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The surprising complexity of peroxisome biogenesis

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Abstract

Peroxisomes are small organelles with a single boundary membrane. All of their matrix proteins are nuclear-encoded, synthesized on free ribosomes in the cytosol, and post-translationally transported into the organelle. This may sound familiar, but in fact, peroxisome biogenesis is proving to be surprisingly unique. First, there are several classes of plant peroxisomes, each specialized for a different metabolic function and sequestering specific matrix enzymes. Second, although the mechanisms of peroxisomal protein import are conserved between the classes, multiple pathways of protein targeting and translocation have been defined. At least two different types of targeting signals direct proteins to the peroxisome matrix. The most common peroxisomal targeting signal is a tripeptide limited to the carboxyl terminus of the protein. Some peroxisomal proteins possess an amino-terminal signal which may be cleaved after import. Each targeting signal interacts with a different cytosolic receptor; other cytosolic factors or chaperones may also form a complex with the peroxisomal protein before it docks on the membrane. Peroxisomes have the unusual capacity to import proteins that are fully folded or assembled into oligomers. Although at least 20 proteins (mostly peroxins) are required for peroxisome biogenesis, the role of only a few of these have been determined. Future efforts will be directed towards an understanding of how these proteins interact and contribute to the complex process of protein import into peroxisomes.

Introduction

Peroxisomes have a relatively simple morphology; they are small organelles, roughly 0.5 to 1.5 μm in diameter, with a single boundary membrane, and an electron-dense matrix which sometimes contains a granular or crystalline core. Initially, plant peroxisomes from a wide variety of plants and plant tissues appeared to be very similar to animal microbodies [88, 187]. It has become apparent, however, that although all plant peroxisomes share common features when viewed by electron microscopy, they are specialized for different metabolic functions in various tissues (reviewed in [13, 130, 212, 279, 281]). Perhaps this physiological diversity should have been the first clue that peroxisomes are more complex than their morphology suggests.

The biogenesis of peroxisomes has been a topic of controversy since it was first reviewed nearly twenty years ago [13], and many aspects remain a mystery [76, 268]. Each class of peroxisome can be distinguished by the complement of enzymes present in the matrix. These enzymes define the physiological role of the organelle. Although one might expect all peroxisomal proteins to possess a similar targeting signal, at least two different types of peroxisomal targeting signals (PTS) that direct proteins to the matrix have been identified (see below). Proteins possessing different PTSs appear to follow distinct pathways of import. The insertion of membrane proteins requires yet another type of targeting signal and corresponding pathway. A possible origin of the peroxisomal membrane from the endoplasmic reticulum (ER) has long been considered, but never proven convincingly. Multiple proteins have been identified that are required for peroxisome biogenesis in yeasts and mammalian cells; plant homologs and the functions they perform in plants have not been described. The mechanisms and significance of peroxisomes' ability to import fully folded and assembled proteins are also

unknown. Much progress has been made, but a thorough understanding of how peroxisomal membranes are synthesized, how matrix proteins are targeted to and translocated across the membrane, and how peroxisome proliferation is accomplished, is still beyond our grasp.

This review will focus on two major aspects of peroxisome biogenesis. First, I will discuss the different functional classes of plant peroxisomes and explain how they may be interconverted during different stages of the plant life cycle. Second, I will present some recent advances in our knowledge about the import of matrix proteins into peroxisomes, with mention made of relevant results pertaining to peroxisomal membrane proteins as well. Where possible, results from studies of plant peroxisomes will be included, but a complete discussion of peroxisome biogenesis can no longer be limited to a single group of organisms.

Model systems for studying peroxisome biogenesis

Castor bean endosperm and rat liver are rich sources of peroxisomes commonly used for the early studies on peroxisome biogenesis [13, 150, 151]. More recently, peroxisomes from germinating seedlings (including castor bean) and mammals (primarily rat liver) have been isolated for reconstitution of the protein import process *in vitro* [14, 29, 37, 134, 182, 184, 189, 245]. The use of *in vitro* assays allows investigators to subdivide the import process into separate steps and to biochemically manipulate the conditions for import. It is important to be sure that the *in vitro* assays faithfully represent the process as it exists *in vivo*. Such a correlation is now possible, since Trelease's group has developed a system in which they can study the transient expression of microprojectile-bombarded constructs as the proteins are localized to peroxisomes in suspension-cultured tobacco cells [11]. In addition, several researchers have examined the targeting of proteins to peroxisomes in transgenic tobacco [89, 170, 213, 301] and *Arabidopsis* [121, 143, 210]. The advantage of using suspension-cultured tobacco cells and transgenic plants is primarily limited to the study of targeting determinants for peroxisomal proteins. *In vivo* systems are not appropriate to answer specific mechanistic questions or to identify the individual components required for protein import into peroxisomes because it is difficult to sufficiently control all the factors required for the import process. The greatest challenge with *in vitro* import assays has been to prove that they are reproducible and provide physiologically relevant and consistent results [162, 165], but recent results from my laboratory [29, 30] and Baker's research group [14, 129] indicate that *in vitro* assays with isolated plant peroxisomes meet these criteria.

Although protein import into yeast peroxisomes is also studied using *in vitro* assays [253, 255, 277] and *in vivo* approaches [2, 188], the real advantage of yeasts has come from the isolation of peroxisome-deficient mutant strains [155, 159, 160, 265, 292, 295]. Yeast cells can survive without functional peroxisomes, when grown on complete media. Mutants in peroxisome biogenesis were identified by screening for cells unable to grow on alternative carbon sources, such as oleic acid or methanol. Such peroxisome-deficient mutants (*pex* mutants) appear to mimic the cellular phenotypes diagnostic of certain human peroxisomal disorders [75, 77, 289, 335, 336]. Peroxisomes may be very small or completely absent in mutant cells, or present as membrane ghosts only. These mutants have led to the identification of many components believed to be required for the biogenesis of peroxisomes (see [4, 67, 152, 256] and references in [55]). By a recently unified system of nomenclature, the peroxisome biogenesis factors are now termed peroxins, represented by the PEX gene acronym [55] and summarized in Table 2.

Thus, it seems that the study of peroxisome biogenesis in plants can contribute to an understanding of the mechanisms involved in the process by exploiting the *in vitro* assay that is now available. The energy requirements for peroxisomal protein import and nonessential components of the import pathway cannot be determined using *in vivo* assays or genetic approaches. *Pex* mutants are unlikely to be isolated in plants because peroxisomes appear to be essential organelles, but we can certainly use the information provided by the *pex* mutants from yeasts and mammalian cells to identify the corresponding plant genes. It is reasonable to suspect that plant homologs exist based on the conservation of peroxisomal targeting signals and transport pathways from evolutionarily diverse organisms, which suggests the presence of unifying mechanisms between the lower and higher eukaryotes [76, 265, 266, 268]. Once plant peroxins have been identified, the *in vitro* assays will provide a powerful tool to examine their interactions and their roles in peroxisome biogenesis.

Classes of plant peroxisomes

Peroxisomes have been studied in a wide variety of plant species – from algae to ferns and gymnosperms – in addition to numerous angiosperms (see [7, 88, 91, 92, 111, 112, 187, 260, 262, 280, 321]), but all of the work on peroxisome biogenesis has been done with peroxisomes from angiosperms. Peroxisomes in all organisms are morphologically similar respiratory microbodies which contain hydrogen peroxide-producing oxidases and catalase to inactivate the reactive peroxides [13, 51, 86, 130, 212, 281, 292]. In higher plants, at least four classes of peroxisomes have been defined, based on the specific physiological role of the organelle, which is specified by the set of enzymes sequestered in the matrix. The characteristics of the three specialized classes of plant peroxisomes are summarized in Table 1.

Glyoxysomes

Glyoxysomes were the first peroxisomes to be isolated from plants [27, 28, 43]. All of the enzymes of the glyoxylate cycle, except for cytosolic aconitase [45, 48, 122], are uniquely localized to glyoxysomes [27, 42, 94, 212]. Enzymes for the β-oxidation of very-long-chain fatty acids are also found in glyoxysomes [13, 28, 41, 42, 131, 292], allowing the organelles to mobilize storage lipids to provide nutrition for the growing heterotrophic seedlings. Glyoxysomes are most prevalent in postgerminative seedlings of oilseed plants, including castor bean endosperm and cotyledons from pumpkin, cottonseed, watermelon, cucumber, and *Brassica napus*, to name a few of the most common sources. In addition, glyoxysomes are present in senescent organs [20, 49, 50, 116, 220]; some of the enzymes of the glyoxylate cycle have been detected in pollen [333] and developing seeds [39, 79, 156, 291] as well.

