

Mapping of the cargo protein membrane translocation step into the PEX5 cycling pathway.

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**Mapping of the cargo protein membrane translocation step
into the PEX5 cycling pathway.**

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PRECEITOS LEGAIS

Esclarece-se serem da responsabilidade da autora desta tese a execução das experiências que estiveram na origem dos resultados apresentados neste trabalho, assim como a sua interpretação, discussão e redacção.

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“We know accurately only when we know little, with knowledge doubt increases.”

Johann Wolfgang von Goethe

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ABSTRACT

Peroxisomes are ubiquitous organelles in the eukaryotic kingdom characterized by a single membrane and a dense protein matrix comprising a large set of enzymes [1]. These organelles are responsible for important functions in the metabolism of lipids, ethanol and glyoxylate, in the detoxification of oxygen reactive species and in the synthesis of ether-phospholipids in mammals [2, 3]. Defects in peroxisomal biogenesis or deficiencies in peroxisomal enzymes are the cause of severe genetic diseases [4]. Peroxisomal biogenesis requires the concerted action of a machinery comprising several different proteins- peroxins- which ensure the assembly of its membrane and the import of proteins into the peroxisomal matrix [1, 5]. There are two main signals that target proteins into the peroxisomal matrix: the so-called peroxisomal targeting signal 1 (PTS1), the most abundant type, comprising a short domain present at the C-termini (frequently the SKL sequence) [6], and the PTS2 signal, a degenerated nona-peptide with the sequence (R/K)(L/V/I)X5(H/Q)(L/A) present at their N-termini [7, 8]. In contrast to the PTS1, which is not cleaved upon import into peroxisomes, the PTS2 signal is proteolytically removed in the peroxisomal matrix of many organisms by a peroxisomal processing peptidase [1, 9, 10]. In mammals and many other organisms both PTS1- and PTS2-containing proteins are targeted to the organelle by PEX5, the peroxisomal cycling receptor [11-14]. PTS1 proteins interact directly with the C-terminal half of PEX5, whereas the PTS2 interaction is bridged by the adaptor protein PEX7 [15-21]. Due to alternative splicing, the mammalian PEX5 gene yields two isoforms: the so called large isoform of PEX5 (PEX5L), and the small isoform (PEX5S). PEX5S lacks a 37 amino acid region involved in the PEX7 interaction and so, only PEX5L is competent in targeting PTS2 proteins into peroxisomes [18-20]. Until now, the data on the mechanism of peroxisomal matrix protein import have been obtained using a PEX5-centered *in vitro* system [22-24] and despite the progress accomplished in the understanding of this pathway, there are still many unsolved mechanistic aspects that can not be addressed with this *in vitro* system and that require a cargo protein-centred perspective instead.

This thesis describes the development of a robust peroxisomal *in vitro* import system that provides a cargo centred perspective. With this system, it was shown that when a rat liver post-nuclear supernatant is fortified with selected recombinant proteins, a robust amount of a ³⁵S-labeled PTS2-containing protein can be specifically imported into peroxisomes allowing the mapping of the step of protein translocation across the peroxisomal membrane into the PEX5 cycling pathway.

RESUMO

Os peroxissomas são organelos ubíquos no reino eucariota, caracterizados pela existência de uma membrana única e de uma matriz proteica densa, constituída por um grande conjunto de enzimas [1]. Estes organelos desempenham importantes funções no metabolismo dos lípidos, do etanol e do glioxilato, na destoxificação de espécies reactivas de oxigénio e na síntese de éterfosfolípidos nos mamíferos [2, 3]. Defeitos na biogénese peroxissomal ou deficiências em enzimas peroxissomais são causa de graves doenças genéticas [4]. A biogénese peroxissomal requer a acção concertada de uma maquinaria composta por várias proteínas diferentes – peroxinas - e envolve processos complexos como a organização da membrana e a importação de proteínas para a matriz [1, 5]. Estão actualmente identificados dois sinais de endereçamento das proteínas para a matriz peroxissomal: o PTS1 (Peroxisomal Targeting Signal type 1), que é o sinal mais frequente, caracterizado por um tripéptido conservado no C-terminal da proteína (usualmente com a sequência SKL) [6], e o PTS2 (peroxisomal targeting signal 2), que consiste num nonapéptido degenerado, com a sequência (R/K)(L/V/I)X5(H/Q)(L/A), localizado no N-terminal das proteínas [7, 8]. Em contraste com o PTS1, que não é clivado, o PTS2 é proteoliticamente removido na matriz do peroxissoma de muitos organismos [1, 9, 10]. Nos mamíferos, e em muitos outros organismos, tanto as proteínas PTS1 como PTS2 são endereçadas para o peroxissoma pela PEX5, o receptor peroxissomal cíclico [11-14]. As proteínas PTS1 interagem directamente com a metade C-terminal da PEX5 mas as PTS2 requerem a acção da proteína adaptadora PEX7, que medeia a interacção com a PEX5 [15-21]. Nos mamíferos o gene *PEX5* origina por splicing alternativo duas isoformas: a isoforma longa, PEX5L, e a curta, PEX5S. A PEX5S não tem a região de 37 aminoácidos envolvida na interacção com a PEX7 pelo que só a PEX5L é capaz de endereçar proteínas PTS2 para o peroxissoma [18-20]. Até agora, muitos dos detalhes sobre o mecanismo de importação de proteínas para a matriz peroxissomal têm sido obtidos usando um sistema de importação *in vitro* centrado na PEX5 [22-24] mas, apesar dos vários progressos conseguidos, existem ainda muitos aspectos que requerem uma perspectiva centrada na proteína “carga”.

Esta tese descreve o desenvolvimento de um sistema de importação *in vitro* centrado na proteína “carga”. Os resultados apresentados mostram que, quando um sobrenadante pós-nuclear de fígado de rato é fortificado com determinadas proteínas recombinantes é possível obter uma importação robusta e específica de uma proteína PTS2 marcada radioactivamente (³⁵S) para a matriz peroxissomal e conseguir assim mapear a fase de translocação de proteínas através da membrana peroxissomal na via de importação mediada pela PEX5.

ABBREVIATIONS

AAA	ATPases associated with diverse cellular activities
ACOX	Acyl-CoA oxidase
ADHAS	Alkyl-DHAP synthase
AGT	Alanine glyoxylate aminotransferase
ALD	Adrenoleukodystrophy
ALDP	Adrenoleukodystrophy protein
AMACR	2-Methylacyl-CoA racemase
ATP _γ S	Adenosine 5'-O-thiotriphosphate
BSA	Bovine Serum Albumine
CCCP	carbonyl cyanide m-chloro phenyl hydrazone
cDNA	Complementar deoxyribonucleic acid
CG	Complementation Group
CHO	Chinese hamster Ovary
Cs	<i>COS-7 cells derived from the kidney cells of African green monkey</i>
DBP	D-bifunctional protein
DHAP	Dihydroxyacetone phosphate
DHCA	Dihydroxycholestanic acid
DsRed	<i>Discosoma sp.</i> red fluorescent protein
DTM	Docking Translocation Machinery
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin –conjugating enzyme
E3	Ubiquitin ligase
E-64	<i>N</i> -(<i>trans</i> -epoxysuccinyl)-L-leucine 4-guanidinobutylamide
EDTA	Ethylenediamine tetraacetic acid
EM	Electron Microscopy
ER	Endoplasmatic Reticulum
gMDH	Glyoxysomal malate dehydrogenase
GSH	Glutathione
GST	Glutathione-S-transferase
IAA	Iodoacetamide
IgG	Immunoglobulin
IRD	Infantile Refsum disease
MOPS	4-morpholinepropanesulfonic acid
mPTS	Membrane peroxisomal targeting signal
NALD	Neonatal adrenoleukodystrophy
NSF	N-ethylmaleimide-sensitive factor
NTP	Nucleoside triphosphate
PBD	Peroxisome Biogenesis Disease
PEX	Peroxin
PH1	Primary hyperoxaluria type I
PHYH	phytanoyl-CoA hydroxylase
PIM	Peroxisomal Import Machinery
PMP	Peroxisome Membrane Protein
PMSF	phenylmethylsulfonyl fluoride
PNS	Postnuclear Supernatant
PTS	Peroxisomal Targeting Signal
RCDP	Rhizomelic Chondrodysplasia Punctata
REM	Receptor Export Module
RING	Really Interesting New Gene
ROS	Reactive Oxygen Species
SCP2	Sterol carrier protein 2
SCPx	Sterol carrier protein X
SDS	Sodium dodecylsulfate
SH3	Src homology 3 domain

THCA	Trihydroxycholestanoic acid
TPR	Tetratricopeptide repeats
Ub	Ubiquitin
VLCFA	Very Long Chain Fatty Acid
WD	Tryptophan-aspartate repeat
wt	Wild Type
ZS	Zellweger Syndrome
ZSS	Zellweger Syndrome Spectrum

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I. INTRODUCTION

1 - PEROXISOMAL STRUCTURE

Peroxisomes were first described as microbodies using electron microscopy in mouse kidney, in 1954, by Rhodin [25]. At this time, peroxisomes were regarded as fossil organelles and were only considered to play a role in the disposal of cells metabolites [25]. In 1966, de Duve and Baudhuin accomplished the first isolation of peroxisomes from rat liver [26]. It was also their research that, having discovered the peroxisomal hydrogen-peroxide based respiration reaction, led to the baptism of these structures as **Peroxisomes** [26].

Later morphological studies revealed that peroxisomes, as mitochondria, are present in nearly all eukaryotic cells [27]. They are only absent in red blood cells and spermatids (although present during the early stages of development, when membranes are formed) [28] and also in *A. phylum* and amitochondriate parasites [29]. Peroxisomes are especially abundant in mammalian liver and kidney [30-32]. In developing mammalian fetus, for a few weeks after birth, peroxisomes are also copious in the oligodendrocytes, the cells that surround the central nervous system, and act in the guiding of its growth and in the synthesis of the myelin sheath that insulates it [33].

This organelle is characterized by a single membrane and a dense, amorphous, protein matrix comprising a large set of enzymes [34]. Typical peroxisomes are round or oval with a diameter of 0,1-1 μ m [2] but peroxisomal size and shape can vary broadly depending not only of the species and cell type, but also of environmental factors and developmental conditions [35]. In fact, in mammalian cells, the number of peroxisomes can vary from a few hundreds to a few thousands, and in some yeast and in human liver, they can present themselves as tubular structures and as tubular-reticular networks [36-40]. Different attempts to measure the pH of the peroxisomal matrix found it to be more acidic [41], similar [42] and even more basic [43] than that of the cytosol.

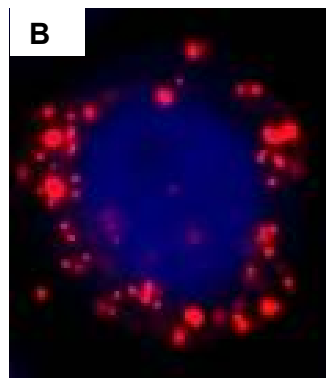
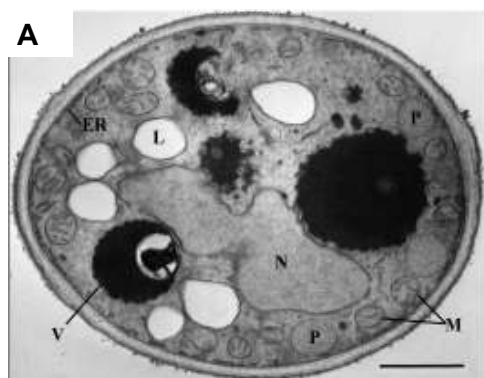


Fig.I-1- Peroxisome morphology. (A) Electron micrograph of wild-type *Saccharomyces cerevisiae* grown on oleic. Bar= 1 μ m; P, peroxisome; M, mitochondrion; N, nucleus; L, lipid droplet; V, vacuole; ER, endoplasmic reticulum (micrograph from [1]). (B) A

CHO cell transfected with the plasmid encoding DsRed-SKL, the red fluorescence protein –DsRed– fused with the peroxisomal targeting signal peptide SKL at the C-terminal. Peroxisomes are shown in red and the nucleus in blue (unpublished work).

In contrast to mitochondria and chloroplasts, peroxisomes do not contain DNA and all their proteins are encoded by nuclear genes, translated in free polyribosomes in the cytosol and imported, post-translationally, into the peroxisome membrane or matrix [44]. The genes required for peroxisome biogenesis are named *PEXn* and their proteins called peroxins –*PEXn*, with *n* being a number simply reflecting the order of discovery [45].

2 - PEROXISOMAL FUNCTIONS

Peroxisomes are very versatile organelles and in mammals their matrix contains at least 50 different enzymes that play key roles in both anabolic and catabolic pathways [34].

Most of the peroxisomal roles involve coordinated interactions between this and other organelles, with each metabolic step being dependent on the successful completion of the previous. The catabolism of VLCFAs and phytanic acid is a process carried out by peroxisomes and mitochondria and the correct functioning of the peroxisomal steps is essential to the overall success of the process [46, 47]. Likewise, the final steps of the synthesis of plasmalogens occur in the endoplasmic reticulum, but the process depends on precursors that are synthesized in peroxisomes [48].

Three peroxisomal functions are found amongst evolutionary diverse organisms: oxidative reactions by using molecular oxygen yielding hydrogen peroxide, defense against oxidative stress and β -oxidation of fatty acids [49]. Other specialized roles depend on the organism and cell type [49].

Peroxisomal function is tightly linked to oxygen metabolism due to the high concentration of hydrogen peroxide generating oxidases in peroxisomes and their set of antioxidant enzymes, namely catalase. The H_2O_2 produced is decomposed by the action of the antioxidant enzymes that convert it to water and oxygen or use it to oxidize other organic compounds [50].

Fatty acid α -oxidation is exclusively peroxisomal and an essential step for the oxidation of fatty acids with a methyl group at carbon 3. These methylated fatty acids must undergo α -oxidative decarboxylation by peroxisomal enzymes in order to become a substrate for further degradation by β -oxidation [34].

Fatty acid β -oxidation is an ubiquitous property of peroxisomes and provides a major source of metabolic energy for the cells. In plant cells [51] and in many eukaryotic microorganisms [52], peroxisomes are the only site of β -oxidation making them essential for the utilization of fat in these organisms. In mammals, β -oxidation of fatty acids is a process shared by both peroxisomes and mitochondria serving different functions and catalyzing the oxidation of different fatty acids and fatty acid derivatives [53]. Peroxisomes catalyze the β -oxidation of a set of substrates that can not be oxidated by mitochondria

[53]: 1) VLCFAs, derived from the diet and from the elongation of long-chain fatty acids; 2) branched fatty acids, like the pristanic acid (that in humans is solely derived from dietary sources either as pristanic acid or via α -oxidation of its precursor phytanic acid); 3) di- and trihydroxycholestanoic acid (DHCA and THCA), that are produced from cholesterol in the liver and constitute intermediates in the formation of primary bile acids - cholic acid and chenodeoxycholic acid - after one cycle of β -oxidation in the peroxisome; 4) certain polyunsaturated fatty acids such as the tetracosahexaenoic acid (C24:6), which is β -oxidized in peroxisomes to produce C22:6; 5) long-chain dicarboxylic acids, that are produced by omega-oxidation of long-chain monocarboxylic acids; 6) some prostaglandins and leukotrienes; 7) some xenobiotics and also 8) vitamins E and K [34].

Peroxisomes also play a vital role in the detoxification of glyoxylate in humans [54-56]. The enzyme alanine glyoxylate aminotransferase (AGT), that catalyses the conversion of glyoxylate into glycine using alanine as the preferred amino donor, is exclusively peroxisomal in humans, rabbits, and guinea pigs, has a dual peroxisomal and mitochondrial location in rats and marmosets, and is mitochondrial in cats [54-56]. This reaction is crucial for preventing the conversion of glyoxylate to the toxic metabolite oxalate, which would precipitate in the liver and in other organs, including kidneys, ultimately causing kidney failure and loss [54-56].

Mammalian peroxisomes act in the oxidation of D- isomers of amino acids (by the action of the peroxisomal enzymes D-amino acid oxidase and D-aspartate oxidase) and in the oxidation of some L-amino acids [57]. Peroxisomes also operate in the oxidation of polyamines [58, 59].

Adding up to providing a compartment for oxidation reactions, peroxisomes are reported to be involved in the biosynthesis of ether-phospholipids, isoprenoids and cholesterol [34]. The biosynthesis of plasmalogens, a family of ether-phospholipids that are important membrane components in some organs as the heart and brain, requires the concerted action of enzymes localized in both the peroxisomes and the endoplasmatic reticulum, with the peroxisome being the site of introduction of the ether linkage by the peroxisomal enzyme alkyl-DHAP synthase [34]. The peroxisome involvement in the synthesis of isoprenoids and cholesterol is still controversial. Since 1985, reports stated that many of the enzymes required for isoprenoid and cholesterol synthesis were partly or even exclusively peroxisomal [60] but recent studies refuted those conclusions and, presently, it is accepted that the evidences for a direct peroxisomal involvement in isoprenoid and cholesterol biosynthesis are still incipient [34].

Interestingly, peroxisomes in plants, yeasts and protozoa generally have a wider spectrum of activities than those in vertebrates. The peroxisomes of the fungi *Penicillium* and *Aspergillus* produce penicillin and plant peroxisomes are responsible for the glyoxylate

cycle and plant hormone biosynthesis/metabolism [61-63]. In plants the glyoxylate cycle is exclusively peroxisomal and represents such an important metabolic path that plant seedlings peroxisomes are named glyoxysomes [64]. The peroxisomes from the plant leaf are named leaf peroxisomes and are the site for part of the glycolate and glycerate pathways of photorespiration [64]. Glycosomes are specialized peroxisomes of *Trypanosomes* and *Leishmania* that contain glycolytic enzymes [65]. In yeasts, peroxisomes house several steps of the glyoxylate cycle, the biosynthesis of lysine and the degradation of amino acids, methanol and hydrogen peroxide [5].

Table I-1: Peroxisomal Functions

	Yeast	Fungi	Plants	Mammals
Biosynthesis	•lysine	•<i>penicillin</i>		•ether phospholipids •cholesterol •bile acids •polyunsaturated fatty acids
Catabolism	•decomposition of hydrogen peroxide •glyoxylate cycle •amino acids •methanol •β-oxidation of fatty acids	•decomposition of hydrogen peroxide •glyoxylate cycle •β-oxidation of fatty acids	•decomposition of hydrogen peroxide •glyoxylate cycle •purines •reactions in photorespiration •β-oxidation of fatty acids	•decomposition of hydrogen peroxide •amino acids •purines •prostaglandin •polyamines •α-oxidation of fatty acids •β-oxidation of very long-chain fatty acids

3 - PEROXISOMAL DISORDERS

Peroxisomes perform crucial functions in many biochemical pathways as highlighted by the existence of severe and fatal diseases caused by errors in the biogenesis of this organelle [66]. It was only in 1973 that peroxisomal associated disorders were identified, when Sidney Goldfischer recognized that kidney and liver tissue from Zellweger syndrome patients were devoid of peroxisomes [67]. It took until 1984 to discover a specific biomarker for the screening of patients [68], and the first gene defect associated with a peroxisomal biogenesis disease (PBD) was only identified in 1992 [69]. Although the study of PBD is just now reaching adulthood, they constitute the first description of a malformation syndrome resulting from a metabolic defect [70].

