in the intermembrane space, illustrate a second pathway for targeting to the intermembrane space. Tim9 and Tim10 do not contain an N-terminal matrix-targeting sequence and are delivered directly to the intermembrane space via the general import pore without involvement of any inner-membrane translocation factors (see Figure 13-28, path B). Translocation through the Tom40 general import pore does not seem to be coupled to any energetically favorable process; however, once located in the intermembrane space, Tim9 and Tim10 proteins acquire two disulfide bonds each and fold into compact, stable structures. Apparently, the mechanism that drives their unidirectional translocation through the outer membrane involves passive diffusion through the outer membrane followed by folding and disulfide bond formation, which irreversibly traps the proteins in the intermembrane space. The process of disulfide bond

formation in the intermembrane space resembles that in the ER lumen and involves a disulfide bond–generating protein, Erv1, and a disulfide transfer protein, Mia40.

Outer-Membrane Proteins

Many of the proteins that reside in the mitochondrial outer membrane, including the Tom40 pore itself and mitochondrial porin, have a β-barrel structure in which antiparallel β strands form hydrophobic transmembrane segments surrounding a central channel. Such proteins are incorporated into the outer membrane by first interacting with the general import pore, Tom40; they are then transferred to the SAM (*s*orting and *a*ssembly *m*achinery) complex, which is composed of at least three outer-membrane proteins. Presumably it is the very stable hydrophobic nature of β-barrel proteins that ultimately causes them to be stably incorporated into the outer membrane, but precisely how the SAM complex facilitates this process is not known.

Import of Chloroplast Stromal Proteins Is Similar to Import of Mitochondrial Matrix Proteins

The most abundant proteins found in the chloroplast stroma are the enzymes of the Calvin cycle, which function in fixing carbon dioxide during photosynthesis (see Chapter 12). The large (L) subunit of ribulose 1,5-bisphosphate carboxylase (rubisco) is encoded by chloroplast DNA

and synthesized on chloroplast ribosomes in the stromal space. The small (S) subunit of rubisco and all the other Calvin cycle enzymes are encoded by nuclear genes and transported to chloroplasts after their synthesis in the cytosol. The precursor forms of these stromal proteins contain an N-terminal *stromal-import* sequence (see Table 13-1).

Experiments with isolated chloroplasts, similar to those with mitochondria, have shown that the general process of stromal import depicted in the top half of Figure 13-29 is similar to that of protein import into the mitochondrial matrix. At least three chloroplast outer-membrane proteins are known to be essential for directing proteins to the stroma. These include a receptor that binds the stromal-import sequence made up of proteins, known as *Toc159* and *Toc34*, each of which has a GTPase domain on the cytosolic side of the outer membrane reminiscent of SRP and SRP receptor. The receptor transfers the stromal-import sequence to a translocation channel made up of Toc75. A complex of at least five chloroplast inner-membrane proteins known as the *Tic complex* forms the translocation channel for passage of proteins into the stromal space. Although these proteins are functionally analogous to the receptor and channel proteins of the mitochondria, they are not structurally homologous. The lack of sequence similarity between these chloroplast and mitochondrial proteins suggests that they may have arisen independently during evolution.