Leaf peroxisomes

In photosynthetic tissues, leaf peroxisomes participate in the reactions of photorespiration, in cooperation with chloroplasts and mitochondria [87, 208, 279]. This class of peroxisomes is characterized by enzymes such as glycolate oxidase, hydroxypyruvate reductase, and serine:glyoxylate aminotransferase, but the enzymes of β-oxidation and catalase, of course, are also present.

Root peroxisomes

A third type of peroxisome is found in the uninfected cells of nodules on legume roots, including soybean, black locust, and cowpea [141, 142, 198, 313]. Peroxisomes in these cells are much enlarged compared to peroxisomes in infected cells of legume nodules and contain the enzyme uricase [17, 141, 142, 199, 313]. Uricase is one of the final enzymes of ureide biosynthesis (reviewed in [246]). Thus, nitrogen is fixed in the infected cells of the nodule, metabolized into ureides in the uninfected cells, and then transported to the rest of the plant.

Unspecialized peroxisomes

Other plant tissues also contain peroxisomes, but perhaps because they have been studied less and their specific metabolic roles are unknown, they are usually designated as ‘unspecialized’ peroxisomes [130, 212]. These organelles are often smaller than glyoxysomes and leaf peroxisomes. Unspecialized peroxisomes contain catalase and, usually, hydrogen peroxide-producing oxidases.

Algal peroxisomes

The distribution of typical peroxisomal enzymes in algae is unusual. Enzymes of the β-oxidation pathway, such as thiolase and acyl-CoA oxidase, may be found in peroxisomes only (*Mougeotia*), in mitochondria only (*Bumilleriopsis*, *Vaucheria*, *Pyramimonas*), or in both peroxisomes and mitochondria (*Eremosphaera*, *Platymonas*, *Heteromastix*, *Pedinomonas*) [7, 260, 321]. Peroxisomes in the alga *Vaucheria* contain catalase and some enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase) as well as the photorespiratory enzyme glycolate oxidase [7]. Mitochondria in this alga also possess some enzymes of the glyoxylate cycle and all the enzymes for fatty acid β-oxidation. Thus, algal peroxisomes may have physiological roles quite different from their higher-plant counterparts.

Transitions between classes of peroxisomes

Mature seeds contain numerous glyoxysomes that persist during desiccation and imbibition and then enlarge dramatically during germination [156]. Cottonseed glyoxysomes obtain the membrane lipids they need for this rapid enlargement from nearby lipid bodies [36].

Table 1. Characteristics of specialized classes of higher-plant peroxisomes.

	Glyoxysomes	Leaf peroxisomes	Root peroxisomes
Location	oilseed cotyledons, senescent organs	photosynthetic cotyledons, leaves	root nodules
Specialized function	fatty acid metabolism	photorespiration	nitrogen transport
Specialized enzyme system	glyoxylate cycle	glycolate metabolism	ureide metabolism
Representative enzymes	isocitrate lyase, malate synthase	glycolate oxidase, hydroxypyruvate reductase	uricase, allantoinase
Common enzyme systems	peroxide metabolism	peroxide metabolism	peroxide metabolism
Representative enzymes	catalase, oxidases	catalase, oxidases	catalase, oxidases
	fatty acid β-oxidation	fatty acid β-oxidation	fatty acid β-oxidation?
	thiolase, multifunctional enzyme	thiolase, multifunctional enzyme	thiolase, multifunctional enzyme

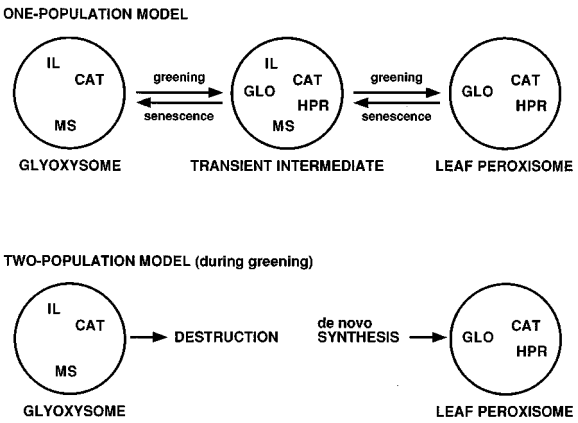

Figure 1. Two major models proposed to explain the transition between classes of higher-plant peroxisomes. In the one-population model, there is a continuum during the conversion between classes, represented by the organelle labeled 'transient intermediate' that contains enzymes representative of both classes of peroxisomes. During greening, light stimulates the conversion of glyoxysomes into leaf peroxisomes in oilseed cotyledons. A reversal of this conversion is seen during senescence, when leaf peroxisomes become glyoxysomes. The two-population model is illustrated only for the transition that occurs during greening; glyoxysomes in heterotrophic cotyledons degrade, leaf peroxisomes arise *de novo* in photosynthetically active cotyledons. Overwhelming evidence supports the one-population model (see text). CAT, catalase; GLO, glycolate oxidase; HPR, hydroxypyruvate reductase; IL, isocitrate lyase; MS, malate synthase.

As seedlings undergo the transition from heterotrophic to autotrophic growth, glyoxysomes in the cotyledons are converted into leaf-type peroxisomes. There were at least two models tested to explain this process (Figure 1). The two-population hypothesis proposed that glyoxysomes disappear during the transition stage of growth and are replaced by newly generated leaf-type peroxisomes. Despite significant controversy (see [13]), the most convincing evidence has been presented for the one-population, or repackaging, hypothesis [15, 203, 205, 244, 278]. The one-population model describes a direct transition from glyoxysomes to leaf peroxisomes through a brief intermediate stage. During this transition stage, glyoxylate cycle enzymes are detected in the same organelle with photorespiratory enzymes. As cotyledons become green, the glyoxylate cycle enzymes are degraded [15, 20, 144, 189, 190] and the membrane composition changes somewhat [300, 327]. Light activates the synthesis of photorespiratory enzymes such as glycolate oxidase [20], which are then post-translationally transported into the transition peroxisomes. A variety of transcriptional and post-transcriptional control mechanisms act to ultimately stop expression of glyoxysome-specific enzymes and begin production of leaf peroxisome proteins [39, 79, 110, 200, 203], leading to a complete and direct con-

version of glyoxysomes into leaf-type peroxisomes in photosynthetically active cotyledons.

During senescence of mature cotyledons and leaves, a reverse transition from leaf-type peroxisomes to glyoxysomes occurs [49, 50, 109, 116, 157, 203, 204, 219, 220, 299]. During this conversion process, there is a decrease in photorespiratory activity and an increase in glyoxylate cycle metabolism as the matrix enzymes involved in these pathways are exchanged. Double-labeling experiments with gold particles of different sizes show that enzymes of leaf peroxisomes and glyoxysomes coexist in the same organelle [204, 244, 278]. In fact, when senescence of mature green *B. napus* cotyledons is induced, a marked increase in both uricase activity (probably indicating increased purine metabolism for mobilization of RNA) and glyoxylate cycle enzymes (for gluconeogenesis from fatty acids) is observed, while glycolate oxidase is still present [299]. Vicentini and Matile [299] concluded that these senescent microbodies, which they termed 'gerontosomes', are multifunctional organelles involved in all three major peroxisomal functions: glyoxylate cycle, photorespiration, and purine catabolism.

Peroxisomes become more abundant during the senescence of cut carnations [62, 63]; enzymes from the glyoxylate cycle, purine catabolism, and β-oxidation are detected in pumpkin petals after blooming is finished [49]. This suggests that versatility of peroxisome function may also occur during flower senescence.

In each case, however, it is unlikely that protein import plays a determinative role in specifying organelle function. Leaf-type and root peroxisomes are capable of importing glyoxysomal enzymes when they are expressed ectopically in transgenic plants [89, 121, 143, 210, 213]. This seemingly universal peroxisomal protein import capacity extends to peroxisomes from other organisms; the peroxisomal protein firefly luciferase can be imported into peroxisomes from plants, yeasts, mammals, and insects [147]. In addition, isocitrate lyase from cottonseed glyoxysomes is imported into cultured mammalian cells [282] and, conversely, rat liver catalase is targeted to glyoxysomes in tobacco suspension cultured cells [284]. Thus, the mechanisms of peroxisomal protein import are conserved in these evolutionarily diverse organisms and do not, by themselves, define the different classes of peroxisomes found in higher plants.