Peroxisomal disorders are a heterogeneous group of inborn errors of metabolism involving the absence or dysfunction of one or more peroxisomal enzymes that result in impairment of peroxisome function. In most cases, these disorders cause severe neurologic dysfunction that varies according to the extent of the impairment [70].

Peroxisomal diseases are divided into two main groups: i) Peroxisomal biogenesis disorders and ii) Single peroxisomal enzyme deficiency [71].

Peroxisomal Biogenesis Disorders

Peroxisomal biogenesis disorders constitute a genetically heterogeneous group of autosomal recessive disorders that result from mutations in genes that encode peroxisomal proteins necessary for peroxisome biogenesis, frequently leading to the complete loss of peroxisomal functions [2, 66]. Patient cells with severe mutations in the proteins PEX3, PEX16 or PEX19 entirely lack peroxisomal structures because these peroxins act early in the peroxisomal biogenesis [72-75]. On the other hand, cells lacking PEX1, PEX2, PEX5, PEX6, PEX10, PEX12, PEX13, PEX14 or PEX26, contain peroxisomal membrane structures, so called ghosts, that lack most of the matrix proteins because they cannot be properly imported [2]. Disfunction of PEX7 leads to the absence of a small group of matrix proteins involved in three biochemical pathways [76, 77].

There are two sub-types of peroxisomal biogenesis disorders, the Zellweger Syndrome Spectrum (ZSS) and the Rhizomelic Chondrodysplasia Punctata (RCDP). The ZSS comprises three phenotypically similar diseases that decrease in the severity of the symptoms: the Zellweger syndrome (ZS) (most severe disease), the neonatal adrenoleukodystrophy (NALD) and the infantile Refsum disease (IRD) (milder form of ZSS). Patients with ZS (also named cerebrohepatorenal syndrome) present, in the neonatal period, profound hypotonia, craniofacial dysmorphism, eye abnormalities, neuronal migration defects, psychomotor retardation and hepatomegaly. Infants with this condition are significantly impaired and usually die during the first year of life. NALD patients have less severe clinical phenotypes than ZS and usually die during late infancy. Patients with IRD, the mildest PBD, can survive longer than the second decade of life [2]. Despite the increased survival rate, both NALD and IRD patients exhibit multiple organ dysfunction, hearing loss, pigmentary retinal degeneration, and psychomotor impairments reflecting the crucial roles of peroxisomes in neuronal migration, proliferation, differentiation and survival [78, 79]. Approximately 80% of PBD patients fall within the Zellweger syndrome spectrum. These disorders occur worldwide but a variation is observed among populations. The prevalence of ZSS is estimated to be 1:50000 in the United States and 1:500000 in Japan [80-82]. About 90% of PBD-ZSS patients show mutations in the *PEX1*, *PEX6*, *PEX10*, *PEX12* or *PEX26* genes. *PEX1* mutations account for about 70% of ZSS patients, and the *PEX6* mutations for approximately 10%. [82].

The other sub-type of PBD, the RCDP type I, is caused by defects in the *PEX7* gene that encodes the peroxisomal targeting signal 2 (PTS2) receptor [83]. These patients show

only PTS2 protein dysfunction and thus the plasmalogen synthesis and the phytanic acid oxidation are affected. RCDP I is a fatal disease characterized by joint calcification, short stature with symmetrical shortening of proximal extremities, craniofacial dysmorphism and severe mental and growth retardation. Most patients die in the first couple of years but some can survive into the second decade of life [2].

Presently, there are 13 known complementation groups in PBD [84] (see table I-2).

PBDs pathologies start at a very early time, still *in utero*, and thus, the potential of postnatal treatment is quite limited. At the moment, there are a few therapeutic trials aiming at improving the developmental outcome and survival that include dietary supplementation or restriction of peroxisomal metabolic products or substrates. So far, these trials were only performed in a limited number of patients and no curative therapy is presently available for PBDs [85].

Table I-2: Complementation Groups (CG) of Peroxisomal Biogenesis Disorders

Gene	CG GiFu ^a	CG KKI ^a	Ghost Peroxisomes	Phenotypes ^b	% ^c	Refs.
PEX1	E	1	+	ZS, NALD, IRD	57%	[86]
PEX2	F	10	+	ZS, IRD	3%	[69]
PEX3	G	12	-	ZS	1%	[87]
PEX5		2	+	ZS, NALD, IRD	1%	[17]
PEX6	C	4	+	ZS, NALD	9%	[88]
PEX7	R	11	+	RCDP	17%	[76, 77]
PEX10	B	7	+	ZS, NALD	2%	[89, 90]
PEX12	G	12	+	ZS	4%	[91]
PEX13	H	13	+	ZS, NALD	1%	[92, 93]
PEX14	K	-	+	ZS	-	[94]
PEX16	D	9	-	ZS	<1%	[74, 95]
PEX19	J	14	-	ZS	>1%	[73]
PEX26	A	8	+	ZS, NALD, IRD	-	[96, 97]

a Nomenclature: Jap: Gifu University, Japan; KKI: Kennedy Krieger Institute, USA; b Phenotypes in each CG are from (Gould et al., 2001); c Proportion of the PBD patients are from (Gould et al., 2001).

Single Peroxisomal Enzyme Deficiency

Single peroxisomal enzyme deficiencies result from mutations in one specific gene leading to the loss of an enzyme or peroxisomal transporter that causes the impairment of a specific metabolic pathway. These defects are inherited in an autosomal or X-chromosomal recessive manner. Most peroxisomal disorders belong to this group, which can be subdivided further into distinct subgroups depending upon which peroxisomal function is impaired. So far, 10 of the more than 50 enzymes present in the peroxisomal matrix were found to be deficient and the cause of a peroxisomal disorder.

The most common is the X-linked adrenoleukodystrophy (X-ALD), a disorder caused by errors in ALDP which is a peroxisome membrane protein member of the ATP-binding cassette family of molecular transporters. In this disease there is an accumulation of very long chain fatty acids in plasma and tissues and, clinically, X-ALD is characterized by a highly variable phenotype ranging from asymptomatic to severe leukodystrophy with progressive demyelization of the central nervous system [98-100]. Another prevalent peroxisomal metabolic disorder is Refsum's disease that is caused by defects in phytanoyl-CoA hydroxylase, a protein required for the metabolism of phytanic acid, causing elevated levels of phytanic acid in plasma and tissue and, at the clinical level, retinitis pigmentosa, peripheral neuropathy, and cerebellar ataxia [101-103]. A list of the other known Single Peroxisomal Enzyme Deficiencies is shown below.

All peroxisomal enzyme deficiencies have severe clinical consequences and defects in enzymes within the same metabolic pathway may show different disease phenotypes [34].

Table I-3: Peroxisomal Single Enzyme Deficiencies

Single peroxisomal enzyme disorder	Protein deficiency	Impaired metabolic pathway
X-linked adrenoleukodystrophy (ALD)	ALD	β -Oxidation of fatty acids
Acyl-CoA oxidase (ACOX1) deficiency	ACOX1	
D-bifunctional protein (DBP) deficiency	DBP	
Sterol carrier protein X (SCPx) deficiency	SCPx	
2-Methylacyl-CoA racemase (AMACR) deficiency	AMACR	
Dihydroxyacetone phosphate (DHAP) acyltransferase deficiency (RCDP type II)	DHAPacyltransferase	Etherphospholipid biosynthesis
Alkyl-DHAP synthase deficiency (RCDP type III)	ADHAP synthase	
Refsum disease	Phytanoyl-CoA hydroxylase	α -Oxidation of fatty acids
Acatalasaemia	Catalase	Hydrogen peroxide metabolism
Hyperoxaluria type I	Alanine glyoxylate aminotransferase (AGT)	Glyoxylate detoxification
Glutaric aciduria type 3	Glutaryl-CoA oxidase deficiency	
Mevalonic aciduria	Mevalonate kinase deficiency	

Adapted from [4].

4 - PEROXISOME BIOGENESIS

Cellular regeneration requires peroxisomes to be continuously formed and removed according to the cell needs.

Different species show a good similarity in the factors involved in peroxisome biogenesis, and the analysis of various model organisms has revealed general rules underlying peroxisomal assembly: i) all known membrane and matrix proteins are targeted to the peroxisome post-translationally; ii) the peroxisomal import machineries and targeting signals for membrane and matrix proteins are distinct; iii) membrane and matrix protein import is mediated by receptors that interact with the peroxisomal targeting signals of their cargo proteins; and iv) both folded, oligomeric and monomeric polypeptides can be imported into the peroxisomal matrix [104]. Despite these general rules, the formation and maintenance of peroxisomes still presents some organism-specific differences [104].

Presently, there are about 85 genes identified in *Homo sapiens* and 61 genes in *Saccharomyces cerevisiae* that codify for peroxisomal proteins [105, 106]. Many of these proteins are enzymes, whereas others are proteins involved in the biogenesis and maintenance of functional peroxisomes. Until now, there are 32 peroxins identified in yeast, 16 of which with mammalian homologues and about 23 with plant homologues (see Table I-4) [105, 106].

In conceptual terms, peroxisome biogenesis can be divided into formation of the peroxisomal membrane, import of proteins into the peroxisomal matrix, and the division of the organelles. This last topic goes beyond the scope of this thesis and will not be address here (for recent reviews see Refs. [107] and [108]). The first two processes are summarized below.

Table I-4: List of peroxins.

Peroxin	Organism	Characteristics and Function
PEX1	M,P,F,Y	AAA ATPase required for matrix protein import, involved in the recycling of the PTS1-receptor PEX5 from the membrane to the cytosol.
PEX2	M,P,F,Y	RING-finger domain-containing integral PMP required for matrix protein import, putative E3 ligase.
PEX3	M,P,F,Y	Integral PMP required for membrane assembly, membrane anchor for PEX19.
PEX4	P,F,Y	Peripheral PMP required for matrix protein import; E2-like ubiquitin-conjugating enzyme.
PEX5	M,P,F,Y	TPR-motif-containing protein required for matrix protein import, PTS1-receptor. In mammals there is a short (PEX5S) and a long (PEX5L) splice isoform of PEX5. Only PEX5L binds PEX7.
PEX6	M,P,F,Y	AAA ATPase required for matrix protein import, involved in the recycling of the PTS1-receptor PEX5 from the membrane to the cytosol.
PEX7	M,P,F,Y	WD40 motif. PTS2-receptor.
PEX8	F,Y	Peripheral PMP required for matrix protein import.
PEX10	M,P,F,Y	RING-finger domain-containing integral PMP required for matrix protein import, putative E3 ligase.
PEX11	M,P,F,Y	PMP involved in peroxisome proliferation (mammals contain three isoforms (α , β , and γ) encoded by different genes).
PEX12	M,P,F,Y	RING-finger domain-containing integral PMP required for matrix protein import, putative E3 ligase.
PEX13	M,P,F,Y	SH3-domain-containing integral PMP required for matrix protein import.
PEX14	M,P,F,Y	PMP required for matrix protein import.
PEX15	Sc	Integral PMP required for matrix protein import, peroxisomal membrane anchor for PEX6.
PEX16	M,P,F,YI	Integral PMP required for peroxisomal membrane assembly.
PEX17	Y	PMP required for matrix protein import.
PEX18	Sc	Predominantly cytosolic protein acting as a co-receptor for PEX7 in <i>Saccharomyces cerevisiae</i> .
PEX19	M,P,F,Y	Predominantly cytosolic protein required for membrane assembly, import receptor and/or chaperone for PMPs.
PEX20	F,Y	Predominantly cytosolic protein acting as an adaptor protein for PEX7 in most fungi.
PEX21	Sc	Predominantly cytosolic protein acting as an adaptor protein for PEX7 in <i>Saccharomyces cerevisiae</i> .
PEX22	P,F,Y	Integral PMP required for matrix protein import, membrane anchor for PEX4.
PEX23	F,Y	Integral PMP involved in proliferation, growth regulation of peroxisomes in <i>Yarrowia lipolytica</i> .
PEX24	F,Y	Integral PMP.
PEX25	Y	Integral PMP.
PEX26 ^a	M,F,Y	Integral PMP required for matrix protein import, membrane anchor for Pex6p.
PEX27	Sc	Peripheral PMP involved in proliferation, elongation and constriction of peroxisomes.
PEX28	Sc	Peripheral PMP involved in proliferation, elongation and constriction of peroxisomes.
PEX29	Sc	Integral PMP involved in the regulation of peroxisome size, number, and distribution.
PEX30	Sc	Integral PMP involved in proliferation.
PEX31	Sc	Integral PMP involved in proliferation.
PEX32	Y	Integral PMP involved in proliferation.

M., mammals; P., plants; F. filamentous fungi; Y. yeasts; YI, *Yarrowia lipolytica*; Sc, *Saccharomyces cerevisiae*. ^a PEX26 is not found in Sc and other related yeasts. Table adapted from [106]

4.1-Peroxisomal Membrane

Peroxisomes are semiautonomous organelles, like mitochondria and chloroplasts, that multiply by growth and division [44]. However it seems that peroxisomes can also form *de novo* via the ER. Indeed, complementation studies using mutant cells lacking any pre-existent peroxisomes demonstrated that the reintroduction of the deleted gene could induce *de novo* formation of peroxisomes [109]. The contribution of this pathway in normal peroxisome containing cells is, however, probably a minor one [110].

The first step of peroxisome biogenesis consists on the synthesis and targeting of proteins into the peroxisomal membrane. Like peroxisomal matrix proteins, membrane proteins are synthesized in free polyribosomes in the cytosol and posttranslationally targeted and inserted into the peroxisomal membrane [44]. In order to be successful, this process requires the protection of the hydrophobic transmembrane segments of membrane proteins from aggregation, the maintenance of their import-competent conformations during and after synthesis in the cytosol, the targeting of these proteins to the peroxisomal membrane via membrane targeting signals, the docking to the membrane, and the insertion of these proteins into the peroxisomal membrane [111-113].

Knowledge on the membrane peroxisomal targeting signals (mPTSs) is still limited but is known to be very different from the signals that target peroxisomal proteins into the matrix of the organelle. The first description of a mPTS was accomplished in 1996, for the peroxisomal membrane protein PMP47 of *Candida boidinii*. This was described as a basic amino acid cluster in front of a transmembrane segment [114]. mPTSs are now admitted to comprise one or more transmembranar segments located nearby a positively charged amino acid cluster [115-119] comprising stretches of at least five amino acids [119, 120]. Many PMPs have multiple and redundant mPTSs, with any one of which being sufficient to direct proteins to the peroxisome membrane [119-121]. mPMP are large, 50–100 amino acid in length, and the combined size of the all targeting signals in several PMPs comprise a significant fraction of the entire protein [119, 120, 122].

In mammals, there are only three known peroxins involved in the peroxisomal membrane assembly: PEX3, PEX19 and PEX16. In yeast, only PEX3 and PEX19 were identified and no PEX16 homologue was found [123].

PEX19 is an acidic protein that functions as a soluble PMP receptor and chaperone delivering newly synthesized PMPs from the cytosol to PEX3 [122, 124]. This PEX19 binding of newly synthesized PMPs in the cytosol immediately after translation serves to prevent PMPs from aggregating and also to direct them to PEX3 [125-127]. It was demonstrated that this PEX19-PMP complex presents a higher affinity for PEX3 than PEX19 alone [125]. In agreement with its function, PEX19 shows a dual subcellular

distribution with 95% being localized in the cytosol and 5% in association with peroxisomes [122]. In solution, human PEX19 is a monomeric protein with a largely disordered N-terminal and a structured C-terminal [128, 129]. The C-terminal domain of PEX19 binds numerous PMPs, including several peroxins such as PEX3 and PEX13 [130-132], and contains a CAAX box where PEX19 is farnesylated [126, 133, 134]. It's the only peroxin known to be farnesylated, and this lipid modification is necessary, although not essential, for its function in peroxisome biogenesis [135, 136]. In fact, in some organisms like *trypanosomes*, PEX19 lacks this farnesylation motif [137].

PEX3 is an intrinsic membrane protein of the peroxisome that serves as the membrane anchor for PEX19 cargo-loaded PMP complexes. Its N-terminal domain (amino acids 1-46) house the necessary information for its targeting to the peroxisomal membrane, while its C-terminal domain is exposed to the cytosol and interacts with the cargo loaded PEX19 [116, 124, 131].

PEX16 is an intrinsic membrane protein identified in many fungi, plants and mammals but absent in most yeasts [138, 139]. Its role in peroxisomal membrane biogenesis is still not very clear but it has been proposed to serve as a recruitment factor for PEX3, or as a component of the putative membrane translocase [139].

4.2-Peroxisomal Matrix

Presently there are more than 50 peroxisomal matrix proteins identified in mammals that are targeted into peroxisomes by one of two different peroxisomal targeting signals (PTSs): the peroxisomal targeting signal 1 (PTS1), or the peroxisomal targeting signal 2 (PTS2) [34].

One interesting feature of peroxisomes is the fact that, unlike other organelles, peroxisomal matrix proteins can translocate across the peroxisomal membrane fully folded and even co-factor bound. Even oligomeric proteins can be imported into the peroxisomal matrix [140, 141]. Early experiments suggested that peroxisomal oligomeric matrix proteins can also reach the matrix of the organelle still as monomers [164, 165]. Indeed, the comparison of the kinetics of rat liver catalase assembly (a tetramer) with its translocation into an organelle fraction suggested that catalase was imported as monomers lacking the heme group [142, 143]. Similar results, consistent with the idea that protein oligomerization occurs only during or after import, were found for malate synthase in cucumber glyoxysomes [144] and for alcohol oxidase in *Candida boidinii* (an oligomeric protein of about 600kDa) [145], [146].

Overall, the peroxisomal membrane capacity to support the import of oligomeric proteins attests for the large plasticity of the peroxisomal membrane but the prevalence and

relevance of such an import under normal physiological conditions is still not clear as most of the observations of oligomeric protein import were obtained using over-expressed proteins [140, 141, 147-150].

Peroxisomal matrix protein import can be divided into five general steps: i) binding of cargo by the receptor, ii) docking and translocation of the receptor/cargo complex at the peroxisome membrane, iii) receptor ubiquitination, iv) recycling of the receptor back into the cytosol and v) receptor deubiquitination, yielding free receptor that can start another round of import.

i) Receptor-cargo recognition and binding.

Most matrix proteins are targeted to peroxisomes by a single PTS1 or PTS2 signal. There are rare cases of proteins that have both (e.g. PEX8 in *Pichia pastoris*) and also peroxisomal matrix proteins with no conventional targeting signal that are directed into the organelle by, yet poorly defined, internal PTSs [151]. The identification of the PTS1 at the C-terminus of luciferase of the firefly *Photinus pyralis* in 1987 [148, 152] was a major breakthrough in the elucidation of the mechanism of protein import into peroxisomes.