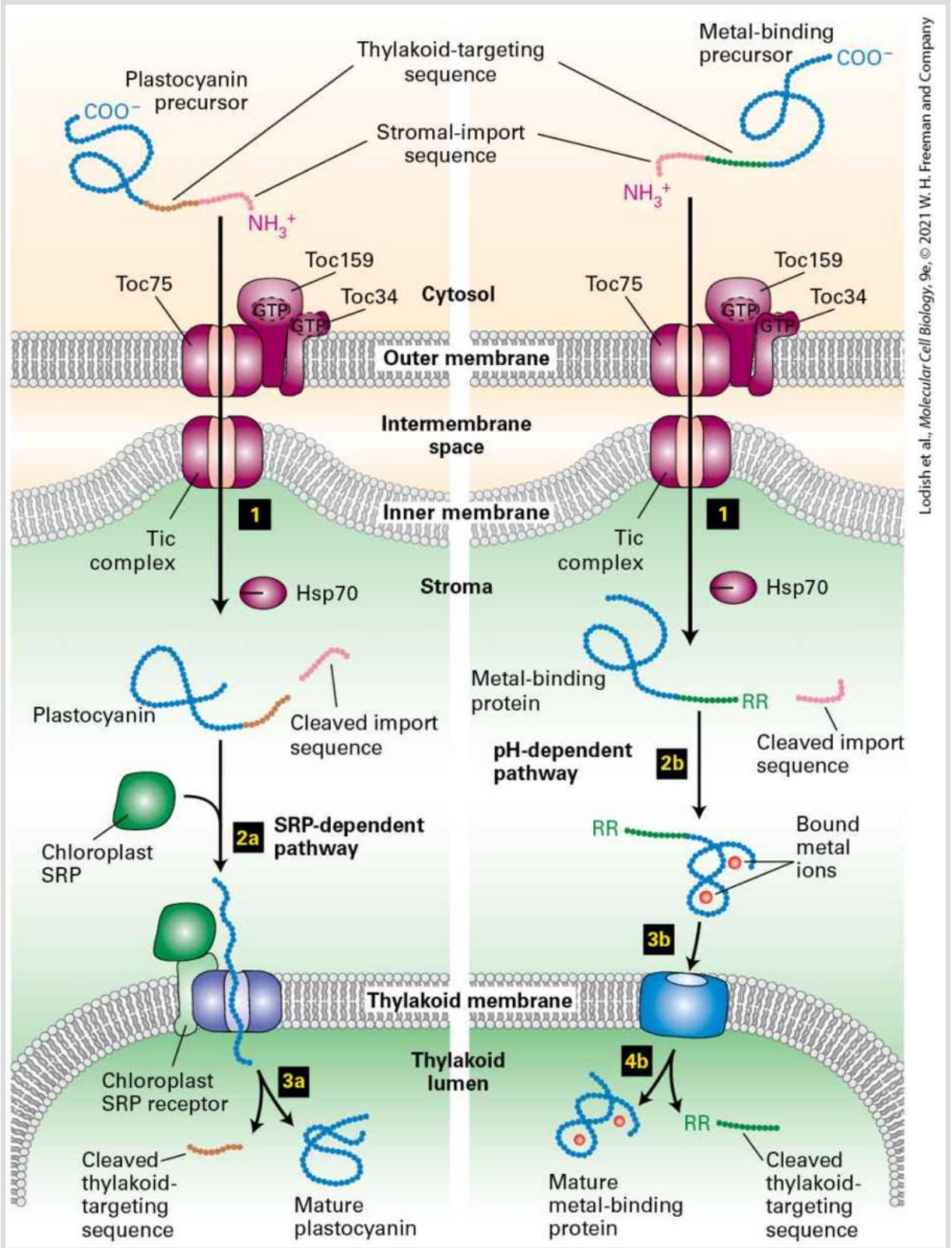


FIGURE 13-29 Transporting proteins to chloroplast thylakoids. Two of the four pathways for transporting proteins from the cytosol to the thylakoid lumen are shown here.

In these pathways, unfolded precursors are delivered to the stroma via the same outer-membrane proteins that import stromal-localized proteins. Cleavage of the N-terminal stromal-import sequence by a stromal protease then reveals the thylakoid-targeting sequence (step 1). At this point the two pathways diverge. In the SRP-dependent pathway (*left*), plastocyanin and similar proteins are kept unfolded in the stromal space by a set of chaperones (not shown), and the thylakoid-targeting sequence binds to proteins that are closely related to the bacterial SRP, SRP receptor, and SecY translocon, which mediate movement into the thylakoid lumen (step 2a). After the thylakoid-targeting sequence is removed in the thylakoid lumen by a separate endoprotease, the protein folds into its mature conformation (step 3a). In the pH-dependent pathway (*right*), metal-binding proteins fold in the stroma, and complex redox cofactors are added (step 2b). Two arginine residues (RR) at the N-terminus of the thylakoid-targeting sequence and a pH gradient across the inner membrane are required for transport of the folded protein into the thylakoid lumen (step 3b). The translocon in the thylakoid membrane is composed of at least four proteins related to proteins in the bacterial plasma membrane. The thylakoid-targeting sequence containing the two arginine residues is cleaved in the thylakoid lumen (step 4b). See R. Dalbey and C. Robinson, 1999, *Trends Biochem. Sci.* **24**:17; R. E. Dalbey and A. Kuhn, 2000, *Annu. Rev. Cell Dev. Biol.* **16**:51; and C. Robinson and A. Bolhuis, 2001, *Nat. Rev. Mol. Cell Biol.* **2**:350.