Peroxisomal protein targeting signals

Peroxisomes contain no DNA [59, 139], have neither matrix- nor membrane-associated ribosomes, and lack an internal membrane system. All peroxisomal proteins are nuclear-encoded, and at least the matrix proteins are synthesized on free ribosomes in the cytosol and are post-translationally transported to the organelle [24, 100, 117, 162, 165, 212, 237, 334, and reviews cited below]. The pathway of peroxisomal membrane protein transport is not as well established (see below). A discussion of peroxisomal targeting signals (PTSs) has been included in several recent general reviews, with emphasis on yeast and mammalian systems [52, 76, 183, 215, 230, 238, 263, 265–267]. The focus here is to integrate our knowledge of PTSs from higher plants into the bigger picture.

The majority of peroxisomal proteins are synthesized at their final, mature size and are not detectably processed during or after import into the matrix. These proteins are targeted to peroxisomes by a carboxyl-terminal PTS1. Some peroxisomal matrix proteins possess an amino-terminal PTS2 which is often proteolytically cleaved after import, though this processing event has not been shown to be tightly coupled to translocation. A few peroxisomal proteins, including most peroxisomal membrane proteins, contain neither a PTS1 nor a PTS2, and may be sorted by an internal signal. Each of these types of targeting signals will be discussed below.

PTS1

The first PTS identified (PTS1) is a tripeptide, serine-lysine-leucine (SKL), that is present at the carboxyl terminus of firefly luciferase and is both necessary and sufficient to target the protein to peroxisomes in insect and mammalian cells [106, 146]. Antibodies raised against an SKL-COOH synthetic peptide recognize PTS1 proteins in cells from yeasts, plants, and trypanosomes, providing further evidence of a common evolutionary origin for microbodies from these organisms [108, 147]. A survey of peroxisomal proteins from plants, yeasts, and mammals, shows that most, but not all, contain the PTS1 tripeptide, or conserved variants of it [1, 3, 52, 76, 108, 211, 225].

Several groups have studied the ability of certain conserved substitutions in the SKL signal sequence to preserve peroxisomal protein targeting [1, 21, 71, 105, 121, 181, 194, 196, 197, 227, 257, 271, 282, 284]. The range of acceptable substitutions in the PTS1 se-

quence appears to be much greater for plants than for mammals and yeasts [196]. In some cases, a degenerate PTS1 is sufficient to target the passenger protein chloramphenicol transferase to plant or mammalian peroxisomes, but the efficiency of transport is compromised compared to more conserved signals [196, 197, 265, 271]. Despite a high degree of species specificity, it appears that the general consensus sequence consists of a small, neutral amino acid in the first position, a positively charged residue in the second position, and a hydrophobic amino acid in the third, carboxyl-terminal, position.

The carboxyl-terminal location of the PTS1 is critical for its function. When a single amino acid is added to the carboxyl terminus of firefly luciferase, the protein is no longer targeted to peroxisomes [52, 105, 186]. The peroxisomal protein amine oxidase has a PTS1-like sequence near but not at the carboxyl terminus; this tripeptide is not involved in targeting [33, 238]. Placement of a PTS1 at the amino terminus or at several internal locations in the cytosolic protein dihydrofolate reductase (DHFR) does not redirect it to peroxisomes [265]. Hundreds of cytosolic proteins have internal PTS1-like tripeptides that obviously do not mistarget the proteins to peroxisomes [265]. The fact that the carboxyl terminus is the last part of the protein released from the ribosome during synthesis is consistent with the post-translational nature of import of peroxisomal matrix proteins with a PTS1 [265, 267].

Catalase is the classic marker enzyme for peroxisomes. Yet the carboxyl-terminal tripeptide on most peroxisomal catalase sequences does not constitute a typical PTS1 [101, 107, 153, 201, 211, 214, 227, 229, 233, 248, 269]. Until recently, the targeting signal for catalase was thought to be an SKL-like peptide located six to fourteen amino acids away from the carboxyl terminus. Trelease *et al.* found that rat liver catalase is targeted to peroxisomes by the carboxyl-terminal tripeptide (ANL); the internal SKL is not functional in its location eight amino acids upstream [284]. Cottonseed catalase is sorted to plant peroxisomes by another non-consensus PTS1 (the tripeptide PSI) [197]. The context of this signal is important. The tripeptide PSI is necessary for proper catalase localization, but it is not sufficient to target a passenger protein to glyoxysomes [197]. The inclusion of additional upstream residues between the carboxyl-terminal tripeptide and the passenger protein causes the chimeric protein to be localized to glyoxysomes [197]. Similarly, Purdue and Lazarow [227] found that the carboxyl-terminal tetrapeptide KANL is both necessary and sufficient to direct import into human fibroblast and yeast peroxisomes, although the tripeptide ANL by itself is not sufficient. Kragler *et al.* [153] found two independent regions of catalase A, each of which is sufficient to function as a PTS. The carboxyl-terminal hexapeptide alone is sufficient, but a construct lacking this hexapeptide is still peroxisomal, indicating that the carboxyl-terminal signal is not necessary. A region in the amino-terminal third of the protein is also sufficient to target catalase A to yeast peroxisomes. Thus, it appears that a non-consensus carboxyl-terminal PTS1 targets catalase to peroxisomes, but its function is context-dependent and does not involve the SKL-like tripeptide usually located slightly upstream in the polypeptide.

There has also been some recent confusion about the PTS for the glyoxylate cycle enzyme isocitrate lyase, despite the fact that the carboxyl terminus represents an apparently acceptable PTS1 sequence [12, 39, 52, 211, 274, 290]. The carboxyl-terminal 37 amino acids from *B. napus* isocitrate lyase are necessary for its peroxisomal localization and the carboxyl-terminal five amino acids are sufficient to direct chloramphenicol acetyltransferase to leaf peroxisomes in transgenic *Arabidopsis* [210]. Baker's group, however, found that the carboxyl terminus of castor bean isocitrate lyase is dispensable for targeting to isolated sunflower glyoxysomes (*in vitro*), to leaf peroxisomes in transgenic tobacco (*in vivo*) [14, 89], and to yeast peroxisomes [274]. Trelease and his coworkers showed that the carboxyl-terminal tetrapeptide of cottonseed isocitrate lyase, which has the same sequence (KARM) as castor bean isocitrate lyase, is necessary for *in vivo* peroxisomal import in transiently transfected mammalian cells [282] and suspension-cultured tobacco BY-2 cells [166]. The tripeptide ARM is sufficient to target a reporter protein to glyoxysomes in suspension-cultured cells [166]. These seemingly conflicting results may be at least partially explained by the piggybacking experiments in which isocitrate lyase lacking its carboxyl-terminal tripeptide associates with subunits possessing a PTS1 and the entire multimer is localized to glyoxysomes *in vivo* [166]. Alternatively, modified derivatives of isocitrate lyase may, in some cases, be unstable in the cytoplasm *in vivo* [210, 212]. It is also possible that, *in vitro*, isocitrate lyase deletion mutants assume a nonnative conformation such that one of the internal SKL-like tripeptides or another previously unrecognized sequence is exposed and able to function as a potential redundant PTS. It

seems unlikely that species differences are responsible for differential targeting signal properties for isocitrate lyase from various plant species, although this possibility cannot yet be completely discounted. Like catalase (see discussion above) and other peroxisomal proteins [56, 71, 76, 106, 283], it appears that the context of the PTS1 and the conformation of the protein may be important for proper targeting of isocitrate lyase to glyoxysomes.

PTS2

Although the carboxyl-terminal tripeptide PTS1 is used by the vast majority of peroxisomal matrix proteins, other signals serve in a similar capacity. An amino-terminal region on some peroxisomal matrix proteins constitutes a second type of PTS, termed a PTS2 [52]. The PTS2 is a nonapeptide with a consensus sequence of (R/K)(L/I/Q/V)X_5(H/Q)(L/A) located near the amino terminus of the peroxisomal protein [52, 76, 94, 178, 212, 238, 263-265]. The arginine residue and both leucines are critical for PTS2 function in *Saccharomyces cerevisiae* [95, 98]. Unlike the PTS1, the PTS2 can function at internal positions in the polypeptide: the PTS2 is usually near, but not at, the amino terminus of the protein [266, 267].

The only PTS2 protein identified in mammals is the fatty acid β-oxidation enzyme thiolase [25, 126, 127, 216, 270, 285]. An 11-residue region from the amino terminus of rat thiolases A and B is both necessary and sufficient for peroxisomal targeting [216, 270]. The rat sequence is nearly identical to the PTS2 of human thiolase [25] and very similar to the predicted PTS2 of yeast [132, 270] and plant thiolases [23, 145, 209, 223]. Until recently, the only other known PTS2 protein in yeasts was amine oxidase from *Hansenula polymorpha* [33, 81, 82]. The amino-terminal PTS2 of pumpkin citrate synthase is sufficient to target a passenger protein into glyoxysomes, leaf peroxisomes, and unspecialized microbodies of transgenic *Arabidopsis* [143]. Citrate synthase uses a PTS1 in *S. cerevisiae* [251]. Similarly, malate dehydrogenase is targeted to yeast peroxisomes by a PTS1 [261], but uses a PTS2 for targeting to glyoxysomes in plants [93, 95, 115, 149]. Surprisingly, the peroxisomal protein Pex8p possesses both a PTS1 and a PTS2 [178, 311]. So far it has only been cloned from *H. polymorpha* [311] and *Pichia pastoris* [167], but presumably a homolog exists in mammalian cells; this would represent the second mammalian PTS2 protein.