PTS1 consists of a non cleavable tripeptide located at the extreme C-terminus of proteins, often with the sequence Ser-Lys-Leu or conserved variants [6]. The vast majority of peroxisomal matrix proteins are targeted into peroxisomes by a PTS1 signal [6] whereas only a small number of peroxisomal matrix proteins contains a PTS2 [7, 8]. PTS2 was first identified in thiolase from rat liver and consists of a nonapeptide sequence, located near the N-terminus, usually with the motif R-(L/V/I/Q)-xx-(L/V/I/H)-(L/S/G/A)-x-(H/Q)-(L/ A) [7, 8]. In *Caenorhabditis elegans* there are no PTS2 containing proteins identified. In *Saccharomyces cerevisiae* the only known PTS2 protein is thiolase [153], in mammals, in addition to thiolase, also the alkyl-dihydroxyacetone phosphate synthase and the phytanoyl-CoA hydroxylase have a PTS2 signal, and in plants approximately one third of peroxisomal proteins may contain a PTS2 [8, 154]. In different organisms the same enzyme may use either type of PTS. For example, acyl-CoA oxidase uses a PTS1 in mammals and in the yeast *Pichia pastoris*, but generally uses a PTS2 in plants; the alkyl-dihydroxyacetone-phosphate acyl transferase contains a functional PTS2 in mammals but has a PTS1 in *Trypanosoma brucei* and *Dictyostelium discoideum* and the peroxisomal malate dehydrogenase and citrate synthase enzymes use a PTS1 in *Saccharomyces cerevisiae* but a PTS2 in plants [155].

Both the PTS1- and the PTS2-containing matrix proteins are recognized in the cytosol by soluble, cytosolic receptors/co-receptors that direct them to the docking site at the peroxisomal membrane: PTS1 proteins are recognized by PEX5 and PTS2 proteins by PEX7 [156, 157].

PEX5

PEX5 was first identified in *Pichia pastoris* and is nowadays recognized in all organisms from protozoa to mammals [17, 158-162]. Mutations in this receptor have been found in patients affected with Zellweger syndrome, neonatal adrenoleukodystrophy, or infantile Refsum disease [17], thus demonstrating the crucial role of this peroxin in peroxisome biogenesis.

In mammals, there are two variants of PEX5 resulting from alternative splicing of the *PEX5* transcript: PEX5S, the short form, and PEX5L, the long form of PEX5. PEX5L contains an insert of 37 amino acids that enables PEX5L to bind PEX7 and thus PTS2-proteins (as well as PTS1-proteins). PEX5S binds PTS1-containing proteins but is incapable of interacting with PEX7 and, therefore, with PTS2-proteins [11, 12].

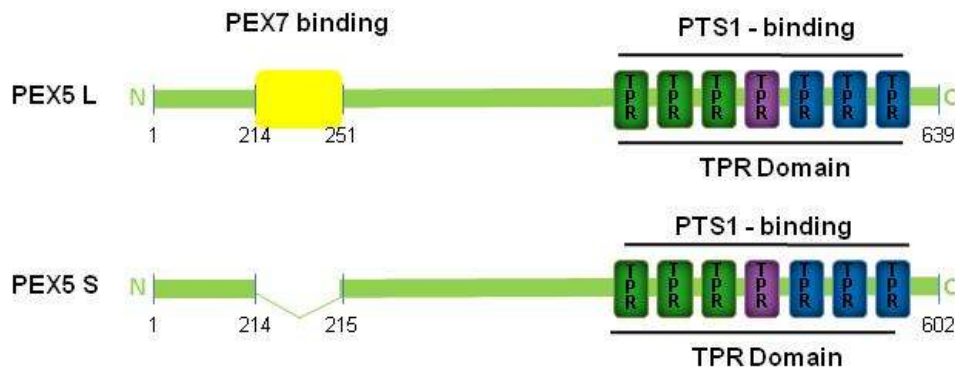


Fig. I-2: Schematic representation of mammalian PEX5 isoforms. In mammals, alternative splicing of the *PEX5* transcript originates two PEX5 isoforms. PEX5L, the long form of PEX5, contains a 37 amino acid insert that enables this form to bind PEX7. PEX5S, the short form, lacks the PEX7 binding domain and binds PTS1-containing proteins but is incapable of interacting with PEX7 and, therefore, with PTS2-proteins [11, 12]. Green, violet and blue boxes represent the seven TPR domains involved in PTS1 binding (color code according to the crystal structure in Fig. I-3). The 37 amino acid region involved in PEX7 binding is indicated by the yellow box.

```

Hs  MAMRELVEA---ECGGA-----NPLMKLAGHFTQDKALRQE-----GLRPGWPWPPGAPASEAASKPLGVASEDEL 62
Dr  MAMRELVEA---ECGGA-----NPLMKLTGHMTQEGGAWRH-----RSTP-TIPP-----TPIEIATEDEL 52
At  MAMRDLVNGGAA-CAVPGSSSSSNPLGALTNALLGSSSKTQERLKEIPNANRSGPRPQFYSEDQQIRSLPGSELDQPL 77
Sc  MDVGS-----CSVG-----NNPLAQLHKHTQQNKSLQFNQ-KNNGRLNESPLQGTNKPGEISAFISNVNAISQEN 64
    *::: : *      *** *      :      * : :

Hs  VAEFLQDQNAFLVSRAPQTFKMDLLAEMQQIEQS----- 97
Dr  VAEFLQGP-----QRPPHSFDMGQLLEEMQQIDQQ----- 82
At  LQPGAQGSSEFFRGFRSVDQNLGAAWDEVQGGPMPPMGPMFEPVQPTFEGPPQVRVLSNFLHSFVESRGGIFPRPAP 155
Sc  MANMQRFINGEPLIDDKRMEIGPSSGRLPPFSNVHS----- 101
    : :      : :

Hs  -----NFRQAPQRAPGVADLALSENWAQEFLL-AAGDAVDVT----QDYN----- 136
Dr  -----NYRQAPQRAPDVAAALALSGDWASEFL-STAASSSGQAALDPA----- 125
At  VPVVLGSLQSDKQCIQRRSSIMARHFFADRGEFFINSQVNALSSLDIDGDIQARGHVPGRFRELDDYWNESQAVVKP 232
Sc  -----LQTSANPTQIKGVNDISHWSQEFQ--GSNSIQNRNADTGNSE----- 141
    : ** :

Hs  ----ETDWSQEFISEVTDPLSVSPARWAEYELEQSEKWLWGEPEG-----TATDR-WYDEYHPEEDLQHTASDFVA 203
Dr  ----DADWTREFINEVAD-----PGRWAEYELEQSEKWLWGLDGE-----REQDKWEAQEYQSGEELRQTANELVA 188
At  NLHPADNWAAEFNQHGMDHG--GPD SWVQSFEQQHGVNGWATEFEQQS QLMSSQMRSMQNI AAMEQTRKLAHTLS 308
Sc  ----KAWQRGSTTASSRFQY--PNTMMNNYAYASMNSLSGSRQLSPAFMNQQQSGRSKEGVNEQEQQPWTDFEFKLE 212
    * : : * : : : :

Hs  KVDDPKLANSEFLKFVRQIGEGQVSLSGAGSGRAQAEQWAAEFIQQQGTSDAWVDQFTRPVNTSA----- 269
Dr  KVDDPKLQN-----TEVSAES-----AESWVDEFAT----- 214
At  QDGNPKFQNSRFLQFVSKMSRGELIIDENQVKQASAPGEWATEYEQQYLGPSPSWADQFANEKLSHGPEQWAEFASGR 386
Sc  KEVSENLDIN-----DEIEKEENVSEVEQNKPETVE----- 243
    : : : : :

Hs  LDMEFERAKSAIESDVFWDKLQAELEEMAKRDAEAHPWLSDYDDLTS-----ATYDKGYQFEEENPLRDHPQPFEEG 342
Dr  YGPDFQQAKAIVESDVFWEKLQWEWEEMAKRDAEAHPWLSDFDQMLS-----SSYDKGYQFEEDNPYLSHEDPFAEG 287
At  GQQETAEDQWNEFSLNVDWDWIDEFAEGPVGDSADAWANAYDEFLEKNAGKQTSGVYVFSMDNPYVGHPEPMKEG 464
Sc  -KEEGVYGDQYQSDQFQEVWDSIHKDAEEVLPSSELVNDLNLGEDYLYKYLGRVNGNIEYAFQSNNEYFNPNAYKIG 319
    : : : : * : : * * * : : : *

Hs  LRLRQEGD-LPNAVLLEFAAVQQDPKHMEAWQYLGTTQAEENEQELLAISALRRCLELKPNDQTALMALAVSFTNESLQ 419
Dr  VKRMEAGD-IPGAVRLFESAVQRQPDNQLAWQYLGTCQAEENEQEFAAISALRRCIELKKNLTALMALAVSFTNESLR 364
At  QELFRKGL-LSEAALALEAEVMKNPENAEGWRLLGVTAEENDDQQAIAAMMRAQEADPTNLEVLLALGVSHSTNELEQ 541
Sc  CLLMENGAKLSEAAALAEFAAVKEKPDHVDLRLGLVQTQNEKELNGISALEECLKLDPKNLEAMKTLAISYINEGYD 397
    * : * : : * : * * : : * : : : * : : : *

Hs  RQACETLRDLWLRYPAYAHLVTPAEEGAGGAGLGPSKR---ILGSLSDSLFLEVKEFLAAVRLDPTSIDPDVQCGL 494
Dr  RQACETLRDWMHNPYRIILEQHEREKQREGAREKESERFGSLPEALFGEVQTLFLNAAAAPSQVDPQLQCGL 442
At  ATALKYLYGWLNRNHPKYGAIAIPP-----ELADSLYHADIARLFNEASQLNPE--DADVHIVL 596
Sc  MSAFTMLDKWAETKYPEIWSRIKQDDKFQ-----KEKGFTHIDMNAHITKQFLQLAN-NLSTIDPEIQLCL 463
    * * * : : : : : : : : * : : *

Hs  GVLFNLSGEYDKAVDCFTAALSVRPNDYLLWNKLGATLANGNQSEEAVAYRRALELQPGYIRSRYNLGISCINLGAH 572
Dr  GVLFNLSGEYDKAVDCFSAAALSVTPQDYLLWNKLGATLANGNRSEEAVAYRRALELQPGFVRSRYNLGISCYNLGAH 520
At  GVLNLSREFDRAITSFQTALQLKPNDYSLWNKLGATQANSVQSADAI SAYQQALDLKPNYVRWANMGISYANQGM 674
Sc  GLLFYTKDDFDKIDCFESALRVNPNDELMMWNLGASLANSRNEEAIQAYHRAQLKPSFVRARYNLAVSSMNIGCF 541
    * : : : : * * : * : : : * : * : * : * : * : * : * : *

Hs  REAVEHFLEALNMQRKS-----RGPRGEGGAMSENIWSTLRLALSMLG--QSDAYGAADA-RDLSTLLTMFGLP 638
Dr  REAVEHFLEALSLQQAAGDGEAGAGRGPGAAATIMSDNIWSTLRMALSMG--ESSLYSAADR-RDLDTLLTHFSHR 595
At  KESIPYYVRALAMNPKA-----DNAWQYLRSLSCAS--RQDMIEACES-RNLDLLQKEFPL- 728
Sc  KEAAGYLLSVLSMHEVN---TNNKKGDVGSLLNTYNDTVIETLKRVIAMN--RDDLLQEVKPGMDLKRFGGEFSF- 612
    * : : * : : : : : : : : * : *

Hs  Q---- 639
Dr  EGAE 600
At  -----
Sc  -----

```

Fig. I-3: Sequence alignment of PEX5 proteins from *Homo sapiens* (Hs), *Danio rerio* (Dr), *Arabidopsis thaliana* (At) and *Sacharomyces cerevisiae* (Sc). The shaded "C" marks the ubiquitination site of PEX5. "-" residues identical in all sequences in the alignment; "." conserved substitutions, ":" semi-conserved substitutions. (ClustalW2, manually edited).

Structurally, PEX5 has two distinct domains: the N-terminal domain and the C-terminal domain. The N-terminal domain is an unstructured region comprising the binding sites for other peroxins that is considered to mediate the binding of PEX5 with components of the import machinery during the receptor cycle [163, 164]. This N-terminal region contains several repeats of a pentapeptide WxxxF/Y motif [161, 165-167] responsible for the interaction with PEX13 and PEX14 [20, 166, 168]. In the long isoform of mammalian PEX5, and in plant PEX5, this region also contains the PEX7 binding site [13, 18]. The functional role for being a disorder domain is just a matter of speculation. It is hypothesized that it may have kinetic importance in the regulation of the interactions with ligands (e.g. docking/translocation complex proteins PEX13 and PEX14 and cargo proteins).

In contrast to the N-terminal region, the C-terminal half of PEX5 is highly conserved. This domain contains seven tetratricopeptide repeats (TPRs) that specifically interact with the PTS1 sequence of the cargo-proteins [16]. Crystal structures of the receptor in the absence and presence of the cargo-protein revealed some conformational changes in PEX5 upon substrate binding [169]. The crystal structures of the PEX5 receptor/peptide complex show that upon cargo binding, the overall conformation of the C-terminal half of the receptor changes from an open snail-like conformation into a closed ring-like structure formed by the two TPR triplets motifs 1–3 and 5–7 and the distorted TPR4 motif that connects them [170, 171]. Comparative analyses of PEX5 C-terminal structures in the presence of either a PTS1 peptide or a fully functional cargo protein show that the receptor acquires a virtually identical conformation, suggesting that it is the C-terminal PTS1 motif, and not other parts of the cargo, the responsible for the structural changes [171, 172]

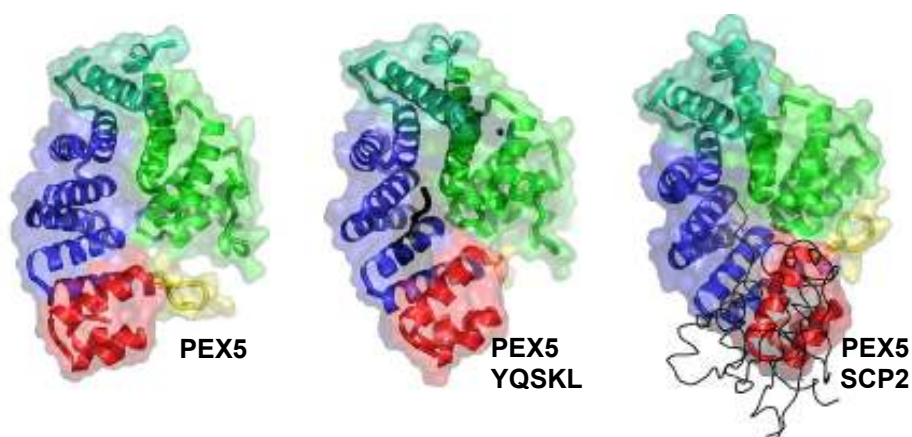


Fig. I-4: PEX5 structures in the absence of ligands (left,[171]), in the presence of the consensus PTS1 peptide YQSKL (central, [172]), and in the presence of the functional PTS1 cargo protein SCP2 (right, [171]). Green regions represent TPR segments 1–3; violet regions represent TPR4 segment; blue regions mark TPR segments 5–7; yellow segments show loop connecting the TPR segment and the C-terminal helical bundle, referred as to '7C-loop' in [171]; red domains represent C-terminal α -helical bundle; black regions show PTS1 ligands. (from [169])

PEX5 is a cycling receptor. At steady-state, the majority of PEX5 is localized in the cytosol but a considerable portion of PEX5 is also found at the peroxisomal membrane, with a significant fraction being protease protected, thus behaving as a membrane protein [173]. When at the membrane, a small N-terminal fragment of mammalian PEX5 was shown to be exposed to the cytosol, while the C-terminus of the protein, containing the PTS1-binding site, faces the peroxisomal matrix [173]. This way, during the protein transport cycle PEX5 acquires a transmembrane topology.

PEX7

PEX7 is a peroxin first identified in *Saccharomyces cerevisiae* [174] but homologues of PEX7 have been identified in other fungi, plants, and humans [76, 77, 175]. It has not been identified in nematodes, in *Caenorhabditis elegans* (which appears to rely exclusively on PTS1s for targeting to the peroxisomal matrix) [155]. In humans, the absence of PEX7 function causes the RCDP I disease [76, 77].

This peroxin is a member of the WD40 protein family and contains 6 repetitions of a conserved sequence of 40 amino acids each (WD40 motifs) with a tryptophane –aspartic acid, as a central domain [174]. WD40 domains are found in a large number of eukaryotic proteins and cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly [176]. With the exception of the 60 N-terminal amino acids, the WD40 motifs make up for almost the whole PEX7 peroxin [175]. PEX7 is a soluble, hydrophilic protein, without transmembrane domains, that recognizes peroxisomal targeting signal type 2 (PTS2)-containing proteins in the cytosol [157, 177].

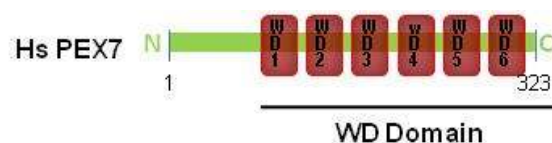


Fig. I-5: Schematic representation of mammalian PEX7. Red boxes indicate the 6 WD motifs.

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Hs      MSAVCGGAARMLRTPGRHGYAAEFSPYLPGRACATAQHYGIAGCG--TLLILDPEAG- 57
Rn      MS-----AARTLRVPGRHGYAVEFSPYLPGRACAAAQHYGIAGCG--TLLVLDQNESG- 52
At      -----MPVFKAP-FNGYSVKFSPFYESRLAVATAQNFILGNNGRIHVLELAPGAPG- 50
Sc      -----MLRYHMQGFGSGYGVQYSPFFDNRLAVAAGSNFGLVGN--KLFLEIDRSGR 50
          :      **.....: .*** *:.....: * *   :: * . .*

Hs      LRLFRSFDWNDGLFDVTWSENNEHVLITCSGDGSLQLWDTAK--AAGPLQVYKEHAQEVY 115
Rn      LQIFRSFDWNDGLFDVTWSENNEHVLVTCSGDGLQLWDTAK--ATGPLQVYKEHTQEVY 110
At      VTESVSYDTADAVYDVCWSESHDSVLIAAIGDGSVKIYDTALPPPSNPFRSQEHAREVQ 110
Sc      IVEVNSFLTQDCLFDLAWNESHENQVLVAQGDGTLRLFDITF--KEFPIAIFKEHEREVF 108
          :      *:      * ::*: *.*: :... ***:::***:      *: :*** :**

Hs      SVDWSQTRGEQLVVSGSWDQTVKLWDPTVGKSLCTFR----- 152
Rn      SVDWSQTRDEQLVVSGSWDQTVKWDPTVGNLCTFR----- 147
At      SVDYNPTRRDSFLT-SWDDTVKLWAMDRPASVRTFK----- 146
Sc      SCNNNLVNRQNFLSS-SWDGSIKIWSPLRKQSLMTLTPRPLEITKMVDPLNAILKKKSF 167
          * :.. .. ::: * *** :*: *      *: *:

Hs      ----GHESIYSTIWSPHIPGCFASASGDQTLRIWDVKAAG---VRIVIPAHQAEILSCD 205
Rn      ----GHESVIYSTIWSPHIPGCFASASGDQTLRIWDVKITG---VRIVIPAHQAEILSCD 200
At      ----EHAYCVYQAVWNPKHGDVFASASGDCTLRIWDVREPG---STMIIPAHDFEILSCD 199
Sc      TGISKNRNCVYQAQFSPHDQNLVLSCSGNSYASLFDIRLPSGKNQNNFLVHSGLEALTC 227
          :      :*. : :*. : . . *.*: :*: :.. .:      * :***

Hs      WCKYNENLLVTGAVDCSLRGWDLNRVRQPVFELLG-----HTYAIR 246
Rn      WCKYNENLLVTGAVDCSLRGWDLNRVRQPVFELLG-----HTYAIR 241
At      WNKYDDCILATSSVDKTVKVDVRSYRVPLAVLNG-----HGAVR 240
Sc      FNKYRPYVVAITGGVDNAIRIWDIRMLNKNESATIKRTVPGQLHNSSCINEIPNAHGLAIR 287
          : **      :*. :.*** ::: **:* .      * :*:

Hs      RVKFSFPFHASVLASCSYDFTVRFWNFSPKPSLLETVEH-----HTEFTCG 291
Rn      RVKFSFPFHASVLASCSYDFTVRFWNFSPKPSLLETVEH-----HTEFTCG 286
At      KVKFSPPHRSLIAASCSYDMSVCLWDYMDALVGRYDH-----HTEFVAVG 285
Sc      KVTWSPHHSNILMSASYDMTCRIWRDLSDNGAKETYKTNSTDATKGSIFNFTQHSEFVFG 347
          :*. :*. : :. :*. :*: :*      *      .      * :*. *

Hs      LDFSLQS-PTQVADCSWDETIKIYDPACTIPA 323
Rn      LDLSLQS-PTEVADCSWDETVKIYNPVC LAVPG 318
At      IDMSVLV-EGLMASGTWDELVVVWQGMDFRAS 317
Sc      ADWSLWKGKPGYVASTANDGNLFVWNLG----- 375
          * *:      :*. :.*** : :::

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Fig. I-6: Sequence alignment of PEX7 proteins from *Homo sapiens* (Hs), *Rattus norvegicus* (Rn), *Arabidopsis thaliana* (At) and *Sacharomyces cerevisiae* (Sc). "-"-residues identical in all sequences in the alignment; ":"- conserved substitutions, "."- semi-conserved substitutions. (ClustalW2).