Description

The illustration from top to bottom shows cytosol, outer chloroplast membrane, intermembrane space, inner chloroplast membrane, stroma, thylakoid membrane, and thylakoid lumen. The outer chloroplast membrane has Toc 75 channel attached to Toc 159-G T P and Toc 34-G T P; and the inner chloroplast membrane has Tic complex channel.

The first part shows a plastocyanin precursor chain with stromal-import sequence at N-terminal followed by a thylakoid-targeting sequence in cytoplasm. Step 1: The precursor chain enters stroma through Toc 75, intermembrane space, and Tic complex. H s p 70 acts on the chain and cleaves stromal-import sequence leaving behind plastocyanin with thylakoid-targeting sequence. Step 2 a, S R P-dependent pathway: Chloroplast S R P binds to chloroplast S R P receptor embedded in thylakoid membrane next to a channel. This binding cause plastocyanin chain to enter thylakoid lumen. Step 3 a: The thylakoid-targeting sequence is cleaved from now matured plastocyanin.

The second part shows a metal-binding precursor chain with stromal-import sequence at N-terminal followed by a thylakoid-targeting sequence in cytoplasm. Step 1 is similar to first part except the thylakoid-targeting sequence of metal-binding protein has R R terminal. Step 2 b, p H-dependent pathway: This protein chain binds to two metal ions. Step 3 b: This enters thylakoid lumen via pore. Step 4 b: The thylakoid-targeting sequence with R R terminal is cleaved from now matured metal-binding protein bound to metal ions.

The available evidence suggests that chloroplast stromal proteins, like mitochondrial matrix proteins, are imported in the unfolded state. Import into the stroma depends on ATP hydrolysis catalyzed by a stromal Hsp70 chaperone whose function is similar to that of Hsp70 in the mitochondrial matrix and BiP in the ER lumen. Unlike mitochondria, chloroplasts do not generate an electrochemical gradient (proton-motive force) across their inner membrane. Thus protein import into the chloroplast stroma appears to be powered solely by ATP hydrolysis.

In addition to Hsp70, the stroma contains two other ATPases that may participate in protein import. These are a chloroplast version of the chaperone Hsp90, and a ring forming AAA+ ATPase known as *Hsp93*. Homologs of both proteins are known to catalyze energy-dependent protein-folding reactions in other contexts and it may be that chloroplast Hsp90 and Hsp93 participate in protein folding in the stroma, protein import into the stroma, or both.

Proteins Are Targeted to Thylakoids by Mechanisms Related to Bacterial

Protein Translocation

In addition to the double membrane that surrounds them, chloroplasts contain a series of internal interconnected membranous sacs, the **thylakoids** (see Figure 12-33). All of the chemical reactions of photosynthesis take place in the thylakoid membrane or lumen and are catalyzed by the proteins that are localized to this specialized subcompartment. Many of these proteins are synthesized in the cytosol as precursors containing multiple targeting sequences. For example, plastocyanin and other proteins destined for the thylakoid lumen require the successive action of two targeting sequences. The first is an N-terminal stromal-import sequence that directs the protein to the stroma by the same pathway that imports the rubisco S subunit. The second sequence targets the protein from the stroma to the thylakoid lumen. The role of these targeting sequences has been shown in experiments measuring the uptake of mutant proteins generated by recombinant DNA techniques into isolated chloroplasts. For instance, mutant plastocyanin that lacks the thylakoid-targeting sequence but contains an intact stromal-import sequence accumulates in the stroma and is not transported into the thylakoid lumen.