In higher eukaryotes, including plants and mammals, the amino-terminal presequence is proteolytically processed following import [25, 95, 115, 127, 143, 209, 216, 223, 270, 285]. The PTS2 is not cleaved from the mature protein in yeasts [18, 72, 81, 82, 98, 270]. A 110 kDa metalloprotease from mammalian peroxisomes was identified that degrades a synthetic presequence of thiolase, but does not process full-length *in vitro* synthesized thiolase [6]. Gietl and her colleagues, however, have isolated from germinating castor bean endosperm a plant cysteine endopeptidase that specifically processes glyoxysomal malate dehydrogenase to its mature form *in vitro*; it is unable to cleave the presequence from thiolase at the proper site [96].

Internal signals

Some peroxisomal matrix proteins contain neither a recognizable PTS1 nor a PTS2 consensus sequence. Two regions of *Candida tropicalis* acyl-CoA oxidase have each been shown to be sufficient to target a passenger protein to peroxisomes *in vitro* [255] and *in vivo* [140]. One domain is amino terminal, but not cleaved, the other domain is internal; neither have sequences that resemble a PTS1 or PTS2. (Acyl-CoA oxidase from rat uses a PTS1 [185, 186].) As mentioned above, catalase A from *S. cerevisiae* has a functional internal PTS [153]. *S. cerevisiae* carnitine acetyl transferase also appears to possess a PTS localized internally [70].

In plants, peroxisomal targeting of the enzyme hydroxypyruvate reductase may be species-specific. Two forms of hydroxypyruvate reductase may be produced by alternative splicing in pumpkin; only one of the isozymes has a PTS1 [123]. *Arabidopsis* hydroxypyruvate reductase contains a PTS1 [169], but the cucumber homolog does not, so it may use an internal PTS [114, 247]. Baker's group contends that castor bean isocitrate lyase [14] and spinach glycolate oxidase [129] also can be imported into isolated sunflower glyoxysomes via unidentified internal targeting signals, though both possess acceptable (and apparently functional) carboxyl-terminal PTS1 tripeptides [12, 301, 302]. A true role for each of the putative internal PTSs cannot be confirmed until a specific interaction between the targeting domain and a peroxisomal protein receptor is proven.

Targeting of membrane proteins

Much less is known about the targeting of membrane proteins to peroxisomes, primarily because fewer membrane proteins have been characterized. Integral membrane proteins seem to use signals other than PTS1 or PTS2 for targeting to peroxisomes [76, 178, 212, 265]; these signals are termed mPTSs, though there is no evidence for a consensus sequence, structure, or even location within the protein yet. None of the known peroxisomal integral membrane proteins possess recognizable PTS1 or PTS2 motifs [76, 178, 212, 265]. Antibodies against PTS1-containing peptides recognize many peroxisomal proteins from a variety of organisms, but decorate only the matrix of the organelle, not the membrane [147, 265]. Several peroxisome assembly mutants in yeasts are impaired in their ability to import peroxisomal matrix proteins, but peroxisomal membrane ghosts are present [69, 75, 76, 159, 168, 175, 226, 265, 268, 295]. Similar peroxisomal membrane ghosts are observed in fibroblasts from patients with Zellweger syndrome and other peroxisome biogenesis disorders [159, 160, 163, 191, 193, 239, 242, 243, 252, 254, 268, 319]. Thus, the targeting signals for peroxisomal membrane proteins are different from the PTSs for matrix proteins, and peroxisomal membrane proteins follow an independent assembly pathway.

The first mPTS to be defined is a 20-residue hydrophilic loop between two of the six transmembrane domains of *C. boidinii* Pmp47p. This region, which is predicted to protrude from the membrane surface into the matrix, is both necessary and sufficient for targeting to the peroxisome membrane [64, 174]. Mutation of either the carboxyl-terminal tripeptide or an internal SKL did not prevent proper targeting and insertion of Pmp47p into the membrane [102]. The only plant mPTS studied so far, cottonseed ascorbate reductase, is similar to the signal in Pmp47p (R.N. Trelease and R.T. Mullen, unpublished results). The mPTS of *S. cerevisiae* Pas21p is present within the carboxyl-terminal 82 amino acids [76]. In contrast, an amino-terminal region of 40–45 amino acids of Pex3p from *S. cerevisiae* and *P. pastoris* is sufficient to target and anchor a passenger protein in the peroxisomal membrane [76, 317]. Surprisingly, the first 16 amino acids of *H. polymorpha* Pex3p target a reporter protein to the ER [8, 76]. The peroxisomal integral membrane protein PMP70 appears to contain an internal sequence that is required for membrane insertion in mammals [133]. The only thing all mPTSs seem to have in common is that they are different from the PTS1 and PTS2 sequences.

PTS receptors and components of the translocation machinery

At least two pathways for the import of peroxisomal matrix proteins have been defined; each PTS interacts with a different specific recognition factor to determine separate transport pathways. A great deal of biochemical and genetic evidence has led to the identification of these recognition factors and suggests their interactions with other components of the translocation machinery. The discovery of yeast and mammalian mutants blocked in either or both of the import pathways has greatly facilitated the cloning and characterization of at least 20 proteins involved in peroxisome biogenesis (reviewed in [76, 178, 266–268]). Most of these proteins are termed peroxins (acronym PEX) according to a unified system of nomenclature established to simplify the classification of homologs from a number of species [55]. A summary of the peroxins and their characteristics is provided in Table 2.

The PTS1 receptor, Pex5p

The PTS1 receptor was first cloned by complementation of the yeast mutants *pas8* in *P. pastoris* [175] and *pas10* in *S. cerevisiae* [294]. These mutants are unable to import PTS1-containing proteins, but PTS2 proteins, such as thiolase, are imported correctly. In humans, a defect in PEX5 (HsPxr1p, HsPTS1R) causes the fatal peroxisome biogenesis disorders assigned to complementation group 2, including some cases of Zellweger syndrome and neonatal adrenoleukodystrophy [57, 85, 266, 268, 318].

The PEX5 gene has been cloned from humans, mouse, and four species of yeasts [9, 57, 85, 175, 207, 272, 293, 294, 318]. In a recent review, Gietl mentions that her group has cloned PEX5 from watermelon using degenerate PCR primers homologs to conserved regions of the reported PEX5 sequences (unpublished results cited in [94]). Though this work is not yet published, it is likely that plant PEX5 genes will be identified in the very near future.

The sequence of Pex5p suggests a bipartite organization. The carboxyl terminus of Pex5p contains 7 consecutive tetratricopeptide repeat (TPR) motifs of 34 amino acids each [57, 85, 94, 175, 207, 265, 272, 293, 294, 318]. Other members of the TPR protein

Table 2. Peroxins[a]