Its subcellular localization during the cargo transport cycle is still a matter of debate. Its known to act as a cycling receptor (such as PEX5), picking up cargo in the cytosol and dropping it off at the peroxisome membrane [178] but whether is retained there or if it goes entirely inside the matrix is not understood so far. Experiments using N-terminally Myc-tagged PEX7 in *Saccharomyces cerevisiae* and human fibroblasts, showed PEX7 to be predominantly in the cytosol [76, 174, 179], whereas PEX7, C-terminally tagged with three haemagglutinin sequences, was localized entirely inside the peroxisomes in *Saccharomyces cerevisiae* [180]. Depending on its subcellular localization, it has been

proposed that PEX7 may function not only as a cytosol-to-peroxisome shuttling receptor [157, 181] but also as an intraperoxisomal pulling receptor [180].

PTS1 Targeting

PTS1 proteins in the cytosol are recognized and bound directly by PEX5 [16, 17]. Most cargoes that bind PEX5 do so via interaction of the TPR repeats on PEX5 with the PTS1, but a few PEX5-dependent cargoes, lacking canonical PTS1 sequences, may interact with PEX5 by other poorly-defined mechanisms [182]. The dissociation constant of the PEX5 cargo complex is in the 18–100 nM range [171, 183].

A specific feature of the peroxisomal translocation machinery is that PEX5 cycles between the cytosol and the peroxisomal membrane, and switches between a soluble and a membrane-bound state [106].

PTS2 Targeting

The PTS2 pathway is responsible for the targeting of a much smaller number of proteins than the PTS1 pathway. Nevertheless, it is still crucial, as demonstrated by the disease RCDP I, caused by a failure to import PTS2 proteins [66].

In contrast to PTS1-proteins import, where only PEX5 is required for the targeting and import into the peroxisome matrix, the import of PTS2-protein entails not only PEX7 but also other auxiliary proteins. In fungi, such as *Yarrowia lipolytica*, *Pichia pastoris*, *Neurospora crassa*, and *Hansenula polymorpha*, PEX7 associates with the peroxisomal membrane via PEX20 [181, 184]. In *Saccharomyces cerevisiae* the import requires the PEX20 functionally redundant orthologues, PEX18 and PEX21 [185]. Humans and plants do not have orthologues of these co-receptors. In mammals, PTS2-mediated targeting of proteins into the peroxisomal matrix requires not only binding of PTS2 to PEX7, but also binding of PEX7 to the long isoform of PEX5 [18, 186]. In *Arabidopsis*, PEX5 and PEX7 form a PTS1/PTS2 receptor complex by interaction between the amino-terminal domain of PEX5 and the carboxyl-terminal domain of PEX7 [13]. Experiments have shown that PEX5L mutants, where the PEX7 interaction is impaired, have a PTS2 protein import defect but are still competent to import PTS1 proteins [13]. Additionally, the down regulation of PEX5 also resulted in a PTS2 import defect, suggesting that the PTS1 and PTS2 import pathways are coupled in plants as they are in mammals [187]. Cells lacking both PEX5 isoforms are deficient in PTS1 and PTS2 import [13].

PEX5L and the PTS2 co-receptors in fungi and yeasts (PEX20, PEX18 and PEX21) share not only a common function but also several structural similarities: a PEX7-binding motif; several diaromatic pentapeptide repeats (WXXXF/Y); and an amino-terminal domain of approximately 30 residues. The role of these co-receptors in the PEX7-cargo interaction is yet not clear but it was reported that PEX18 and PEX21 promote the formation of an

import competent PEX7-PTS2 protein complex before the docking step in *Saccharomyces cerevisiae* [188]. While PEX5L independently and directly binds to PEX14 and PEX13 [20, 166], PEX18/PEX21 require the presence of PEX7 for the binding to the docking/translocation complex [189]. In mammalian cells, PEX7 requires PEX5L for the interaction with PEX14 but binds to PTS2 proteins in the absence of PEX5L [186, 189].

In *Caenorhabditis elegans*, where there are no PTS2 containing proteins identified, PEX7 appears to be absent and PEX5 lacks the PEX7 interaction motif. Homologs of mammalian PTS2 proteins in *Caenorhabditis elegans* lack a PTS2 signal and are imported via a PTS1 instead, indicating that the PTS2 pathway is absent in this organism [155].

Presently there is no structural information on the interaction between PTS2 proteins and PEX7.

ii) Docking and translocation of the receptor/cargo complex at the peroxisome membrane

Following cargo recognition, cargo-loaded receptors are delivered to the peroxisome membrane where they interact with conserved membrane peroxins: PEX13 and PEX14 [5].

PEX13 and PEX14

PEX13 and PEX14 are integral membrane peroxins with a central role in the cargo docking and translocation across the peroxisomal membrane. Both peroxins were identified in all organisms and were shown to be essential for both PTS1- and PTS2-mediated protein import.

PEX13 is structurally characterized by two transmembrane domains and one SH3 domain [190, 191]. The SH3 domain is exposed to the cytoplasm and plays an essential role for PEX13 function (its deletion in yeasts leads to a similar phenotype as that of a complete *PEX13* deletion) [191]. SH3 domains have approximately 60 amino acids in length and retain a high level of sequence similarity, especially among residues involved in ligand recognition. They are known to mediate protein–protein interactions involved in several cellular processes including cytoskeletal organization, signal transduction and protein localization [192].

PEX14 is an integral membrane protein exposing its carboxyl-terminal two thirds into the cytosol, whereas its N-terminal third is either embedded in the peroxisomal membrane or even exposed into the matrix of the organelle [193]. Pex14 interacts with itself. The homooligomerization interface comprises a 16 amino acids transmembrane helix motif (from amino acids 109 to 127) and a putative coiled-coil located in the C-terminal region (amino acids 140 to 278) [194, 195]. It has been demonstrated that in the absence of a functional PEX14, peroxisomal matrix proteins are retained in the cytosol [196].

PEX13 and PEX14 can interact directly with each other [197]. In *Saccharomyces cerevisiae*, PEX14 has two domains of interaction with PEX13. A proline-rich SH3-ligand motif (PxxP) located in its N-terminal region, that establishes the interaction with the SH3 domain of PEX13, and a second interaction region located in the luminal domain of PEX13 [197]. Mammalian PEX14 does not contain a PxxP domain and the interaction with PEX13 is accomplished by a different N-terminal region of PEX14 [197]. Studies in yeast showed that when the PEX14 binding sites in PEX13 are mutated, the import of both PTS1 and PTS2 proteins is considerably less efficient but not completely abolished, suggesting that the PEX13–PEX14 interaction, although important, is not essential for import [197].

The association of both PEX13 and PEX14 with PEX5 is mediated by the WxxxF/Y motifs present in the N-terminal half of PEX5. In the case of PEX13, this association is achieved by the interaction with either a novel binding cleft on the SH3 domain of yeast PEX13, or with both the PEX13 N-terminus and SH3 domain in the case of mammalian PEX13 [21, 164]. The interaction of PEX5 with PEX14 involves a conserved AX₂FLX₇SPX₆FLKGKGL/V motif, present in the first 80 amino acids of PEX14 [166, 198]. In *Saccharomyces cerevisiae* there is an additional interaction region described in the C-terminal domain of PEX14, between amino acids 250-308 [199] but so far, such an additional binding region appears to be absent in mammalian PEX14 [196]. Mammalian PEX5 has seven WxxxF/Y motifs all capable of interacting independently with PEX14 [166]. The dissociation constant for PEX5-PEX14 interaction is in the low nanomolar range [166, 200]. During the PEX14-PEX5 interaction, PEX14 undergoes conformational changes, especially in its hydrophobic domains [198].

It has been suggested that PEX14 binds more strongly to cargo-loaded PEX5, than to free PEX5, whereas PEX13 interacts more strongly with cargo-free PEX5 [20, 200]. On the other hand, over-expression of PEX14 in CHO cells, but not of PEX13, leads to an increase in the amount of PEX5 associated with the peroxisomal membrane [20, 200]. These observations led to the proposal that PEX14 is the docking protein for PEX5. However, it is now clear that the role of PEX14 goes well beyond this function. Indeed, the membrane topology of this peroxin (which has its strongest PEX5-binding domain embedded in the peroxisomal membrane or even exposed into the matrix of the organelle) together with the observation that PEX5 remains associated with PEX14 during all the steps occurring at the membrane [23] strongly suggest that PEX14 is involved in the translocation step as well [196].

The specific function of PEX13 in the PEX5-mediated import pathway is still unknown but a role in the cargo release step has been suggested [21].

Based on the membrane topologies of PEX14 and DTM-embedded PEX5 as well as on the energetic of this process (see section iv) “Recycling of the receptor back into the cytosol” bellow) our laboratory proposed, a few years ago, that cargo proteins are translocated across the peroxisomal membrane by PEX5 itself [164, 201]. The driving force for this process derives from the strong protein interactions that are established at the DTM involving PEX5, on one side, and components of the DTM (probably PEX14), on the other [193, 196, 202]. According to this model, PEX5 never crosses the organelle membrane completely. A derivative of this model was published later [203]. Those authors proposed that several molecules of PEX5 assemble around cargo proteins and that this complex then becomes inserted into lipid membranes, as bacterial pore forming toxins do. Although this so-called transient pore model was proposed half decade ago, no convincing evidence supporting it is presently available. Actually, although the authors subsequently claimed that PEX5 interacts with lisosomes [204], our laboratory could not find any evidence for this phenomenon [205].

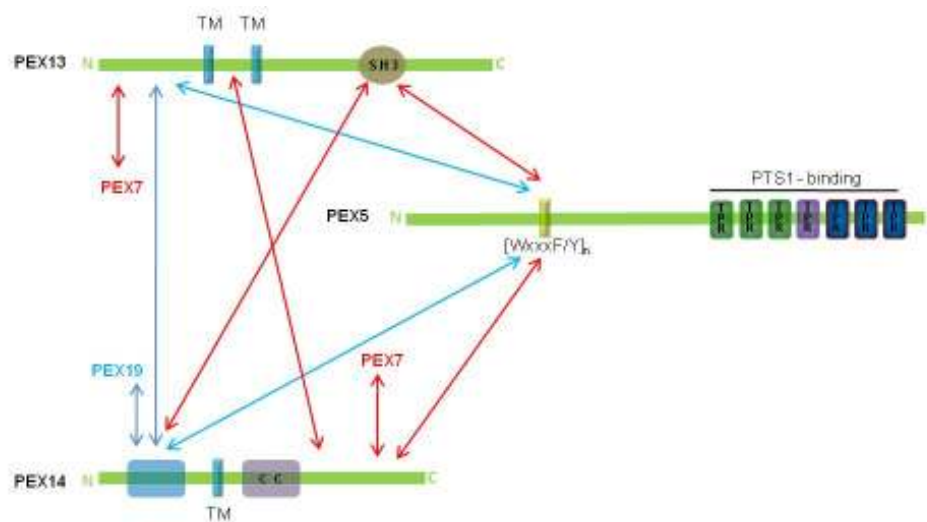


Fig. I-7: Schematic representation of PEX5, PEX13 and PEX14 interactions. Arrows indicate interactions among the three proteins in yeasts (red) and mammals (blue). Binding sites that are not precisely mapped are indicated by question marks. Only one WxxxY motif in the N-terminal half of Pex5p is shown, but the number (n) can vary between 2 (yeasts) and 7 (mammals). TPR, tetratricopeptide repeat; TM, transmembrane domain; CC, coiled-coil domain; PxxP, proline-rich SH3 binding motif; SH3, Src homology 3 domain (adapted from [21]).

PEX2, PEX10 and PEX12

PEX2, PEX10 and PEX12 are intrinsic membrane proteins composed of two transmembranar regions and a RING domain at the C-terminal exposed to the cytosol [206]. Impaired function of these three peroxins results in the failure of matrix protein import [70]. RING domains consists of a cross-braced structure in which two Zn^{2+} ions are coordinated by the side chains of cysteine and/or histidine residues. There are hundreds of proteins with RING finger domains in mammals [207]. Several studies have implicated this domain as an important element in the function of various proteins such as the breast cancer 1 (BRCA1) [208]. Functionally, the RING domain is now known to be involved in ubiquitination reactions and actually, it defines a large family of ubiquitin ligases (E3). PEX10 and PEX12 interact directly with the PTS1-receptor PEX5 throughout their RING finger [209]. The RING finger of PEX10 also interacts with PEX2 and PEX12 [209, 210]. Evidence that PEX5 [209, 211, 212] and PEX20 [213], accumulate in peroxisomes in cells with a disrupted RING-complex suggested that these peroxins are involved in PEX5 release from the peroxisome. Recent data showing that DTM-embedded PEX5 has to be monoubiquitinated in order to be supported back into the cytosol provides the molecular basis to understand this phenomenon [206, 214]. The specific RING peroxin, or the combination of them, that catalyses this monoubiquitination remains unknown.

iii) Receptor ubiquitination

DTM-embedded PEX5 is exported back to the cytosol for further rounds of import [57, 59, 60] in a process that requires ubiquitination [214].

Ubiquitination consists in the covalent attachment of a 76 amino acid ubiquitin (Ub) polypeptide to a substrate and this post-translational protein modification is highly conserved throughout evolution [215]. Interestingly, poly- and monoubiquitination serve very different functions. Polyubiquitination is a well known process that targets proteins to degradation by the 26S proteasome [216]. In contrast, monoubiquitination functions as a transient modification to modulate protein biological properties [206, 217-220]. In some cell surface proteins monoubiquitination has been shown to function as a sorting marker for plasma membrane internalization and as a signal for the internalization of membrane proteins into multivesicular bodies [221].

Protein ubiquitination is achieved throughout a three-step enzyme cascade: first, the E1 (Ub activating enzyme) activates the Ub, then this activated-Ub is transferred to E2 (the Ub conjugation enzyme) and finally, an E3 (Ub ligase) conjugates the ubiquitin moiety to the substrate protein [215, 222]. Generally, ubiquitin is covalently attached through an amide bond involving the carboxyl group of the last glycine of ubiquitin on one hand, and an amino group of the targeted protein on the other, but recent findings showed that

proteins can also be ubiquitinated at serines and threonines (yielding oxyesters) or even at cysteines (forming thiol esters) [206].

Three peroxins have been shown to be ubiquitinated at the peroxisomal membrane: the PTS-receptor PEX5 [223-226] and the PTS2-co-receptors PEX18, PEX21 [227] and PEX20 [213].

Two types of ubiquitination can occur in PEX5: monoubiquitination on a conserved cysteine residue (Cys11) and mono- or poly-ubiquitination on two adjacent lysine residues (Lys18 and Lys24) in the yeast protein [206, 224-226]. The use of a cysteine as the ubiquitination target in PEX5 is not only unusual (at the actual state of knowledge) but also thermodynamically less favorable and much more labile, when comparing with the ubiquitination of other residues such as lysines. In fact, thiolester conjugates are easily disrupted by nucleophilic attack of molecules such as glutathione (GSH) and the ubiquitination of PEX5 through a thiolester ligation may serve the purpose of creating an ubiquitin-PEX5 intermediate that can be rapidly disrupted, yielding the free receptor [206]. PEX5-monoubiquitination depends on the E2 enzyme PEX4 in yeast, or on the UbcH5a/b/c enzyme in mammals, and appears to be conserved among species [223, 224, 228]. PEX4 is a peroxin found in yeast and plants that is membrane-anchored via PEX22 [118, 229].

PEX5 polyubiquitination has been described in yeast. It is not PEX4 dependent and is only observed in certain *PEX*-deletion strains, namely *PEX4*, *PEX22*, *PEX1*, *PEX6*, and *PEX15* [225, 230]. This polyubiquitination usually occurs at lysine residues and requires the Ub-conjugating enzymes UBC4 [225, 230] or the partly redundant UBC5 [230] and UBC1 [226]. Evidence suggest that this UBC4-dependent ubiquitination has a quality control function, priming dysfunctional and accumulating PEX5 for release from the membrane and subsequent proteasomal disposal [225, 230].

iv) Recycling of the receptor back into the cytosol

In vitro import studies revealed that the insertion of PEX5 is ATP-independent, but its export back to the cytosol requires ATP [202, 231]. Later experiments using *in vitro* systems in *Saccharomyces cerevisiae* [232] and in human fibroblast cells [22] identified the peroxisomal AAA (ATPase associated with various cellular activities) ATPases PEX1 and PEX6 as the motor proteins of PEX5 export.

PEX1 and PEX6

PEX1 and PEX6 are the only two peroxins known to be members of the large AAA protein family. Mutations in the *PEX1* and *PEX6* genes are responsible for PBDs of CG1 and CG4 [70].

AAA proteins serve a wide range of different cellular processes, including vesicular transport, DNA repair, proteolysis, microtubule movement, disassembly of membrane-associated protein complexes and protein folding or unfolding in an ATP-dependent manner [233]. This type of proteins, share one or two AAA cassettes (known as D1 or D2 domains) that are characterized by a conserved sequence of 200–250 amino acids, which includes the Walker A and B motifs for ATP binding and ATP hydrolysis respectively [234, 235]. Both PEX1 and PEX6 contain two AAA domains in their C-terminal part and form a complex displaying a dual localization: cytosolic and at the peroxisomal membrane [22, 232]. Membrane association is achieved by binding to the integral peroxisomal membrane protein PEX15 in yeast, or its ortholog PEX26 in humans. PEX26 recruits PEX1–PEX6 complexes to peroxisomes, via PEX6 [2, 236], and the three proteins were shown to be critical for PEX5 export. PEX26 dysfunctions are responsible for PBDs of CG8 [2, 96].