Four separate pathways for transporting proteins from the stroma into the thylakoid have been identified. All four pathways have been found to be closely related to analogous transport mechanisms in bacteria, illustrating the close evolutionary relationship between the thylakoid membrane and the bacterial plasma membrane. Transport of plastocyanin and related

proteins into the thylakoid lumen from the stroma occurs by an SRP-dependent pathway that uses a translocon similar to SecY, the bacterial version of the Sec61 complex (Figure 13-29, *left*). A second pathway for transporting proteins into the thylakoid lumen involves a protein related to bacterial protein SecA, which uses the energy from ATP hydrolysis to drive protein translocation through the SecY translocon. A third pathway, which targets proteins to the thylakoid membrane, depends on a protein related to the mitochondrial Oxa1 protein and the homologous bacterial protein (see Figure 13-27, path B). Some proteins encoded by chloroplast DNA and synthesized in the stroma or transported into the stroma from the cytosol are inserted into the thylakoid membrane via this pathway.

Finally, thylakoid proteins that bind metal-containing cofactors follow another pathway into the thylakoid lumen (see Figure 13-29, *right*). The unfolded precursors of these proteins are first targeted to the stroma, where the N-terminal stromal-import sequence is cleaved off, and the protein then folds and binds its cofactor. A set of thylakoid-membrane proteins assists in translocating the folded protein and bound cofactor into the thylakoid lumen. This process is powered by the H^+ electrochemical gradient normally maintained across the thylakoid membrane. The thylakoid-targeting sequence that directs a protein to this pathway includes two closely spaced arginine residues that are crucial for recognition. Bacterial cells also have a mechanism for translocating folded proteins with a similar arginine-containing sequence across the plasma membrane, known as the *Tat (t*win-*a*rginine *t*ranslocation) pathway. The molecular mechanism whereby these large folded globular proteins are translocated across the thylakoid membrane is not fully understood, but

the presence of a folded protein with an appropriate twin arginine signal appears to induce the oligomerization of Tat proteins in the membrane to form pore-like structures. In this respect, the Tat pathway resembles the pathway for the import of folded proteins into peroxisomes, described in the next section.

KEY CONCEPTS OF SECTION 13.4

Targeting of Proteins to Mitochondria and Chloroplasts

- Most mitochondrial and chloroplast proteins are encoded by nuclear genes, synthesized on cytosolic ribosomes, and imported post-translationally into the organelles.
- All the information required to target a precursor protein from the cytosol to the mitochondrial matrix or chloroplast stroma is contained within its N-terminal targeting sequence. After protein import, the targeting sequence is removed by proteases within the matrix or stroma.
- Cytosolic chaperones maintain the precursors of mitochondrial and chloroplast proteins in an unfolded state. Only unfolded proteins can be imported into the organelles.
- Proteins destined for the mitochondrial matrix bind to receptors on the outer mitochondrial membrane and are then transferred to the general import pore (Tom40) in the outer membrane. Translocation takes place at sites where the outer and inner membranes of the organelles are close together and occurs through the outer and inner membranes concurrently. Translocation is driven by ATP hydrolysis by Hsp70 in the matrix (see Figure 13-24) and by the proton-motive force across the inner membrane.
- Proteins sorted to mitochondrial destinations other than the matrix usually contain two or more targeting sequences, one of which may be an N-terminal matrix-targeting sequence (see Figure 13-26).
- Some mitochondrial proteins destined for the intermembrane space or inner membrane are first imported into the matrix and then redirected; others never enter the matrix, but go directly to their final location.
- Protein import into the chloroplast stroma occurs through outer-membrane and inner-membrane translocation channels that are analogous in function to mitochondrial

channels, but composed of proteins unrelated in sequence to the corresponding mitochondrial proteins.

- Proteins destined for the thylakoid have secondary targeting sequences. After entry of these proteins into the stroma, cleavage of the stromal-targeting sequences reveals the thylakoid-targeting sequences.
- The four known pathways for moving proteins from the chloroplast stroma to the thylakoid closely resemble translocation across the bacterial plasma membrane (see Figure 13-29). One of these systems can translocate folded proteins.