Name[b]	Characteristics	Size (kDa)	Species[c] former names[d]	Refs.
Pex1p	AAA-ATPase; two AAA ATP-binding domains; cytosolic and vesicle-associated; may cause infantile refsum disease, neonatal adrenoleukodystrophy, Zellweger syndrome CG1[e]	117–127	ScPas1p PpPas1p HsPex1p	78 125 221, 234
Pex2p	C_3HC_4 zinc-finger motif; integral peroxisomal membrane protein; may cause Zellweger syndrome CGg5, CG10	35–52	RnPAF1 HsPAF1 ChPAF1 PaCar1p PpPer6p YlPay5p LdPex2p	286 249 288 19 309 65 84
Pex3p	integral peroxisomal membrane protein; mPTS in first 40 amino acids	51–52	ScPas3p HpPer9p PpPas2p	128 8 317
Pex4p	related to ubiquitin-conjugating enzymes; associated with the cytosolic surface of the peroxisomal membrane	21–24	ScPas2p PpPas4p	316 46
Pex5p	PTS1 receptor; contains 7–8 tetratricopeptide motif repeats; localized to peroxisomal membrane, matrix, and cytoplasm; may cause neonatal adrenoleukodystrophy, Zellweger syndrome CG2	64–69	PpPas8p ScPas10p HsPxr1p HsPTS1R HpPer3p HpPah2p YlPay32p MmPxr1p	175 294 57 85, 318 293 207 272 9
Pex6p	AAA-ATPase; 1–2 AAA ATP-binding domains; import not completely inhibited in disruption; some homology to myosin; cytosolic and vesicle-associated; may cause Zellweger syndrome CG4	112–127	PpPas5p ScPas8p YlPay4p RnPAF2 HsPXAAA1	259 303 206 287 326
Pex7p	PTS2 receptor; 6 WD-40 repeats; PTS in amino-terminal 56 amino acids; responsible for rhizomelic chondrodysplasia punctata in humans; localized to cytosol, peroxisomal membrane and matrix; may cause Zellweger syndrome CG11	37–42	ScPas7p ScPeb1p HsPex7p KlPex7p MmPex7p	173, 232 330, 331 26, 195, 228 in 268 26
Pex8p	contains both a PTS1 and a PTS2; localized to peroxisomal matrix and inner surface of peroxisomal membrane	71–81	HpPer1p PpPer3p ScPas6p YlPex8p	311 167 in 76 256
Pex9p	integral peroxisomal membrane protein with a cysteine-rich region	42	YlPay2p	66
Pex10p	C_3HC_4 zinc-finger motif; integral peroxisomal membrane protein; involved in peroxisome proliferation or lumen formation	34–48	HpPer8p PpPas7p	237 137
Pex11p	peroxisomal membrane protein; involved in peroxisome proliferation; deficiency results in giant peroxisomes	27–32	ScPmp27p CbPmp30p	73, 171, 172 240
Pex12p	C_3HC_4 zinc-finger motif (degenerate?); integral peroxisomal membrane protein; may cause Zellweger syndrome CG3	40–48	PpPas10p ScPex12p HsPex12p	136 in 268 35, 208a
Pex13p	membrane receptor for PTS1 receptor (Pex5p); carboxyl-terminal SH3 domain; integral peroxisomal membrane protein; putative docking protein for peroxisomal protein import	40–43	PpPex13p HsPex13p ScPex13p	104 104 68, 74

Table 2. Continued

Name[b]	Characteristics	Size (kDa)	Species[c] former names[d]	Refs.
Pex14p	membrane receptor for both the PTS1 and PTS2 receptors; putative point of convergence of the PTS1- and PTS2-dependent protein-import pathways; peripheral peroxisomal membrane protein, attached to the outer surface	38–39	HpPex14p ScPex14p HsPex14p	152 4, 32 in 268 in 76
Pex15p	phosphorylated integral peroxisomal membrane protein; overexpression causes proliferation of the endoplasmic reticulum membrane	43.6	ScPas21p	68a
Pex16p	peripheral protein on matrix face of peroxisomal membrane; overexpression leads to enlarged peroxisomes	44.5	YlPex16p	67
Pex17p	localized to cytoplasmic surface of peroxisomes	23	ScPas9p	130a, in 268
Pex19p	probably cytosolic; contains carboxyl-terminal farnesylation signal	39.7	ScPas12p	103a, in 268
Pas22p	cytosolic DnaJ homolog	48		f

[a]Adapted from similar tables in [55, 76, 178, 268, 308], and updated appropriately.
[b]Based on the unified nomenclature [55]; these proteins are now called peroxins, with the PEX acronym.
[c]Species: Cb, *Candida boidinii*; Ch, Chinese hamster ovary cells; Hp, *Hansenula polymorpha*; Hs, *Homo sapiens*; Kl, *Kluyveromyces lactis*; Ld, *Leishmania donovani*; Mm, *Mus musculus*; Pa, *Podospora anserina*; Pp, *Pichia pastoris*; Rn, *Rattus norwegicus*; Sc, *Saccharomyces cerevisiae*; Yl, *Yarrowia lipolytica.*
[d]Protein names before the nomenclature unification.
[e]Complementation Group number (CG) of peroxisome biogenesis disorders, including Zellweger syndrome.
[f]53 Y. Elgersma, Ph.D. thesis, Amsterdam, 1995; in [268].

family are involved in transcription, cell cycle control, chromosome segregation, RNA processing, and mitochondrial protein import (reviewed in [99, 250]). SKL peptides bind specifically to the TPR region of Pex5p [31, 57, 175, 275, 318]; a peptide lacking the SKL signal does not bind to PpPex5p [175]. This provides functional confirmation that Pex5p is the PTS1 receptor. The amino terminus is less conserved and may confer species specificity to Pex5p from different organisms [94]. This may explain why PpPEX5 was unable to complement an *H. polymorpha pex5* mutant [293].

The subcellular localization of Pex5p is surprisingly controversial. Although no transmembrane domains are predicted by the sequence, PpPex5p behaves like an integral membrane protein that faces the cytosol [175, 275]. ScPex5p is found mainly in the cytosol [68, 294]. HpPex5p is detected both in the cytosol and in peroxisomes [207, 293]; YlPex5p is primarily intraperoxisomal [272]. Finally, the human Pex5p homolog has been reported to be cytosolic and partially [57, 318] or fully peroxisome associated [85]. These apparent differences may be explained by (1) the different techniques by which the localization was determined, (2) species differences, or (3) a model in which the PTS1 receptor shuttles between the cytosol and the peroxisome, as shown in Figure 2. In this model, Pex5p picks up its PTS1-protein cargo in the cytosol, takes it to the peroxisomal membrane, releases the cargo at the membrane surface or inside the organelle, and then recycles back to the cytosol [76, 178, 267, 268, 308].

The PTS2 receptor, Pex7p

Mutations in the PTS2 receptor specifically prevent the import of the PTS2 protein thiolase, while the import of PTS1 proteins proceeds normally [173, 295, 329, 332]. Complementation of the yeast mutants *pas7* and *peb1* (from *S. cerevisiae*) led to the identification of ScPEX7 [173, 232, 330, 331]. Defects in the human ortholog, HsPEX7, are responsible for the autosomal recessive disease rhizomelic chondrodysplasia punctata [26, 195, 228].

Pex7p is smaller than Pex5p and is comprised almost entirely of six WD-40 (β-transducin-related) repeats [26, 173, 228, 330, 331]. Specific interactions between the amino-terminal targeting signal on thiolase and Pex7p have been demonstrated *in vivo* and *in vitro*, using the two-hybrid system and coimmunoprecipitations [173, 232, 331]. Although not a PTS2, the amino-terminal 56 residues of ScPex7p are necessary and sufficient for peroxisomal targeting [331].

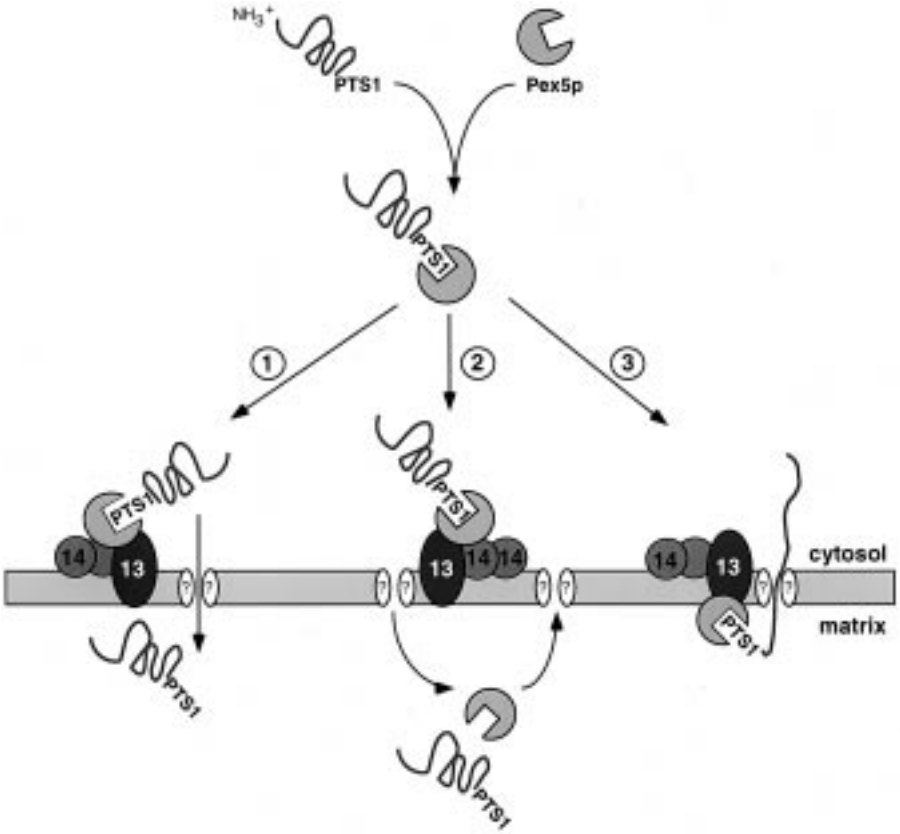

Figure 2. Import of PTS1 proteins. 0Proteins possessing a carboxyl-terminal PTS1 are thought to interact with a soluble PTS1 receptor, Pex5p. There are at least three models proposed for the import of PTS1 proteins after binding to Pex5p. The models differ by the proposed location and action of the PTS1 receptor. In the first pathway, Pex5p binds to the membrane-bound Pex13p receptors Pex13p and Pex14p. Pex5p releases its PTS1 protein cargo at the membrane surface for subsequent translocation into the peroxisome matrix. Model 2 proposes that Pex5p binds to Pex13p and Pex14p, but does not release the PTS1 cargo until both Pex5p and the PTS1 protein arrive in the matrix. Presumably, Pex5p is then recycled back out to the cytosol, or degraded within the matrix. Finally, the third mechanism proposed is based on an intraperoxisomal location for Pex5p, in which the PTS1 receptor somehow uses a 'pulling mechanism' to achieve PTS1 protein import. The translocation channels depicted are purely hypothetical. 13, Pex13p; 14, Pex14p.