The exact mechanism of substrate recognition and extraction from the peroxisomal membrane by the AAA peroxins is still unsettled. Although PEX5 and the AAA ATPases may form a complex at the peroxisomal membrane, no direct interaction of the receptor with either PEX1 or PEX6 has yet been reported [232, 237, 238]. The comparison of these AAA peroxins with other structurally similar members of the AAA family, lead to the supposition that the ATP-binding and hydrolysis induces conformational changes that generate the driving force to pull the receptor out of the membrane [239]. The X-ray structure of the N-terminal domain of PEX1 revealed remarkable similarities to the corresponding domains of other AAA proteins, such as Cdc48p or Sec18p (NSF), namely the shared presence of a double-psi beta barrel fold [240, 241]. Recent data from Cdc48p and its adaptor protein Ufd1p, identified such a fold as an ubiquitin-binding domain with two binding sites for mono- and polyubiquitin [242]. Whether this domain of PEX1 is also capable of binding ubiquitin remains to be elucidated.

v) PEX5 deubiquitination yielding free receptor

Once released from the peroxisomal membrane, the soluble Ub-PEX5 species (stage 4) undergo deubiquitination, in a process that is thought to combine both an enzymatic processes and a simple nucleophilic attack of the thiol ester bond by GSH, thus regenerating free PEX5 [22, 214, 232].

Peroxisomal Processing Proteases

In mammals and plants, the PTS2 is usually proteolytically removed upon import into the peroxisome [9, 154, 243, 244]. Until now, the implication of this PTS2 processing is still unknown because the catalytic properties of processed and unprocessed enzymes are very similar [245, 246] and the PTS2 is not processed in lower eukaryotes like yeasts [9]. Recently, DEG15 and TYSND1 proteases, both containing a PTS1 signal, and both believed to be Serine proteases, were found to be responsible for peroxisomal protein processing in plants [10] and in mammals [9], respectively.

DEG15 from watermelon exists as a dimer of 144 kD and as a monomer of 72 kD with different substrate specificities: the dimer form serves as the peroxisomal processing protease and the monomer as a general degrading protease [10]. The two forms are interconvertible depending on the Ca^{2+} concentration [10].

The mammalian TYSND1 catalytic protease domain is homologous to that of DEG15 (lacking a 67-amino acid loop domain) and it was demonstrated to be responsible for both the removal of PTS2-containing precursor peptide from pre-thiolase and for the processing of some PTS1 proteins involved in the peroxisomal β -oxidation pathway [9]. The only other known peroxisomal proteases are the insulin-degrading enzyme (IDE) [247], that cleaves peptides with less than 50 amino acid in length and does not work on large proteins [248, 249]; and the ATP-dependent peroxisomal Lon protease, that is likely to function as a chaperone and degrade unfolded proteins [250].

The cycling receptor model

Summing up, it is evident from the data described above that there are still many details of the PEX5-mediated protein import pathway that we do not fully understand. Indeed many of the protein-protein interaction data are quite complex and frequently it is difficult to assess how physiologically relevant some of the reported interactions are. Furthermore, as emphasized in the previous sections there are significant differences between the peroxisomal import machinery (PIM) of lower eukaryotes and mammals, a fact that may complicate data extrapolation between different organisms. Nevertheless, it is clear that many components of the PIM have been conserved throughout evolution and these are essential for the function of the PIM. These components are: the cycling receptors PEX5 and PEX7, the membrane-embedded peroxins of the docking/translocation machinery (DTM) comprising PEX13, PEX14 and the RING finger proteins PEX2, PEX10 and PEX12, and the receptor export module (REM) peroxins PEX1, PEX6 and PEX26 [251, 252]. With the recent discovery of PEX5 ubiquitination, the E1 and E2 enzymes of the ubiquitin-conjugating cascade can also be considered components of the PIM [214].

Collectively, the data obtained using PEX5-centered *in vitro* systems [22-24], protein-protein interaction studies [253] and cell biology experiments [19, 20, 212, 254] support the pathway schematized in Fig. I-8. First, cytosolic PEX5 (stage 0) binds newly synthesized peroxisomal matrix proteins in the cytosol. The PEX5-cargo protein complex (stage 1) then docks at the DTM and ultimately gets inserted into it (stage 2) (an ATP-independent but cargo protein-dependent insertion) [201, 202, 255]. Next, the DTM-embedded PEX5 is mono-ubiquitinated at a conserved cysteine residue (stage 3) [214, 224] and is then dislocated from the peroxisomal membrane in an ATP-dependent process by the REM, a protein complex containing PEX1 and PEX6, and PEX26 [22, 214, 232]. Finally, ubiquitin is removed from the cytosolic Ub-PEX5 conjugate (stage 4) probably by a combination of enzymatic and non-enzymatic mechanisms [24] yielding free PEX5 (stage 0) that can then start a new protein transport cycle.

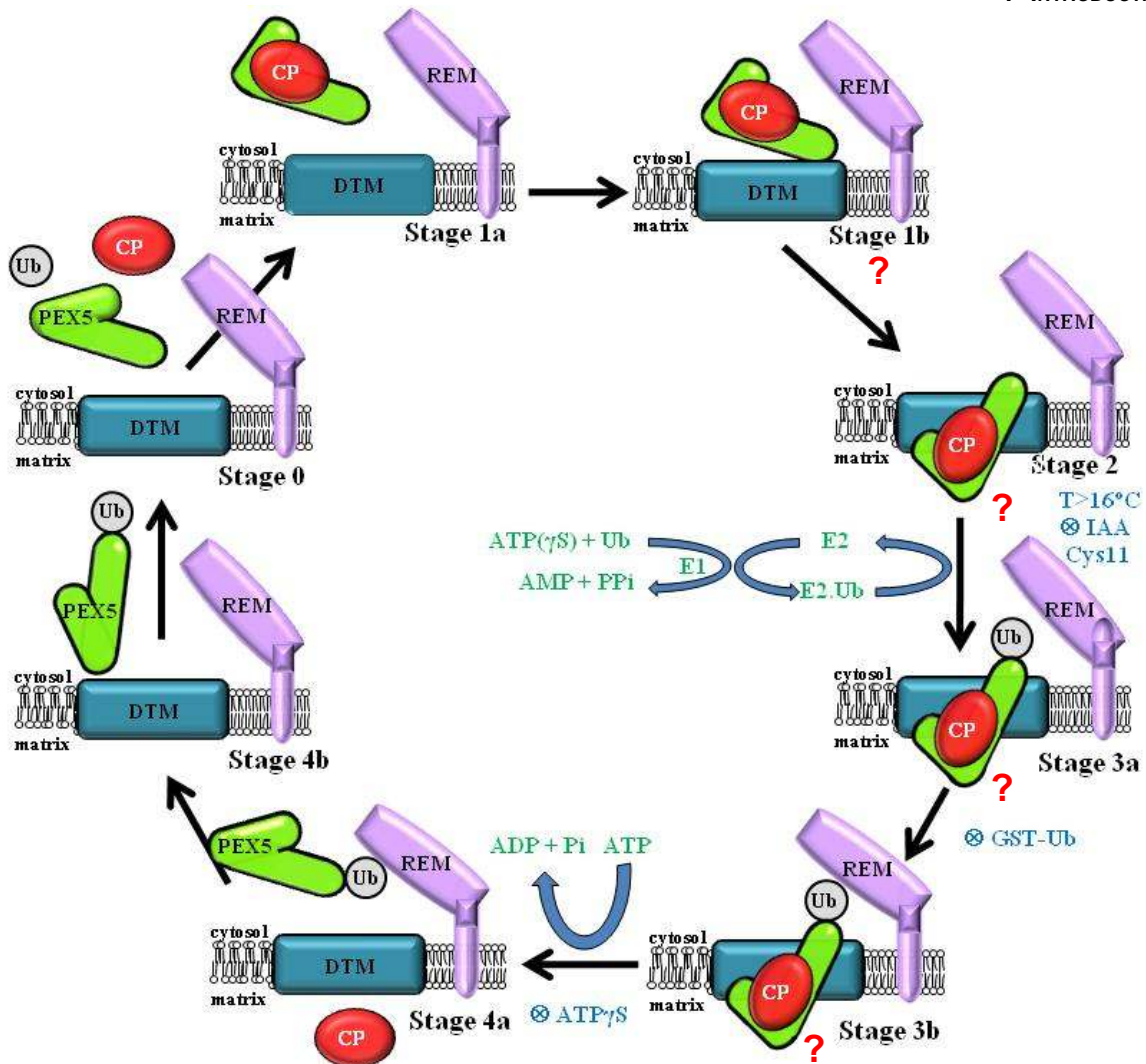


Fig. I-8: The PEX5-mediated peroxisomal protein import pathway. PEX5 protein sorting pathway comprehends five major stages (stage 0–4). Substages (a and b) are mostly of conceptual nature. The different stages have been characterized with a PEX5-centered *in vitro* system, applying several strategies that block ($\otimes$) the pathway at different steps. *Stage 0*, cytosolic cargo-free PEX5. *Stage 1*, cytosolic PEX5-cargo protein complex. *Stage 2*, PEX5 embedded in the peroxisomal docking/translocation machinery (DTM). *Stage 3*, DTM-embedded mono-ubiquitinated PEX5. *Stage 4*, cytosolic mono-ubiquitinated PEX5. Insertion of PEX5 into the DTM is cargo protein-dependent. Mono-ubiquitination of stage 2 PEX5 yielding stage 3 PEX5 requires activated ubiquitin. Activation of ubiquitin by the ubiquitin-activating enzyme (E1) is an ATP-dependent process. Note that this activation, which involves the synthesis of the acyl phosphate AMP-ubiquitin anhydride and the release of pyrophosphate, can also be achieved with ATP γ S (a sulphur atom at a nonbridge position of the γ -phosphate of ATP does not affect the reaction [256]). The ubiquitin analogue GST-Ub is also used efficiently by the ubiquitin-conjugating cascade acting on PEX5. However, this stage 3 species is no longer a substrate for the receptor export module (REM), presumably because of the bulkiness of GST-Ub. Apyrase hydrolyzes ATP and thus blocks PEX5 both at stage 2 and 3b levels. Note that if apyrase is added to the *in vitro* assays before PEX5 (or Δ C1PEX5L), the receptor will not proceed to stage 3a. Stage 4 PEX5 is deubiquitinated yielding stage 0 PEX5 in a process that probably involves deubiquitinating enzymes (DUBs) and glutathione (GSH). The step at which cargo proteins are translocated across the peroxisomal membrane (Red question mark) is the topic of this work. Mammalian PEX7 is not represented in the model because it is presently unknown whether it is retained at the DTM together with PEX5L or translocated across the peroxisomal membrane together with the cargo protein, as proposed for the yeast protein [178].

II. OBJECTIVES

Until now, many of the mechanistic details of the PEX5-mediated protein import pathway in mammals have been obtained using a PEX5-centered *in vitro* system [22-24]. These data, together with information derived from protein-protein interaction studies [253] and cell biology experiments [19, 20, 212, 254], support the model summarized in Fig.I-9. This model, despite aiming at describing the mechanism of protein translocation across the peroxisomal membrane, still lacks experimental information on the cargo-proteins themselves.

The aim of this thesis was to develop a robust *in vitro* import system centered on the cargo protein that should provide a better understanding of cargo translocation across the peroxisomal membrane, namely the steps where the cargo protein is moved from the cytosolic side of the peroxisomal membrane into the DTM, and also the step at which it is released from the DTM into the peroxisomal matrix. Naturally, defining the energetic of the complete translocation process was also a major goal.

III. EXPERIMENTAL PROCEDURES

1- Expression and Purification of Recombinant Proteins

PEX5L, Δ C1PEX5L, PEX5L(N526K) and GST-Ub

The recombinant large isoform of human PEX5 (PEX5L) [164], a protein comprising the first 324 amino acid residues of PEX5L (Δ C1PEX5L) [228], PEX5L containing the missense mutation N526K [PEX5L(N526K); [257]] and the GST-ubiquitin fusion protein [GST-Ub; [214]] were obtained as previously described.

Δ C1PEX5S

For the production of a histidine-tagged recombinant protein comprising the first 287 amino acid residues of the small isoform of human PEX5 (Δ C1PEX5S), the plasmid pGEM4-PEX5S [257] was used as the template in a PCR reaction with the primers Δ C1PEX5S F and Δ C1PEX5S R (see table III-2). The resulting PCR product was digested with NdeI and BamHI and cloned into the NdeI/BamHI sites of pET28c vector (Promega). This protein was then expressed in the *Escherichia coli* strain BL21 and purified using HIS-Select™ nickel affinity gel (Sigma).

2- Synthesis of Radiolabeled Proteins

Plasmids for the synthesis of ^{35} S-Labeled proteins

The plasmid encoding PEX5L(C11K) was described before [24].

pre-Thiolase (p-T) - The cDNA encoding full-length human thiolase precursor (clone IMAGE ID 3860150, from imaGenes) was amplified by PCR using the primers FI_Thio F and FI_Thio R (see table III-2), designed according to the published sequence [258] and cloned into the HindIII and the EcoRI sites of pGEM4 (Promega).

mature Thiolase (m-T) - The cDNA encoding the amino acid sequence of mature thiolase (m-T) preceded by a methionine (Δ PTS2-thiolase) was obtained by PCR using the primers Δ PTS2-thio_F and FI_Thio R and cloned into the HindIII and the EcoRI sites of pGEM4 (Promega).

pre-Thiolase(L4R) (p-T(L4R)) - The cDNA encoding full-length human thiolase precursor (clone IMAGE ID 3860150, from imaGenes) possessing an arginine at position 4 was obtained with QuickChange Site-directed Mutagenesis Kit (Stratagene), using pGEM4-pre-Thiolase as template and the primers FL_Thio_L4R F and FL_Thio_L4R R (see table III-2).

^{35} S-Labeled proteins were synthesized using the TNT® T7 Quick Coupled Transcription/translation kit (Promega) in the presence of [^{35} S] methionine (specific activity >1000 Ci/mmol; PerkinElmer) following the standard conditions of the manufacturer.

The amounts of ^{35}S -labeled proteins obtained with this kit were not determined but according to the manufacturer yields of 3-6 ng/ μl are common.

3- Subcellular Fractionation of Rat Liver

Preparation of Postnuclear Supernatants (PNS)

Livers from male Wistar rats of approximately 2 months old, fasted for about 24h and euthanized after CO₂ anesthesia, were removed into SEM buffer supplemented with 2 $\mu\text{g/ml}$ *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64), and homogenized on ice. The homogenates were centrifuged twice at 600g for 10 min. using the SS34 rotor in a Sorvall® centrifuge from DuPont Instruments. The supernatant was then aliquoted in single use fractions, frozen in liquid N₂ and stored at -70°C. Protein concentration was determined by Bradford protocol with bovine IgGs as protein standards.

Preparation of Cytosol

Livers from male Wistar rats of approximately 2 months old, fasted for about 24h and euthanized after CO₂ anesthesia, were homogenated in 5 volumes of ice cold STE supplemented with E-64. The cytosolic fraction was obtained after sequential centrifugation steps performed at 4°C: 10 min at 600g; 20 min at 12300g and 1h at 100000g. The supernatant was then aliquoted in single use fractions, frozen in liquid N₂ and stored at -70°C.

4- *In vitro* Import Reactions

Standard import reactions were performed using 400 μg of rat liver PNS protein and 1 μl of a rabbit reticulocyte lysate containing ^{35}S -labeled protein. Unless otherwise indicated, the import was allowed to occur for 45 min at 37 °C in 100 μl of import buffer containing 3 mM ATP. Recombinant PEX5 proteins (2 ng/ μl for ΔC1PEX5 versions and 4 ng/ μl for the full-length proteins (approximately 55 nM final concentrations) and GST-Ub or bovine ubiquitin (10 μM) were added to some reactions, as indicated. The ionophores valinomycin (added from a 1 mM stock solution in ethanol), Fluka calcium ionophore II (0.5 mM in ethanol) and carbonylcyanide *m*-chlorophenylhydrazone (1 mM in ethanol) were used at 10 μM , 5 μM and 10 μM , respectively. In the experiments aiming at determining whether or not import of pre-thiolase requires hydrolysis of cytosolic ATP, both the ^{35}S -labeled protein and the PNS were pre-treated as follows: 1) Rabbit reticulocyte lysates were incubated with 40 μg of rat liver cytosolic proteins, in the presence or absence of 200 ng of $\Delta\text{C1PEX5L}$, in 10 μl of import buffer containing 0.3 mM ATP (quantities per μl of lysate) at 37 °C for 10 min. At the end of the incubation the

energetic status of the solution was either reinforced by adding ATP to 3 mM or changed by adding either ATP γ S (3 mM final concentration) or apyrase (20 U/ml final concentration). Incubation proceeded for an additional 10 min; 2) PNS at 8 μ g/ μ l in import buffer was incubated for 5 min at 37 °C in the presence of 0.3 mM ATP. Then, 50 μ l aliquots of this suspension were added to 40 μ l of import buffer containing ATP (6.75 mM), or ATP γ S (6.75 mM) or apyrase (45 U/ml) and either bovine ubiquitin or GST-Ub (22.5 μ M each), as specified in the legends to the figures. After 3 min. at 37 °C, 10 μ l of the energetically matching rabbit reticulocyte lysate solutions were added and the mixtures were incubated for an additional 45 min. Protease-treatment of import reactions was performed on ice for 45 min using 400 μ g/ml of trypsin (final concentration). After inactivation of the protease with 500 μ g/ml PMSF for 2 min on ice, the organelle suspensions were diluted to 500 μ l with SEMK (SEM buffer containing 50 mM KCl) and isolated by centrifugation (11300 g, 20 min at 4 °C). The samples were then subjected to SDS-PAGE, transferred to nitrocellulose, and the radioactive proteins were detected by autoradiography. Sonication of protease treated organelles in SEM buffer containing 1 μ g/ μ l PMSF and 1:500 (v/v) mammalian protease inhibitors cocktail (Sigma) and ultracentrifugation was done as previously described [53].

5- Gradient Density Centrifugation

For the density gradient centrifugation analysis, a 4-fold scale-up of the standard import reaction was used. After trypsin treatment and inactivation of the protease, the complete import mixture was diluted to 1.5 ml with SEM buffer and analysed by Nycodenz step gradient centrifugation exactly as described before [125].

6- Immunoblots

Western blots to nitrocellulose membranes were incubated over night with the relevant antibody (see table III-3) in TBS buffer supplemented with 5% skim milk. Rabbit and mouse antibodies were detected using alkaline phosphatase-conjugated anti-rabbit and anti-mouse antibodies (Sigma).