Like Pex5p, Pex7p lacks a predictable transmembrane spanning region. The subcellular localization of Pex7p is also not clear; one group claims that ScPex7p is primarily cytosolic [173, 232], while another group reports that ScPex7p is mainly intraperoxisomal [330, 331]. An epitope-tagged version of MmPex7p localized to the cytosol of mammalian fibroblasts [26]. Thus, it is proposed that the PTS2 receptor may also shuttle between the cytosol and peroxisomes, similar to the mechanisms suggested for the PTS1 receptor (see Figure 3) [178, 267, 268].

Membrane-bound receptors

The cytosolic localization of at least some of the PTS1 and PTS2 receptors suggests that one or more docking proteins must be present on the peroxisome surface (see Figures 2 and 3). Two peroxisomal membrane proteins have recently been identified that appear to be components of the protein import apparatus.

Pex13p is an integral peroxisomal membrane protein with a src homology 3 (SH3) domain that interacts directly with the PTS1 receptor, but not the PTS2 receptor [68, 74, 104]. This suggests that Pex13p is the membrane-bound receptor for Pex5p. Surprisingly, cells deficient in Pex13p are defective in the import of both PTS1- and PTS2-containing proteins. Identifiable peroxisomal membranes are present, however, providing further evidence that the pathway for integration of peroxisomal membrane proteins is distinct from the pathways for the import of peroxisomal matrix proteins.

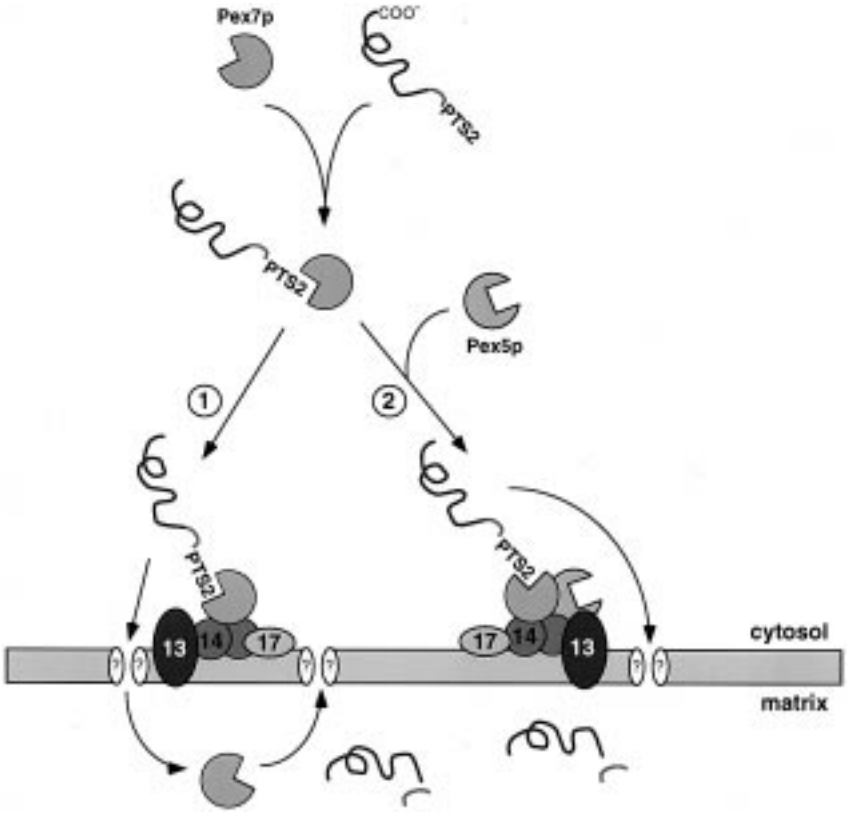

Figure 3. Import of proteins possessing an amino-terminal cleaved PTS2. The PTS2 receptor, Pex7p, binds to peroxisomal matrix proteins possessing an amino-terminal PTS2. Pex7p interacts with the peripheral peroxisomal membrane receptor, Pex14p. In the first pathway, Pex7p binds a PTS2 protein and interacts directly with Pex14p on the peroxisomal membrane. Alternatively, Pex7p may interact with Pex5p, as shown in the second pathway. Similar to the models shown in Figure 2, it is not known whether Pex7p is (1) transported into the matrix to release the PTS2 protein prior to recycling back to the cytosol or degradation in the matrix, or (2) if Pex7p releases its cargo at the surface of the membrane. Although not shown, Pex7p could also use a 'pulling mechanism' to effect PTS2 protein import, as depicted in Figure 2. After import, the amino-terminal PTS2 is proteolytically cleaved from the mature polypeptide. 13, Pex13p; 14 Pex14p; 17, Pex17p.

The peripheral peroxisomal membrane protein, Pex14p, interacts with both the PTS1 receptor and the PTS2 receptor [4, 32, 152]. Thus, Pex14p may act as a docking protein for the cytosolic receptors at a point of convergence between the PTS1 and PTS2 pathways of matrix protein import. Common components of the translocation machinery for matrix protein import are suggested by the many mutants which fail to import any matrix proteins [155]. For example, *S. cerevisiae* and *H. polymorpha pex14* mutants lack normal peroxisomes; peroxisomal membranes are detectable, but matrix proteins accumulate in the cytosol [4, 152].

Interactions between Pex proteins

The discovery and identification of all these peroxins has led to at least three models designed to explain how multiple targeting signals interact with different cytosolic and membrane-bound receptors [4]. The point on which the models differ is whether the PTS1 and PTS2 receptors, Pex5p and Pex7p, respectively, (1) deliver their cargo while bound to the peroxisomal membrane [68, 74, 76, 104, 173, 318], (2) are translocated across the membrane before releasing the cargo in the matrix [58, 232, 293], or (3) are present exclusively in the matrix and promote protein import by a 'pulling mechanism' [272, 331]. One interpretation of these models is shown in Figure 3; see also [308]. Regardless of which mechanism is ultimately shown to be correct, it will have to accommodate all of the interactions that have been detected between the many players required to import peroxisomal matrix proteins.

The PTS1 receptor and the PTS2 receptor are known, from specific yeast mutants, to operate in two independent pathways [173, 232, 294, 295, 330]. Yet

another interaction between these proteins has been shown using a yeast two-hybrid system [232]. It is likely that the TPR motif region of Pex5p interacts with the WD-40 repeats of Pex7p; other proteins with these structures are reported to be functionally related [99, 296]. This may explain why severe mutations in PEX5 (the PTS1 receptor) sometimes abolishes the import of both PTS1 and PTS2 proteins [57, 58, 252].

Additional complications arose with the identification of Pex14p. ScPex14p appears to self-oligomerize, perhaps through interactions between putative coiled-coil domains [4]. In addition, using the yeast two-hybrid system, Pex14p was shown to interact strongly with both of the PTS receptors and with the novel peripheral peroxisomal protein Pex17p (formerly Pas9p); a weak interaction between Pex14p and Pex13p was also detected [4, 32, 130a]. Coimmunoprecipitation confirmed the interactions between Pex14p, Pex17p, and the two PTS receptors, Pex5p and Pex7p.

The yeast two-hybrid system may also identify transient interactions between proteins. Thus we can not yet determine in what order the peroxins interact, whether there is a specific cascade of interactions that occurs as the matrix protein is passed along the peroxins, or whether a single complex forms and all the proteins interact concurrently. In addition, the yeast two-hybrid system may not detect every *in vivo* interaction. For example, the import defect in *pex8* mutants of *P. pastoris* suggests that Pex8p is directly involved in peroxisomal protein transport [167]. Two more potential components of the peroxisomal matrix protein import apparatus are Pex2p and Pex12p [58, 76]; both are integral peroxisomal proteins with zinc-finger domains whose functions are unknown (see Table 2). A combination of further genetic and biochemical studies will be required to unequivocally resolve these mechanistic questions.