Table III-1: Buffers

Name	Composition
SEM	0.25 M sucrose, 20 mM MOPS-KOH pH 7.4, 1 mM EDTA-NaOH pH 7.4
STE	0.25 M sucrose, 20 mM Tris-HCl pH 7.5, 1 mM EDTA-NaOH pH 7.5
SIE	0.25 M sucrose, 5 mM Imidazole-HCl pH 7.4, 1 mM EDTA-NaOH pH 7.4
Import Buffer	0.25 M sucrose, 50 mM KCl, 20 mM MOPS-KOH, pH 7.4, 3 mM MgCl ₂ , 20 µM methionine, 2 µg/ml E-64 and 2 mM GSH, pH 7.2
TBS	10mM Tris, 0,9% NaCl pH 7.4

Table III-2: Primers

Primers Name	Oligonucleotide sequence
ΔC1PEX5S F	5'-GCGAACTGCATATGGCAATGCGGGAGCTGG-3'
ΔC1PEX5S R	5'-GCGCGGATCCTCATTAGTACCCCTTATCATAGGTAGCTG-3'
Fl_Thio F	5'-GCGAAGCTTGCCACCATGCAGAGGCTGCAGGTA-3'
Fl_Thio R	5'-GCGCGAATTCTCAGTTCCCAGGGTATTCAA-3'
ΔPTS2-thio F	5'- GAATACGAAGCTTGCCACCATGCTGAGCGGTGCC-3'
FL_Thio_L4R F	5'-ATGCAGAGGCGGCAGGTAGTGCTGGG-3'
FL_Thio_L4R R	5'-CCCAGCACTACCTGCCGCCTCTGCAT-3'

Table III-3: Antibodies

Antibody	Source
Anti-Thiolase	Prof. Marc Fransen, Katholieke Universiteit Leuven, Belgium
Anti-PEX13	Prof. Marc Fransen, Katholieke Universiteit Leuven, Belgium
Anti-Catalase	catalog n°. RDI-CATALASE abr Research Diagnostics, Inc
Anti-KDEL	catalog n°. ab12223 Abcam
Anti-cytochrome C	catalog n°. 556433 BD Pharmingen
Anti-Rabbit	catalog n° A9919, SIGMA
Anti-Mouse	catalog n°. A-2429, SIGMA

IV. RESULTS

In vitro import assays are powerful tools to study the mechanisms of protein import into organelles (e.g. mitochondria, chloroplasts and endoplasmatic reticulum).

In general terms, these assays consist of incubating a ^{35}S -labeled reporter protein with an organelle suspension under physiological conditions. At the end of the incubation a large amount of a highly active protease is added to the reaction to degrade non-imported ^{35}S -labeled protein and finally, after inactivation of the protease, the organelles are pelleted and analyzed by SDS-PAGE and autoradiography. In the peroxisomal biogenesis field, this kind of approach was not very popular until a few years ago. Indeed, besides some pioneering efforts made many years ago [259-261], this strategy was largely abandoned mainly due to the low yield obtained in these assays [261], a strong limitation when distinguishing specific from unspecific events.

When drawing a strategy to develop a peroxisomal cargo-centered *in vitro* import system, some setbacks were foreseen. A major problem regards the fact that the PTS1 signal is not cleaved upon import [1] and the high fragility of peroxisomes [260, 262]. Indeed, peroxisome suspensions, be it a PNS or a purified organelle fraction, always contain soluble PTS1 proteins that leaked from the organelles during tissue homogenization, organelle purification or even simple manipulation [260, 262]. The presence of these proteins in *in vitro* import reactions means that an *in vitro* synthesized ^{35}S -labeled PTS1-containing reporter protein (3-6 ng/ μl ; see *Experimental Procedures*) will have little chances of binding endogenous PEX5 (approx. 30 ng in our assays; [173]) and be, therefore, imported [173]. At a first sight, this problem could be solved by the supplementation of *in vitro* reactions with recombinant PEX5. However, even with this modification, the *in vitro* import yield for a PTS1 reporter is still very weak probably because addition of the recombinant protein to the reaction also increases the concentration of PEX5-PTS1 protein complexes comprising endogenous proteins, creating now a competition problem at the DTM. In order to overcome these problems, two strategies were used. First, we decided to use a PTS2 protein as reporter because mammalian PTS2 proteins are not only much less abundant than PTS1 proteins [250, 263, 264] but also, and more importantly, because the PTS2 peptide is proteolytically removed upon import [1, 9]. This property, besides providing an extra criterion to assess *in vitro* import, implies that a ^{35}S -labeled PTS2-containing reporter protein will not face much competition from endogenous proteins in the interaction with the endogenous PTS2 receptor PEX7. However, as the PEX7-PTS2 cargo protein complexes are transported to mammalian peroxisomes by PEX5L, the competition with endogenous PEX5-PTS1 protein complexes at DTM level would remain. This led us to introduce the second modification to increase the import efficiency: the supplementation of import reactions with recombinant mutant versions of PEX5L that are unable to bind PTS1 proteins while fully

competent in the targeting of PTS2 proteins to the matrix of peroxisomes [11, 19]. These proteins are: Δ C1PEX5, a protein comprising the first 324 amino acid residues of PEX5L, thus lacking the TPR region responsible for the interaction with PTS1 proteins but still containing a fully functional PEX7 binding domain; and PEX5L(N526K), a protein containing the missense mutation N526K that impairs PTS1 binding but fully competent in PTS2 targeting to the peroxisome.

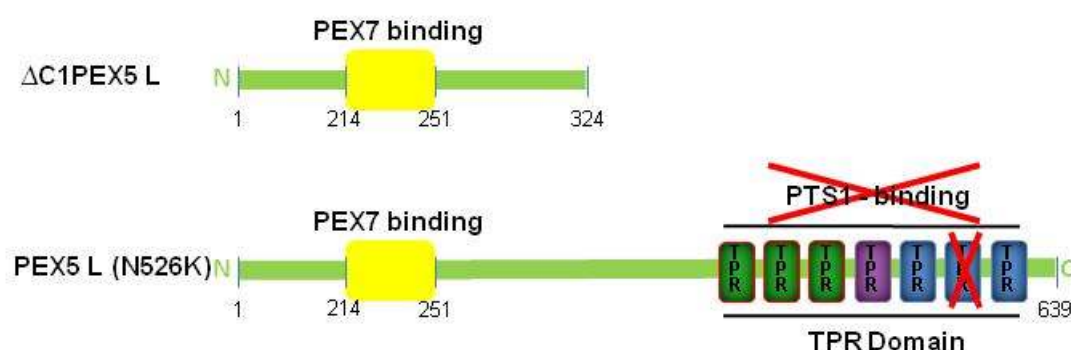


Fig. IV-1: Schematic representation of mammalian PEX5L versions with impaired PTS1 binding. Δ C1PEX5 comprises the first 324 amino acid residues of PEX5L, truncated in the TPR region responsible for the interaction with PTS1 proteins but with a fully functional PEX7 binding domain. PEX5L(N526K) is a protein containing the missense mutation N526K that impairs PTS1 binding but fully competent in PTS2 targeting to the peroxisome. Red boxes represent the seven TPR domains involved in PTS1 binding; the red X shows the mutated TPR and the blue box indicates the PEX7 binding region.

The addition of the above versions of PEX5L recombinant proteins to an *in vitro* import assay with a PTS2 reporter protein would not lead to an increase in the concentration of PEX5 protein complexes containing endogenous PTS1 proteins and would therefore, surpass both competition problems (at the PEX5 binding step and at the DTM). We note that both PEX5L mutant proteins seem to be able to enter the DTM in a cargo protein-independent process [201, 257]. This property could again create a competition problem at the DTM. However, the results presented below indicate that the stimulatory effect obtained with these proteins is much larger than their inhibitory action.

The human 3-ketoacyl-CoA thiolase, a peroxisomal PTS2 protein, was used as reporter protein in our *in vitro* import assays. For simplicity, it will be designated only as thiolase from here forward.

In humans, peroxisomes have only one isoform of thiolase but in rat, two peroxisomal isoenzymes of thiolase have been identified: thiolase A and thiolase B [265-267]. The peroxisomal thiolase A is constitutively expressed whereas the expression of thiolase B is strongly induced by peroxisome proliferators such as hypolipidemic drugs [266, 267]. Both peroxisomal rat thiolases (A and B) are synthesized as larger precursors with amino-

terminal pre-sequences of 36 and 26 amino acids respectively, that are cleaved upon translocation of the protein into peroxisomes [266, 267]. Except for the extra 10 amino acids at the amino-terminus of the thiolase A pre-sequence and 10 amino acid substitutions in the rest of the molecules, both peroxisomal thiolases are almost identical to the human homologue (see Fig. IV-2). Uncleaved thiolase (containing the PTS2 pre-sequence) is designated as pre-thiolase (p-T) and the mature form (after enzymatic cleavage of the pre-sequence) is referred to as mature-thiolase (m-T).

```

Rn_A      MSESVGRITSAMHRLQVVLGHLAGRPSESSALQAAPCSATFPQASASDVVVVHGRRTPIGR 60
Rn_B      -----MHRLQVVLGHLAGRSESSALQAAPCSAGFPQASASDVVVVHGRRTPIGR 50
Hs        -----MQRLQVVLGHLRGPADSGWMPQAAPCLSGAPQASAADVVVVHGRRTAICR 50
          *:***** * :.* ***** : *****:*****:*. *

Rn_A      AGRGGFKDITPDELLSAVLTAVLQDVKLKPECLGDISVGNVLEPGAGAVMARIQFLSGI 120
Rn_B      AGRGGFKDITPDELLSAVLTAVLQDVKPKPECLGDISVGNVLQPGAGAAMARIAQFLSGI 110
Hs        AGRGGFKDITPDELLSAVMTAVLKDVLNLRPEQLGDCVGNVLQPGAGAIMARIAQFLSDI 110
          *****:*****:***: :* ****.*****:***** *****.*

Rn_A      PETVPLSAVNRQCSSGLQAVANIAGGIRNGSYDIGMACGVESMSLSNRGNPGNISSRLLE 180
Rn_B      PETVPLSAVNRQCSSGLQAVANIAGGIRNGSYDIGMACGVESMTLSERGNPGNISSRLLE 170
Hs        PETVPLSTVNRQCSSGLQAVASIAGGIRNGSYDIGMACGVESMSLADRGNPGNITSRLME 170
          *****:*****:*****:*****:*****:*****:*****:***:*

Rn_A      SDKARDCLIPMGITSENVAERFGISRQKQDAFALASQQKAASAQSKGCFRAEIVPVTITV 240
Rn_B      NEKARDCLIPMGITSENVAERFGISRQKQDAFALASQQKAASAQSKGCFRAEIVPVTITV 230
Hs        KEKARDCLIPMGITSENVAERFGISREKQDTFALASQQKAARAQSKGCFQAEIVPVTITV 230
          .:*****:*****:***:***** *****:*****

Rn_A      LDDKGDRKTIITVSQDEGVRPSTTMEGLAKLPAPFKDGGSTTAGNSSQVSDGAAAVLLARR 300
Rn_B      LDDKGDRKTIITVSQDEGVRPSTTMEGLAKLPAPFKDGGSTTAGNSSQVSDGAAAVLLARR 290
Hs        HDDKGTKRSITVTQDEGIRPSTTMEGLAKLPAPFKDGGSTTAGNSSQVSDGAAAILLARR 290
          **** :*:***:***:*****:*****:*****:*****:*****

Rn_A      SKAEELGLPILGVLSRYAVVGVPDIMGIGPAYAIPAALQKAGLTVNDIDIFEINEAFAS 360
Rn_B      SKAEELGLPILGVLSRYAVVGVPDIMGIGPAYAIPAALQKAGLTVNDIDIFEINEAFAS 350
Hs        SKAEELGLPILGVLSRYAVVGVPDIMGIGPAYAIPVALQKAGLTVSDVDIFEINEAFAS 350
          *****:*****:*****:*****:*****:*****:*****

Rn_A      QALYCVEKLGIPAEKVNPLGGAIALGHPLGCTGARQVVTLLNELKRRGTRAYGVVSMCIG 420
Rn_B      QALYCVEKLGIPAEKVNPLGGAIALGHPLGCTGARQVVTLLNELKRRGRRAYGVVSMCIG 410
Hs        QAAVCVEKLRLPPEKVNPLGGAVALGHPLGCTGARQVITLLNELKRRGKRAYGVVSMCIG 410
          ** ***** :*.*****:*****:***** *****

Rn_A      TGMGAAAVFEYPGN 434
Rn_B      TGMGAAAVFEYPGN 424
Hs        TGMGAAAVFEYPGN 424
          *****

```

Fig. IV-2: Sequence alignment of thiolase proteins. 3-ketoacyl-CoA thiolase A and B from *Rattus norvegicus* (Rn), is aligned with 3-ketoacyl-CoA thiolase from *Homo sapiens* (Hs). The shaded "C" marks the position after which the PTS2 signal is cleaved. "*" - residues identical in all sequences in the alignment; "-" - conserved substitutions, "." - semi-conserved substitutions. (ClustalW2).

1- ^{35}S -labeled pre-thiolase acquires a protease-protected and organelle-associated status in *in vitro* import reactions fortified with $\Delta\text{C1PEX5L}$ or PEX5L(N526K) .

Taking into consideration the described limitations, *in vitro* import assays using ^{35}S -labeled pre-thiolase as the PTS2-reporter protein and different versions of recombinant PEX5 proteins were performed.

As shown in Fig.IV-3, when the system is used in its basic configuration (*i.e.*, no recombinant proteins added) the amount of protease-resistant ^{35}S -labeled protein that can be detected in organelle pellets is almost none (lane 2), suggesting that, under these conditions, no significant amount of the reporter protein reaches the matrix of peroxisomes.

Supplementation of the import reaction with recombinant PEX5L consistently results in a small increase in the amount of protease-resistant ^{35}S -labeled protein being pelleted with the organelles (lane 5). About half of the protease-resistant protein migrates on SDS-PAGE exactly as mature thiolase (see bellow) suggesting that ^{35}S -labeled pre-thiolase did reach the correct compartment. However, the yield of the process under these conditions is extremely low (less than 1% of the input). A dramatic improvement was obtained when a previously titrated amount of $\Delta\text{C1PEX5L}$ (data not shown), a recombinant protein comprising amino acid residues 1-324 of PEX5L and unable to bind PTS1 proteins efficiently, was included in the import buffer (lane 3). The stimulatory effect of $\Delta\text{C1PEX5L}$ on pre-thiolase import is clearly dependent on its PEX7-binding domain because negligible amounts of protease-protected ^{35}S -labeled protein were obtained in import reactions supplemented with $\Delta\text{C1PEX5S}$, a truncated PEX5 molecule that lacks the PEX7 – and PTS1- binding sites (lane 4). Essentially the same stimulatory effect was obtained with PEX5L(N526K) (lane 6), a mutated PEX5 version that does not bind PTS1 proteins efficiently [172]; but still contains a functional PEX7 binding site [11].

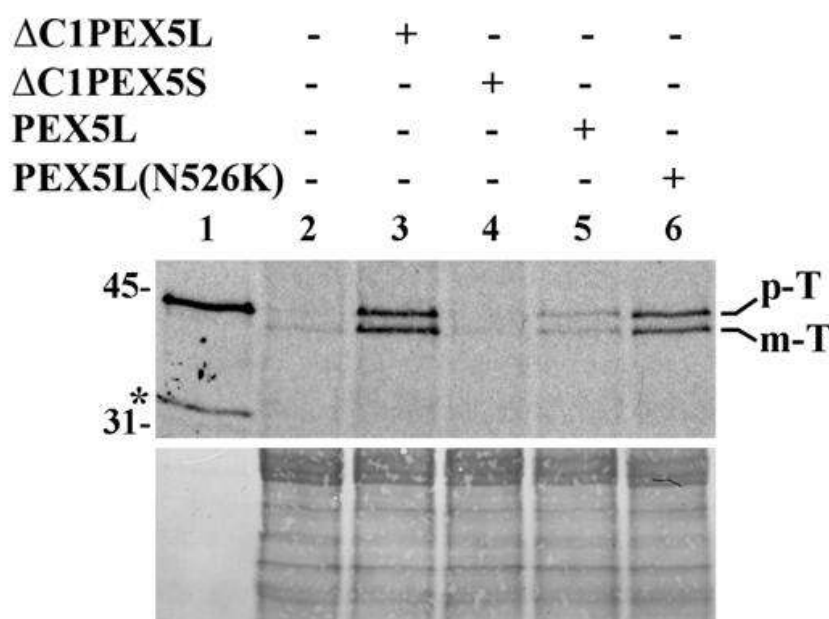


Fig. IV-3: A robust amount of ^{35}S -labeled pre-thiolase acquires a protease-protected and organelle-associated status in *in vitro* import reactions fortified with Δ C1PEX5L or PEX5L(N526K). A rat liver PNS fraction was incubated with ^{35}S -labeled pre-thiolase in import buffer containing ATP in the absence (lane 2) or presence of recombinant Δ C1PEX5L (lane 3), Δ C1PEX5S (lane 4), PEX5L (lane 5) or PEX5L(N526K) (lane 6). After trypsin treatment, the organelles were isolated by centrifugation, subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. The Ponceau S-stained membrane (lower panel) and its autoradiograph (upper panel) are shown. Lane 1, 10% of the reticulocyte lysate containing ^{35}S -labeled pre-thiolase used in each reaction. The asterisk marks a radiolabeled band produced by the *in vitro* translation kit in an unspecific manner. p-T and m-T, precursor and mature forms of thiolase, respectively. The *numbers* at the left indicate the molecular masses of the applied standards in kDa.

2 – Kinetics of Δ C1PEX5L-mediated import of pre-thiolase into peroxisomes.

We next characterized the time-dependence of the Δ C1PEX5L-mediated ^{35}S -labeled pre-thiolase import. A five-fold standard import reaction supplemented with ATP and Δ C1PEX5L was set and aliquots were withdrawn at the indicated time points and processed as described in Fig. IV-3. As shown in Fig. IV-4, the amount of protease-resistant ^{35}S -labeled protein increases steadily up until 30-45 min (lanes 2-6).

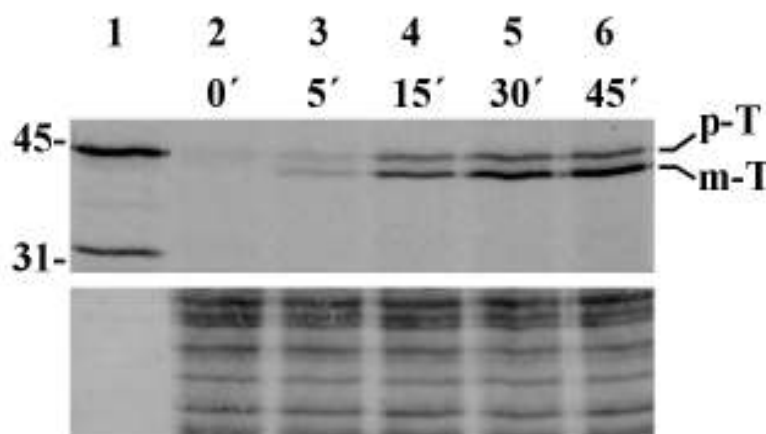


Fig. IV-4: Δ C1PEX5L-mediated import of pre-thiolase is time-dependent. Aliquots from a five-fold standard import reaction were withdrawn at the indicated time points and processed as described in Fig. IV-3. The Ponceau S-stained membrane (lower panel) and its autoradiograph (upper panel) are shown. Lane 1, 10% of the reticulocyte lysate containing ^{35}S -labeled pre-thiolase used in each lane. p-T and m-T, precursor and mature forms of thiolase, respectively. The *numbers* at the left indicate the molecular masses of the applied standards in kDa.

The temperature dependence of the Δ C1PEX5L-mediated ^{35}S -labeled pre-thiolase import, was also characterized by using a PNS pretreated with 0.3 mM ATP for 5min at 37°C (to trigger the release of endogenous PEX5 from the DTM, see *Experimental Procedures*). Reactions were incubated for 45 min. at the indicated temperatures and the samples were processed as described above. As shown in fig. IV-5, the Δ C1PEX5L-mediated import of ^{35}S -labeled pre-thiolase is quite sensitive to the temperature of incubation. For temperatures below 26°C the import efficiency was very low (lanes 2-4), maybe due to a retardation of the receptor-cargo interaction, or a less efficient interaction of the receptor-cargo complex with the DTM, or a decrease in the membrane fluidity (or a combination of these factors). The amount of protease-protected ^{35}S -labeled thiolase is essentially the same at 26°C and 37°C. However, processing of pre-thiolase seems to be more efficient at the higher temperature.

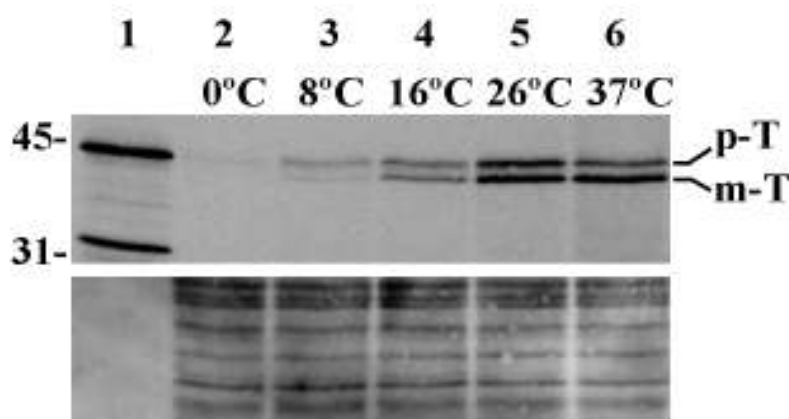


Fig. IV-5: Δ C1PEX5L-mediated import of pre-thiolase is temperature-dependent. A PNS pretreated with 0.3 mM ATP for 5min. at 37°C was used in import reactions containing ATP and recombinant Δ C1PEX5L. After 45 min. at the indicated temperatures the samples were processed as described in Fig.IV-2. The Ponceau S-stained membrane (lower panel) and its autoradiograph (upper panel) are shown. Lane 1, 10% of the reticulocyte lysate containing ^{35} S-labeled pre-thiolase used in each reaction. p-T and m-T, precursor and mature forms of thiolase, respectively. The *numbers* at the left indicate the molecular masses of the applied standards in kDa.

3 – A functional PTS2 signal is required for the import of ^{35} S-labeled pre-thiolase into peroxisomes.

In order to confirm that the PTS2 signal in the reporter protein is required for pre-thiolase import into peroxisomes in our assays, *in vitro* import reactions supplemented or not with recombinant Δ C1PEX5L were performed using either full length ^{35} S-labeled pre-thiolase or ^{35} S-labeled Δ PTS2-thiolase as reporter proteins (see Fig. IV-6).



Fig. IV-6: Schematic representation of pre-thiolase and Δ PTS2-thiolase. Upon entry into the peroxisomal matrix, thiolase is cleaved in its PTS2 domain yielding mature-thiolase. (A) Represents the full length thiolase (pre-thiolase, p-T) containing the PTS2 domain (in violet) and the sequence corresponding to the mature enzyme (in red). (B) Shows the mature version of thiolase, lacking its PTS2 region and containing only the domain corresponding to the mature enzyme (in red).

As shown in Fig. IV-7, in contrast with the behavior of ^{35}S -labeled pre-thiolase (lane 4), a ^{35}S -labeled protein corresponding to mature thiolase does not acquire a protease-resistant status when subjected to *in vitro* import assays (lane 6).

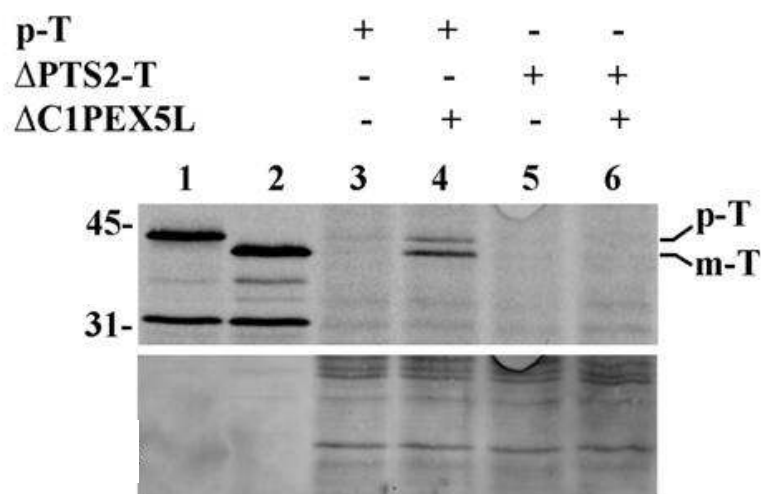


Fig.IV-7: ^{35}S -labeled thiolase lacking its PTS2 does not acquire a protease-resistant/organelle associated status when incubated with $\Delta\text{C1PEX5L}$ -supplemented rat liver PNS. Standard import reactions supplemented with $\Delta\text{C1PEX5L}$ (lanes 4 and 6) or lacking this recombinant protein (lanes 3 and 5) were programmed with ^{35}S -labeled pre-thiolase (lanes 3 and 4) or ^{35}S -labeled thiolase lacking the PTS2 ($\Delta\text{PTS2-T}$; lanes 5 and 6). At the end of the incubation the samples were processed as described in Fig. VI-2. The Ponceau S-stained membrane (lower panel) and its autoradiograph (upper panel) are shown. Lane 1, ^{35}S -labeled pre-thiolase (10% of the input used in lanes 3 and 4); lane 2, ^{35}S -labeled thiolase lacking the PTS2 (10% of the input used in lanes 5 and 6). p-T and m-T, precursor and mature forms of thiolase, respectively.

A similar assay to the above was performed using this time a radiolabeled version of pre-thiolase that contains a missense mutation (L4R) that impairs PEX7 binding – p-thiolase (L4R) [268].



Fig. IV-8: Schematic representation of pre-thiolase with impaired PEX7 binding p-T(L4R). p-T(L4R) is a protein containing the missense mutation L4R that impairs PEX7 binding [268]. The PTS2 domain is shown in violet and the mature sequence of thiolase colored in red. The mutated arginine (4R) is marked in red.

As presented in Fig. IV-9, the ^{35}S -labeled thiolase with the L4R mutation does not acquire a protease-resistant status when subjected to *in vitro* assays in the presence of the recombinant protein (lane 4).

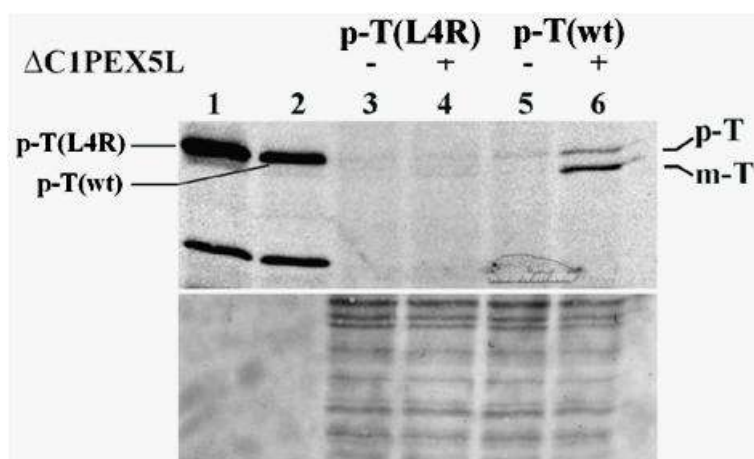


Fig.IV-9: ^{35}S -labeled thiolase with an impaired PEX7 binding domain (p-T(L4R)) does not acquire a protease-resistant/organelle associated status when incubated with $\Delta\text{C1PEX5L}$ -supplemented rat liver PNS. Standard import reactions supplemented with $\Delta\text{C1PEX5L}$ (lanes 4 and 6) or lacking this recombinant protein (lanes 3 and 5) were programmed with ^{35}S -labeled pre-thiolase (lanes 5 and 6) or ^{35}S -labeled thiolase with an impaired PEX7 binding domain (p-T(L4R); lanes 3 and 4). At the end of the incubation the samples were processed as described in Fig. VI-2. The Ponceau S-stained membrane (lower panel) and its autoradiograph (upper panel) are shown. Lane 1, ^{35}S -labeled pre-thiolase (L4R) (10% of the input used in lanes 3 and 4); lane 2, ^{35}S -labeled pre-thiolase (10% of the input used in lanes 5 and 6). p-T and m-T, precursor and mature forms of thiolase, respectively.

Altogether, the data show that a functional PTS2 domain is required for the import of the reporter protein into peroxisomes.

4- ^{35}S -labeled pre-thiolase is specifically imported into peroxisomes.

To clearly determine whether the protease-resistant ^{35}S -labeled reporter protein detected in our assays is inside peroxisomes, a complete protease-treated import reaction (*i.e.*, organelles plus soluble phase) was loaded onto a discontinuous Nycodenz gradient and centrifuged. The gradient was then fractionated and equivalent volumes of each fraction were analyzed by SDS-PAGE and Westernblot/autoradiography. As shown in Fig.IV-10, PEX13, an intrinsic peroxisomal membrane protein, displays a dual behavior in these gradients (see also [125]). One fraction is collected in fractions 2 and 3 of the gradient (the 30-45% (w/v) Nycodenz interface) and represents highly pure peroxisomes; and the other at fractions 9-10 representing peroxisomes/peroxisomal vesicles of lower density [23, 125]. Of significance is that this gradient distribution profile is also displayed by the ^{35}S -labeled pre- and mature-thiolase, leading us to conclude that the ^{35}S -labeled reporter protein is, in fact, imported into peroxisomes.

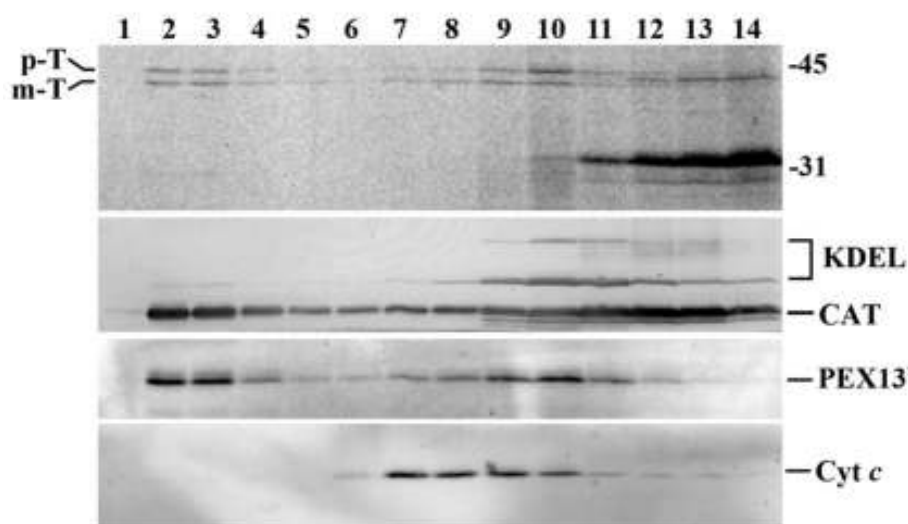


Fig. IV-10: ^{35}S -labeled pre-thiolase is specifically imported into peroxisomes. A $\Delta\text{C1PEX5L}$ -supplemented PNS fraction was incubated with ^{35}S -labeled pre-thiolase in import buffer containing ATP for 45 min. After trypsin treatment and inactivation of the protease, the complete import mixture was diluted with SEM buffer and subjected to Nycodenz gradient centrifugation. The gradient was then fractionated from the bottom (lane 1) to the top (lane 14), and equal aliquots from each fraction were subjected to SDS-PAGE and Western blotting. The nitrocellulose membrane was exposed to an x-ray film to detect the ^{35}S -labeled protein (*top panel*) and afterward probed with the following antisera: anti-KDEL (*KDEL*; recognizes GRP72 and GRP98, two endoplasmic reticulum proteins), anti-cytochrome *c* (*Cyt c*; a mitochondrial marker), anti-catalase (*CAT*; a peroxisomal enzyme) and anti-PEX13. This last serum recognizes on trypsin-treated peroxisomes a 30-kDa fragment of PEX13 (*PEX13'*) [173]. Note that catalase remaining at the top of the gradient (lanes 11-14) results from leakage of peroxisomes during preparation of PNS fractions. p-T and m-T, precursor and mature forms of thiolase, respectively. The *numbers* at the right indicate the molecular masses of the applied standards in kDa.

5 – Membrane potential does not affect the import of pre-thiolase.

Having validated this PTS2-centered *in vitro* import assay, we then assessed whether or not the translocation of pre-thiolase across the organelle membrane requires a membrane potential.

For this purpose, the import efficiencies of pre-thiolase obtained with ATP-pretreated components in reactions supplemented with either valinomycin (a potassium ionophore), CCCP (a proton ionophore) or Ca^{2+} ionophore II, in the presence of bovine ubiquitin and containing 3 mM ATP were compared. As shown in Fig. IV-11, no difference was found when comparing the reaction without ionophores (lane 1) with reactions supplemented with the selected ionophores (lanes 2-4). Thus, potassium, proton or calcium ionophores have no effect on the import of pre-thiolase into peroxisomes

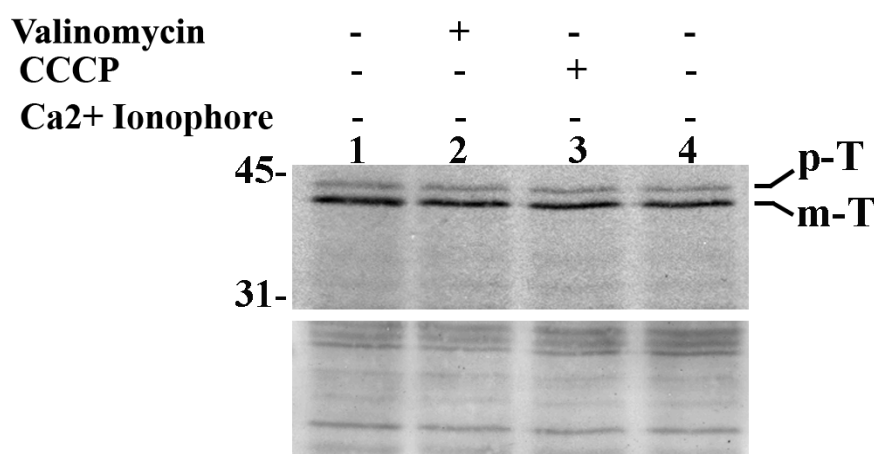


Fig. IV-11. Import of pre-thiolase into peroxisomes is not inhibited by the presence of ionophores.

Import reactions assembled with components pretreated with 0.3 mM ATP (see Experimental Procedures) and supplemented with either 10uM Valinomycin (lane 2), 10uM CCCP (lane 3) or 5uM Ca^{2+} Ionophore II (lane 4) were performed in the presence of 3 mM ATP. After trypsin treatment, the organelles were isolated by centrifugation and analyzed as described in the legend to Fig. IV-3. The autoradiograph (upper panel) and the corresponding Ponceau S-stained membrane (lower panel) are shown.

6 - Pre-thiolase import does not require NTP hydrolysis.

The results presented above exclude the possibility that cargo translocation across the peroxisomal membrane requires the existence of a membrane potential at the organelle membrane. We next determined the ATP requirements of this process. The knowledge of whether or not it requires the hydrolysis of cytosolic ATP would not only allow the characterization of the energetics of this process as it would also enable us to link the cargo translocation step to a specific stage of the PEX5 cycling pathway (see Fig. I-8).

When designing an experiment to address this issue, some intrinsic properties of our *in vitro* system that would compromise any comparison of import efficiencies in the presence/ absence of ATP (or in the presence of poorly hydrolysable ATP analogues) were taken into account.

The first property regards the fact that most of the DTM in PNS fractions is occupied by endogenous PEX5 that is not released in the absence of ATP, or in the presence of ATP γ S, therefore reducing very much the levels of the ^{35} S-labeled reporter protein imported if these conditions are used from the very beginning of the experiment [202] (data not shown). This problem was circumvented by incubating the PNS fractions in import buffer containing 0,3 mM ATP for 5 min at 37 °C before changing the energetic status of the reactions (by adding apyrase or ATP γ S) or reinforcing it with more ATP, and before adding the ^{35} S-labeled reporter protein.

The second property that has to be considered resides in the fact that during the incubation period, both the soluble endogenous PEX5 and the recombinant Δ C1PEX5L species will associate with the DTM. In the absence of ATP, or in the presence of ATP γ S, DTM-embedded PEX5 molecules will no longer be dislocated back into the cytosol and, thus, the import capacity of peroxisomes will decrease over time. To control this variable, a large amount of the ubiquitin analogue GST-Ub was added to some import reactions. GST-Ub, is a good substrate for the ubiquitin conjugating cascade acting on peroxisomal PEX5 or Δ C1PEX5L. However, GST-Ub-PEX5 (or Δ C1PEX5L) conjugates are no longer substrates for the REM [228]., thus the rates at which the amount of free DTM decreases in GST-Ub-containing import reactions will be the same regardless of their energetic status. Finally, because a fraction of the *in vitro* synthesized pre-thiolase may exist in the reticulocyte lysate in a complex with some ATP-dependent chaperone, we pre-incubated the ^{35} S-labeled reporter protein with a rat liver cytosolic fraction containing Δ C1PEX5L recombinant protein in the presence of 0.3 mM ATP for 10 min at 37 °C. After this step, and before starting the import assay, the energetic status of this solution was changed or reinforced as described above.

Two different strategies were used to determine whether or not translocation of pre-thiolase across the peroxisomal membrane requires hydrolysis of cytosolic ATP: 1) inclusion of high amounts of ATP γ S (a non-hydrolysable ATP analog) and 2) depletion of NTPs from the import assays using apyrase.

In the first approach, the import efficiencies of pre-thiolase obtained with ATP-pretreated components in reactions supplemented with bovine ubiquitin or GST-Ub and containing 3 mM ATP or 3 mM ATP γ S were compared. As shown in Fig. IV-12, whereas in the presence of ubiquitin the amount of protease-resistant reporter protein obtained in the presence of ATP is slightly larger than the one obtained in the presence of ATP γ S (lanes 2 and 3, respectively), no significant difference is observed in reactions containing GST-Ub (compare lanes 4 and 5). Thus, under conditions where dislocation of PEX5/ Δ C1PEX5L is blocked, a vast excess of ATP γ S over ATP has no effect on the amount of reporter protein acquiring a protease-resistant status.

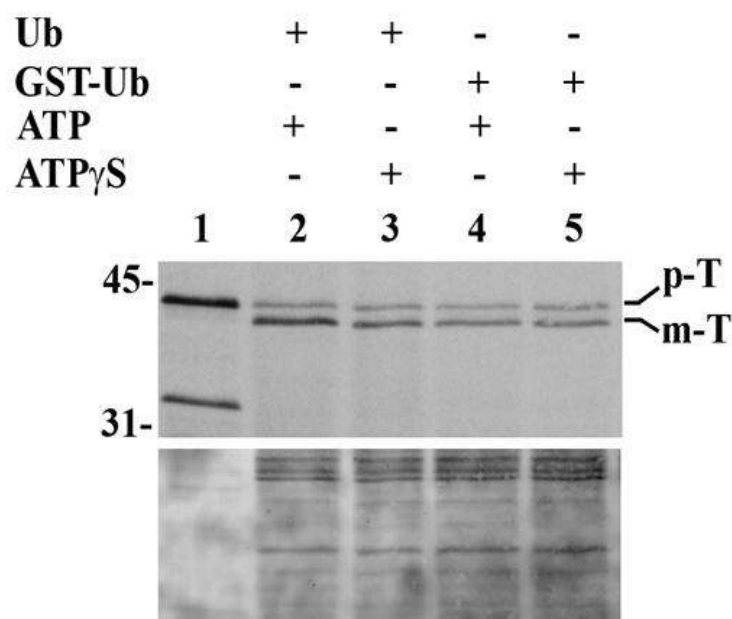


Fig. IV-12: Import of pre-thiolase into peroxisomes is not inhibited by ATP γ S. Import reactions assembled with components pretreated with 0.3 mM ATP (see Experimental Procedures) and supplemented with either bovine ubiquitin (Ub) or GST-Ub were performed in the presence of 3 mM ATP or 3 mM ATP γ S, as indicated. After trypsin treatment, the organelles were isolated by centrifugation and analyzed as described in the legend to Fig. IV-2. The autoradiograph (upper panel) and the corresponding Ponceau S-stained membrane (lower panel) are shown. Lane 1, 10% of ^{35} S-labeled pre-thiolase solution used in each lane.