Biochemical evidence for peroxisomal protein import receptors

Biochemical approaches corroborate the genetic results and are necessary to determine the mechanisms responsible for peroxisomal protein import. For instance, the PTS1 receptor has been localized to the cytosol, the peroxisome membrane, and the peroxisome matrix [57, 68, 85, 175, 207, 272, 275, 293, 294, 318]. Wendland and Subramani [314] provide evidence of SKL-dependent binding sites present in the cytosol of permeabilized mammalian cells. Wolins and Donaldson [322, 323], however, identify SKL-dependent binding sites on glyoxysomal membranes. Using a synthetic SKL peptide (based on the last 12 residues of rat acyl-CoA oxidase), they found that binding is saturable and specific for peroxisomal membranes. Addition of excess unlabeled SKL peptide could compete for binding to the membranes. Recent results suggest that a low-affinity binding site, which is destroyed by protease treatment, specifically binds the carboxyl-terminal tripeptide; a high-affinity site, which is less protease sensitive, may interact with a domain upstream of the carboxyl terminus [323].

In vitro assays of protein import provide powerful tools that allow a precise investigation of the molecular mechanisms involved in peroxisomal protein transport. A recent report from my laboratory establishes the reliability and relevance of an *in vitro* assay for protein import into isolated pumpkin glyoxysomes [29]. We show that import is time-, temperature-, and energy-dependent, as well as specific for peroxisomal proteins. Moreover, import of the peroxisomal protein glycolate oxidase is saturable, and addition of excess non-radiolabeled glycolate oxidase competes with radiolabeled translation products for import. Notably, pretreatment of the glyoxysomes with protease abolishes subsequent import.

Although none of the peroxins have yet been cloned from plants, we expect that homologs exist and will soon be identified, and interpret the *in vitro* assays results accordingly. The saturation, competition, and protease-pretreatment experiments were most likely affecting Pex13p (and possibly, Pex14p) or interfering with the activity of Pex5p. A small amount of Pex5p may be added with the wheat germ lysate (part of the cell-free translation system) and some Pex5p is probably present on the membranes of the isolated peroxisomes. Although only a small amount of Pex5p is usually bound to yeast peroxisomes, Dodt and Gould [58] found that Pex5p accumulates on normal peroxisomes at low temperatures or in the absence of ATP. Thus it is possible that membrane-associated Pex5p is enriched when the glyoxysomes are isolated (on ice, without added ATP) for *in vitro* assays. Finally, only about 10% of the protein presented to glyoxysomes is imported in the *in vitro* assays (up to about 50% efficiency can be achieved under specific conditions, see discussion below [46a]; some of this limitation may be due to suboptimal levels of cytosolic factors required for peroxisomal protein import. Thus, a combination of *in vivo* and *in vitro* approaches will be necessary

to fully understand the process of protein import into peroxisomes.

Energetics of peroxisomal protein import

Several groups have shown that ATP hydrolysis is required for protein import into peroxisomes [14, 29, 30, 129, 134, 314]. GTP hydrolysis also appears to support the import of glycolate oxidase into isolated pumpkin glyoxysomes, though it is not clear whether GTP is acting at the same step(s) as ATP or whether the nucleotides affect different parts of the import process [30]. GTP-binding proteins are involved in other transport pathways [10, 148, 218] and three small GTP-binding proteins have been identified in rat peroxisomal membranes [298]. The ATP requirement may be specific for matrix protein import; insertion of the rat liver peroxisomal membrane protein PMP22 is not ATP-dependent [54].

The existence of a protonmotive force (PMF) across the peroxisomal membrane has not been firmly established, and its role in peroxisomal protein import is equally unclear. Various inhibitors and ionophores that collapse the PMF and abolish import have been used to implicate components of the PMF in protein transport across bacterial membranes [325, 328], thylakoids [38, 276], and mitochondria [217]. Some researchers have found that ionophores inhibit protein import into peroxisomes [16, 30], while other groups observe no effect of ionophores on peroxisomal protein import [134, 314].

It is possible that nonspecific pores in the peroxisomal membrane allow small ions and metabolites to diffuse freely across the membrane, thereby dissipating the PMF [235, 236, 292]. Alternatively, the PMF may indirectly facilitate protein import; an ATPase on the peroxisomal membrane could make the matrix acidic, thus establishing a pH gradient. Yeast peroxisomes maintain a pH gradient across the peroxisomal membrane [310], with a matrix pH 5.8–6.0 [202]. An ATPase analogous to the V-class H^+-ATPase found on vacuolar membranes has been reported on peroxisomal membranes [53, 60, 61, 315, 324]. An acidic matrix might be required for the dissociation of the peroxisomal matrix protein from its PTS receptor, similar to the ligand release observed when the receptor and cargo are exposed to the acid pH in late endosomes of mammalian and yeast cells.

Other peroxisomal proteins may also bind ATP. Two of the peroxins identified in yeast and mammalian cells, Pex1p and Pex6p, are members of the AAA family of ATPases [78, 125, 206, 221, 234, 259, 287, 303, 326]. Other AAA ATPases (ATPases associated with diverse cellular activities) are involved in vesicle fusion, control of cell division, regulation of transcription, and intracellular proteolysis. Pex1p and Pex6p have not been definitively localized, but they appear to be cytosolic and vesicle associated, not membrane proteins. This suggests that their role in peroxisome biogenesis is not to maintain an acid matrix, like the H^+-ATPase proposed above.

ATP may also be required for the activity of the peroxisomal membrane protein PMP70, a member of the ATP-binding cassette (ABC) superfamily of transporters [90, 138]. (The cystic fibrosis transmembrane conductance regulator protein is a notorious member of the ABC transporter superfamily.) Defects in PMP70 are responsible for a subset of complementation group 1 patients with the peroxisome biogenesis disorders Zellweger syndrome and neonatal adrenoleukodystrophy [90, 138]. At least some forms of X-linked adrenoleukodystrophy are caused by mutations in a homolog of PMP70 [40, 192]. As the name suggests, these ATP-dependent proteins may be involved in metabolite transport across the peroxisomal membrane. PMP70 has recently been implicated in interactions between peroxisomes and the cytoskeleton [124]. Thus, it is likely that ATP serves multiple roles during peroxisome biogenesis.

Molecular chaperones

Cytoplasmic chaperones may represent another group of proteins in which ATP hydrolysis functions during peroxisomal protein import. Chaperones such as Hsp70 (heat shock protein of the 70 kDa class) are nucleotide-dependent proteins that bind newly synthesized proteins to facilitate proper folding and prevent non-specific aggregation [118, 176]. This may be especially important during the synthesis of PTS1 proteins since their targeting signal is last to emerge from the ribosome, perhaps leaving the rest of the protein at risk for misfolding. Antibodies raised against bovine cytosolic Hsp73 inhibit the import of microinjected human serum albumin-PTS1 conjugates into mammalian peroxisomes [307]. Similarly, the import of isocitrate lyase into isolated pumpkin glyoxysomes was inhibited by antibodies against cytosolic wheat germ Hsp70 and by antibodies against *Escherichia coli* Hsp90 [46a]. Others have presented additional

evidence of the requirement for cytosolic factors, but these factors were not identified [5, 314].

Two chaperones have been localized to glyoxysomal membranes. A member of the Hsp40 family, DnaJ, is bound to the cytosolic surface of cucumber glyoxysomes, perhaps by farnesylation [224]. PMP73, a glyoxysomal membrane protein from cucumber seedlings, is immunorelated to other members of the Hsp70 family of chaperones [44].

There are two reports of peroxisomal matrix-localized chaperones. Recently, Gietl and her colleagues [320] isolated a watermelon Hsp70 that is present in both plastids and glyoxysomes. It is encoded by a single gene with two in-frame translation start sites; synthesis of the shorter protein produces an Hsp70 that is targeted to peroxisomes by a PTS2 [320]. In a less convincing study, antibodies to the bacterial chaperonins cpn10 and cpn60 (homologs of Hsp10 and Hsp60 class chaperones) from *Chromatium vinosum* cross-reacted with proteins in the mitochondrial inner membrane and the peroxisomal matrix of rat hepatocytes [297]. We have observed 2- to 3-fold higher levels of protein import into glyoxysomes isolated from heat-shocked pumpkin seedlings, perhaps due to increased levels of glyoxysomal Hsps [46a].

Although peroxisomal membrane and matrix chaperones have been found, their function is unknown. The cytosolic chaperones Hsp70 and Hsp90 affect peroxisomal protein import [46a, 307], but their mechanism of action has not been determined. Hsp70 may be required to maintain the targeted protein in an import-competent state, as in chloroplastic and mitochondrial protein import (reviewed in [113, 119, 158, 231]). In yeast, a DnaJ homolog cooperates with Hsp70 to prevent protein aggregation [47, 231]. Hsp90 is often found in a 'superchaperone complex' where it acts in concert with Hsp70 and other components [135, 222]; Hsp90 may interact with the peroxisomal protein directly or, possibly, with the PTS receptor to facilitate stable complex formation before docking on the membrane. These and other possible hypotheses must be tested before solid conclusions can be drawn about the role of chaperones in peroxisome biogenesis.

Import of folded proteins and oligomers

One of the most intriguing aspects of peroxisomal protein transport is that large folded proteins and intact oligomers can be properly targeted and imported. Several studies suggest that at least some peroxisomal proteins fold and assemble in the cytosol prior to import. For example, dimerization of *S. cerevisiae* malate dehydrogenase precedes import [71]. Prefolded proteins and proteins stabilized in a folded conformation by cross-linking or other chemical modifications are imported into mammalian peroxisomes [258, 304–306] and trypanosome glycosomes [120]. The PTS2 protein thiolase may already be folded when it binds to Pas7p in two-hybrid experiments [232]. In addition, 'piggy-backing' experiments, in which epitope-tagged protein constructs lacking a PTS are coexpressed and colocalized with wildtype peroxisomal proteins possessing a PTS, suggest that PTS1- and PTS2-proteins can be imported as oligomers into *S. cerevisiae* peroxisomes [97, 177] and into tobacco suspension culture glyoxysomes [166]. Perhaps the strongest proof of the import of large structures is provided by Walton and his colleagues who show that 9 nm colloidal gold particles conjugated to PTS1-peptides are imported into peroxisomes when microinjected into human fibroblast cell lines [306].

Some studies provide potentially conflicting results. Purified octameric alcohol oxidase from *P. pastoris* microinjected into mammalian cells is incorporated into punctate structures, some of which are peroxisomes [305]. In contrast, alcohol oxidase from *C. boidinii* [103] and from *P. pastoris* [310a] assembles into octamers only after the monomeric subunits are translocated into peroxisomes [103]; binding of the FAD cofactor to *H. polymorpha* alcohol oxidase requires a peroxisomal factor and must occur prior to oligomerization [80]. When cells from two mutant lines of complementing Zellweger fibroblasts were fused, preexisting tetrameric catalase was rapidly relocated in newly generated peroxisomes [34]. The addition of the catalase inhibitor aminotriazole, however, inhibits catalase import in similar experiments [179, 180]. Aminotriazole may prevent dissociation or unfolding of the tetramers, or it could alter the oligomeric structure of catalase such that the protein is not recognized for import [178–180].

Several models have been proposed to explain how proteins cross the peroxisomal membrane [76, 178, 266, 267, 308]; these are illustrated in Figure 4. A preponderance of evidence suggests that peroxisomal proteins may assemble in the cytosol prior to import (Figure 4, model 2). Although the oligomers may be translocated intact (model 2a), we cannot yet eliminate the possibility that the oligomers dissociate at the membrane just prior to import and then reassemble in the matrix (model 2b). Another interesting model

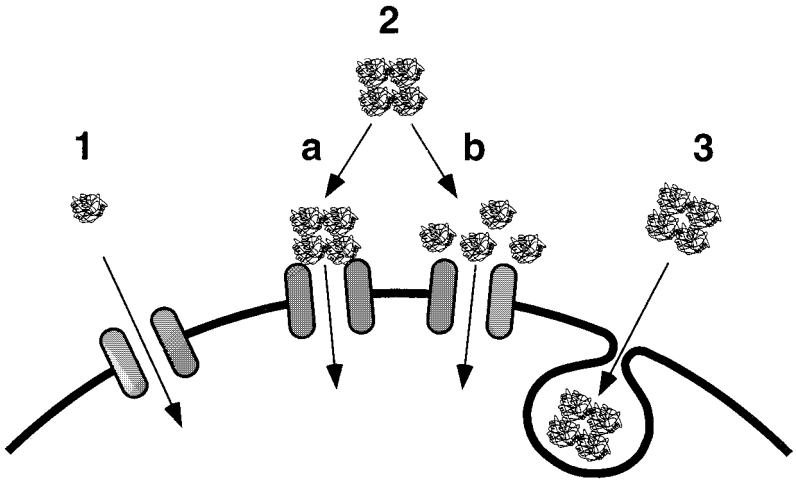

Figure 4. Import of folded proteins and oligomers into peroxisomes. 1. Import of a monomeric protein through an unidentified translocation channel into the peroxisome matrix. 2. Import of a hypothetical tetrameric protein into the peroxisome matrix. The oligomer may be translocated across the membrane intact, as shown in a, or the oligomer may dissociate at the surface of the membrane prior to monomer import into the matrix, as shown in b. 3. An invagination of the peroxisome membrane, in a novel form of endocytosis, may engulf the intact oligomer, and release it into the matrix by an unknown mechanism.

proposes that the proteins assemble at the membrane or in vesicles, followed by import via a novel form of endocytosis caused by invagination of the peroxisome membrane (model 3; see [177, 178]). Finally, some peroxisomal proteins may be translocated as monomers and assembled in the matrix (model 1). This is consistent with reports that catalase [161], malate synthase [154], and alcohol oxidase [80, 103] monomers are imported prior to assembly.

These models are not necessarily mutually exclusive. Recent experiments on the import of the tetrameric protein isocitrate lyase into isolated pumpkin glyoxysomes suggest that though the oligomeric form can be imported, the efficiency of the reaction is much greater for the import of monomeric isocitrate lyase [46a]. *In vitro* import is detectable within 5–10 min ([29], W.J. Crookes, unpublished results); normal *in vivo* import occurs on a similar time scale [102, 103, 161, 164]. Most of the studies discussed above measured oligomeric import only after 12–48 h. Thus, models 1 and 2 may both occur simultaneously during peroxisome biogenesis, with varying frequency or efficiency, or under specific conditions.

Conclusions and future directions

Despite the early observations that peroxisomes are morphologically simple organelles, their assembly has proven to be surprisingly complex. The different classes of plant peroxisomes serve specific metabolic roles (Table 1), requiring a transition between classes to meet the changing needs of the tissues. Thus glyoxysomes become leaf peroxisomes during greening of oilseed cotyledons, and during senescence leaf peroxisomes are converted back into glyoxysomes (Figure 1). The general mechanisms involved in peroxisome assembly, however, appear to be conserved between all types of peroxisomes in higher and lower eukaryotes.

Tremendous progress has been made recently in identifying the receptors and components of the translocation machinery. At least 20 peroxins are required for the import of matrix proteins (Table 2); two pathways defined by two targeting signals also share common components. With the exceptions of a few receptors, the function of each peroxin remains unknown. The subcellular distribution of the signal receptors (Pex5p, Pex7p) must be determined before we can distinguish between the various shuttling models proposed (e.g., Figure 2).

Additional cytosolic factors, including molecular chaperones, may be required to maintain the targeted protein or its soluble receptor in an appropriate conformation for recognition by other factors or to facilitate oligomer assembly. The capability of peroxisomes to import oligomers represents a novel mechanism of protein import, not yet described for other protein transport systems.

Although the functions of a few peroxisomal membrane proteins have been determined, additional unidentified membrane proteins are likely involved in roles that include organelle movement, attachment to the cytoskeleton, and control of organelle inheritance during cell division and checkpoints during the cell cycle [266]. The targeting and insertion of peroxisomal membrane proteins requires different signals and receptors than peroxisomal matrix proteins; the energy requirements for and chaperone involvement in membrane assembly remain completely unexplored. Tantalizing evidence [8, 22, 76, 83, 133, 172, 177, 241, 267, 312] is beginning to accumulate suggesting that the ER may participate in peroxisome membrane formation, which would have a major impact on our views of peroxisome assembly and origins. The ER has long been accepted as the probable source of lipids for most peroxisomes (except that lipid bodies provide lipids for glyoxysomes in germinating oilseeds [36]), but a proposed role for the ER in the transport of some peroxisomal membrane proteins is new and controversial.

Peroxisome biogenesis is not simple. But the tools are in place, and the powerful combination of yeast genetics and biochemical reconstitution experiments now available promises to provide a new understanding of the mechanisms involved in peroxisome formation and allow us to answer the question, 'How *do* you make a peroxisome?'

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