In the second strategy, the import efficiencies of pre-thiolase obtained with ATP-pretreated components in reactions supplemented with GST-Ub and ATP or apyrase (an enzyme that degrades NTPs [269]) were compared. As shown in Fig. IV-13a, the amount of protease-protected ^{35}S -labeled protein is not significantly affected by the presence of apyrase in the reaction (compare lanes 4 and 5). The control experiment, shown in Fig. IV-13b, confirms that the amount of apyrase used in these assays efficiently removes ATP from these reactions because conjugation of GST-Ub to PEX5L(C11K), an ATP-dependent step [24], no longer occurs.

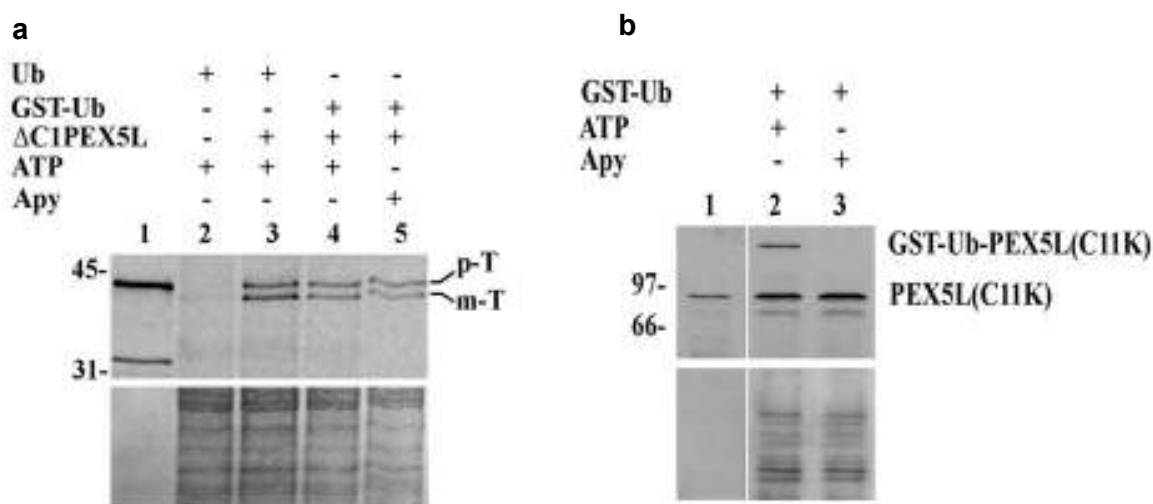


Fig. IV-13: Import of pre-thiolase into peroxisomes does not require cytosolic NTPs. (a) A rat liver PNS was pretreated with 0.3 mM ATP for 5 min. and divided into four equal aliquots (lanes 2-5). The first and second aliquot (lanes 2 and 3; respectively) received bovine ubiquitin and 3 mM ATP; the third aliquot received GST-Ub and 3 mM ATP (lane 4); and the fourth received GST-Ub and apyrase. After 3 min at 37 °C, the first aliquot received ^{35}S -labeled pre-thiolase pre-incubated with ATP in the absence of $\Delta\text{C1PEX5L}$ whereas the second and third aliquots received ^{35}S -labeled pre-thiolase pre-incubated with ATP in the presence of $\Delta\text{C1PEX5L}$ (lanes 3 and 4). The fourth aliquot received ^{35}S -labeled pre-thiolase pre-incubated with ATP plus $\Delta\text{C1PEX5L}$ and treated with apyrase (See materials and methods for details). After 45 min. at 37 °C, the samples were treated with trypsin and processed as described above. Lane 1, 10 % of the ^{35}S -labeled pre-thiolase used in the import reactions. The autoradiograph (upper panel) and the corresponding Ponceau S-stained membrane (lower panel) are shown. p-T and m-T, precursor and mature forms of thiolase, respectively. (b) *In vitro* assays using ^{35}S -labeled PEX5L(C11K). The experimental conditions used in lane 2 and 3 were exactly the ones used in lanes 4 and 5 of the experiment shown in A, respectively, with the exception that recombinant $\Delta\text{C1PEX5L}$ was omitted because it strongly competes with the radiolabeled protein for the DTM. At the end of the 45 min incubation at 37 °C, the organelles were sedimented and processed for SDS-PAGE. Note that PEX5L(C11K) is as functional as normal PEX5L in these assays [24]. It was used here for practical reasons because the GST-Ub-PEX5L(C11K) conjugate, unlike the GST-Ub-PEX5L, is not destroyed by prolonged incubation in the presence of GSH and can be analyzed under normal SDS-PAGE conditions [24]. The complete absence of GST-Ub-PEX5L(C11K) in lane 3 indicates that the amount of apyrase used in these assays efficiently depletes ATP from the reactions. Lane 1, 10 % of ^{35}S -labeled PEX5L(C11K) used in each reaction. Numbers to the left indicate the molecular masses of protein standards in kDa.

Importantly, the fractions of pre-thiolase undergoing processing in these reactions are all very similar suggesting that at least the N-terminus of the reporter protein reaches the matrix of the peroxisome in a process which requires neither dislocation of the ubiquitinated receptor from the DTM (reactions containing ATP γ S plus ubiquitin or ATP plus GST-Ub; see Fig. IV-12) nor even its ubiquitination (reaction containing apyrase, Fig. IV-13a).

7 – The release of *in vitro* imported ^{35}S -labeled thiolase into the peroxisomal matrix occurs before PEX5 ubiquitination.

In order to further characterize the cargo translocation and release steps, *in vitro* import assays of ^{35}S -labeled pre-thiolase in the presence of compounds known to block the PEX5 cycling at different steps were performed.

In these experiments protease-treated organelles from *in vitro* import assays performed in the presence of ubiquitin plus ATP (that allows all steps of the PEX5 cycling pathway to occur; see Fig. 4.2b), ubiquitin plus ATP γ S (that blocks the stage 3b-to-stage 4a transition), GST-Ub plus ATP (that blocks the stage 3a-to-stage 3b transition) or ubiquitin plus apyrase (that blocks the stage 2-to-stage 3a transition) were disrupted by sonication in a low ionic-strength buffer and subjected to ultracentrifugation to separate soluble proteins from membrane-associated ones.

Under these conditions we observed an almost complete extraction of the matrix protein catalase from the organelles (Fig. IV-14, lanes S) whereas mitochondrial cytochrome c, a peripheral membrane protein, remained in the membrane pellets (Fig. IV-14, lanes P). The endogenous rat liver thiolase, accessed by immunoblotting, displayed a dual behavior with comparable amounts of the protein appearing in the membrane and soluble fractions. Of main importance is the fact that the localization of the *in vitro* imported mature thiolase in peroxisomes was the same as the endogenous protein, thus indicating that this fraction of the protease-resistant reporter protein reached its final location (Fig. IV-14). It is interesting to note that a large part of protease-resistant pre-thiolase was found in the membrane pellets (Fig. IV-14, P samples). Whether this fraction represents a population associated with some intraperoxisomal membrane-bound or high molecular mass protein (e.g., the peroxisomal Lon protease [270]), or an intraperoxisomal off-pathway species (e.g., a misfolded pool) is still not known.

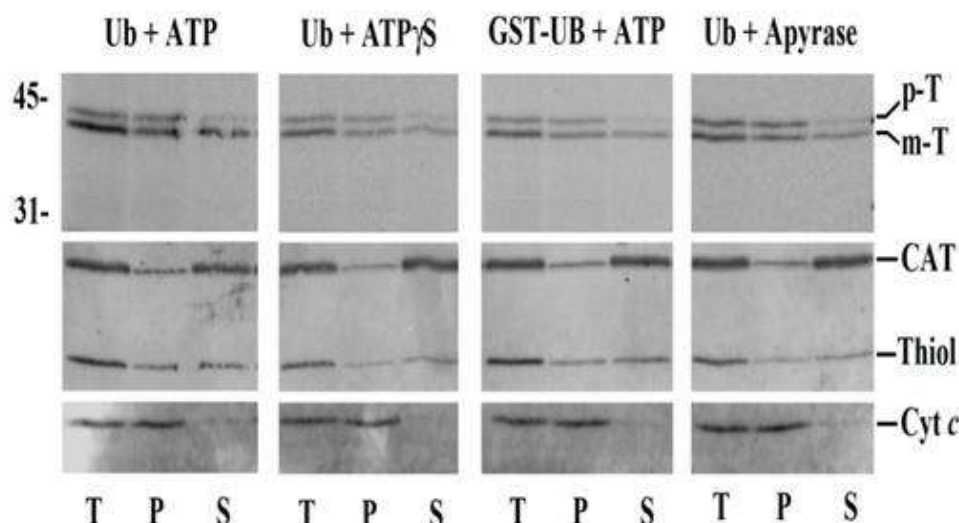


Fig. IV-14: Release of *in vitro* imported ³⁵S-labeled thiolase into the peroxisomal matrix occurs before stage 3. Trypsin-treated organelles from *in vitro* import reactions containing the indicated combinations of bovine ubiquitin (Ub), GST-Ub, ATP, ATP γ S or apyrase and performed as described in Legend to Fig. 4, were disrupted by sonication in a low ionic-strength buffer and divided into two halves. One half (samples T) was kept on ice whereas the other was subjected to ultracentrifugation to separate membranes (samples P) from soluble proteins (samples S). Equivalent portions of samples T, P and S were subjected to SDS-PAGE and blotted onto a nitrocellulose. The membrane was first exposed to an x-ray film to detect the ³⁵S-labeled protein (*top panel*) and afterward probed with the following antisera: anti-thiolase (Thiol); anti-catalase (CAT) and anti-cytochrome c (Cyt c). p-T and m-T, precursor and mature forms of thiolase, respectively. *Numbers* to the left indicate the molecular masses of protein standards in kDa.

V. DISCUSSION

Peroxisomes are fundamental organelles in cell metabolism and there are several severe human disorders caused by errors in the biogenesis of this organelle. Although significant progresses have been made in the elucidation of the molecular mechanisms underlying peroxisome biogenesis, there are still many aspects of this process that remain poorly characterized and one of these aspects concerns the translocation and release of newly synthesized proteins into the peroxisomal matrix.

In mammals, and many other organisms, the import of all cargo proteins into the matrix of the organelle occurs in a process mediated by the peroxisomal cycling receptor PEX5. The mechanism of this PEX5-mediated protein import pathway in mammals has been extensively studied, and many features of this pathway have been characterized using protein-protein interaction analysis [253], cell biology experiments [19, 20, 212, 254]. However most of the mechanistic details regarding this protein sorting pathway have been obtained using a PEX5-centered *in vitro* import system [22-24]. Due to the low abundance of endogenous PEX5 and the presence of import-competent PTS1-cargo proteins in organelle suspensions such approach has been quite successful. Collectively, the data support the mechanism presented in Fig. I-8. It must be noted, nevertheless, that the steps at which the cargo is translocated and released into the matrix were merely speculative and not sustained by experimental data. Obviously, a cargo-centered peroxisomal *in vitro* import system would be useful to address these issues.

Over the last years, several laboratories described cargo-centered *in vitro* import systems [259-261]. However, at least in our hands, the protein import yields obtained with these (“non-fortified”) systems have always been extremely low. Some of the reasons behind this limitation are described in section IV of this thesis and are related to the high fragility of peroxisomes and the fact that the PTS1 signal is not cleaved upon import. Together, these two properties create a competition problem between the ³⁵S-labeled reporter PTS1 protein and endogenous cargoes in the interaction with PEX5.

Taking into consideration these difficulties, we chose to develop a PTS2 cargo-centered *in vitro* import system which allowed us to fortify the *in vitro* reactions with recombinant PEX5 versions unable to bind PTS1-proteins (Δ C1PEX5L and PEX5L(N526K)). In this way, highly reproducible import yields were obtained using ³⁵S-labeled pre-thiolase as a reporter protein (Fig.IV-3 and Fig.IV-9). Similar results were obtained with another PTS2-protein, phytanoyl-CoA hydroxylase (PHYH) (unpublished reports).

Our data clearly suggest that the complete translocation of ³⁵S-labeled pre-thiolase across the peroxisomal membrane, as assessed by its protease-resistant status, its processing into the mature form and its release into the peroxisomal matrix, occurs before stage 3a (see Fig. I-8). Notably, none of these steps requires hydrolysis of cytosolic NTPs (Fig.IV-12 and IV-13).

Although the conclusion that cargos are translocated before stage 3a was somehow expected, the finding that they are also released at this level was surprising. Apparently, cargo release from DTM-embedded PEX5 is not triggered by ubiquitination of the receptor [224] nor by its ATP-dependent dislocation back into the cytosol [206]. So being, the process by which cargo proteins are released from the DTM remains unexplained. One possibility is that the ionic composition of the peroxisomal matrix is somehow different from the cytosolic one (due to a Donnan effect, [271]) thus leading to the disruption of the PEX5-cargo protein interaction. Alternatively, a still-unidentified peroxisomal protein, belonging or not to the DTM, could be the cargo release factor.

Very recently, in contrast to the results presented here, Miyata et al. [231] reported that translocation of proteins across the peroxisomal membrane requires hydrolysis of ATP. Considering that in those *in vitro* import assays both the PNS and the ³⁵S-labeled reporter proteins were supplemented from time 0 with either ATP or a non-hydrolysable ATP analogue, the obtained conclusion was not unexpected. Indeed, as explained in section IV., PNS fractions must be pre-incubated with ATP to release endogenous DTM-embedded PEX5 into the cytosol. Only then can we be sure that peroxisomes under the two different energetic conditions will display the same import capacity.

In conclusion, in this work we developed a robust *in vitro* peroxisomal protein import system centered on a PTS2 cargo-protein. Using this system, we were able to map the cargo translocation step into the PEX5 cycling pathway and demonstrate, for the first time, that translocation across the organelle membrane and release of the cargo into the matrix occur before stage 3 (see Fig. V-1). Importantly, none of these steps require hydrolysis of cytosolic ATP.

This adds another peculiarity to this remarkable protein import machinery.

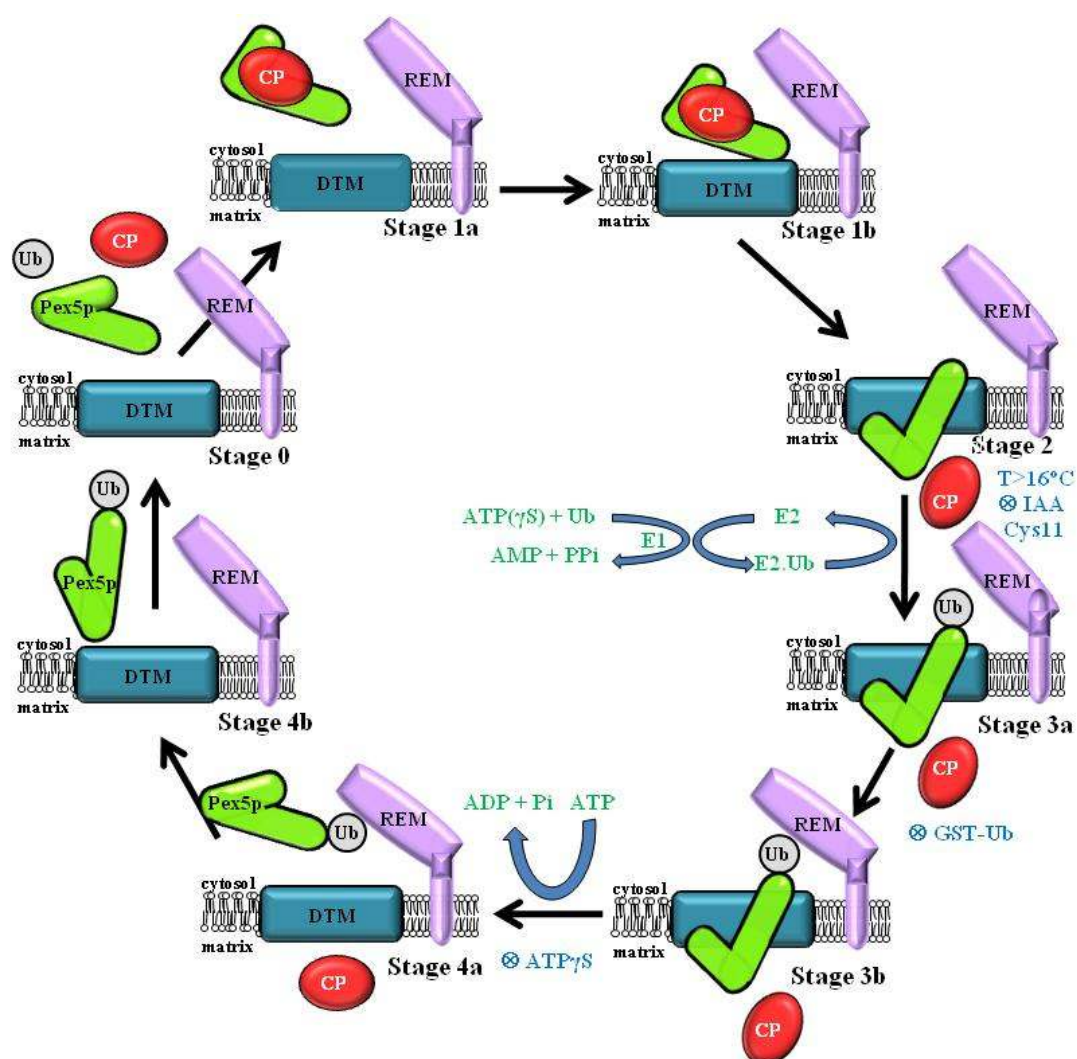


Fig. V-1: The PEX5-mediated protein import pathway. Revised version of Fig I-8 containing the conclusions reported in this work. Membrane translocation of the cargo protein and its release into the organelle matrix occur before stage 3.

VI. FUTURE PERSPECTIVES

Having established a PTS2-centered *in vitro* import system, an obvious step ahead will be to look at PEX7 itself. Actually, preliminary experiments showed that, indeed, supplementation of import reactions with a recombinant PTS2-containing protein and one of the recombinant PEX5L mutant versions used in this work results in a robust peroxisomal association of ³⁵S-labeled PEX7 (unpublished results). Such PEX7-centered *in vitro* system will allow us to address many questions concerning this branch of the peroxisomal protein sorting pathway. Some of the questions that will be addressed are:

- Is PEX7 retained at the DTM together with PEX5 or does it go into the matrix together with its cargo?
- How is PEX7 recycled back into the cytosol? Alone or together with PEX5?
- What are the energetic requirements for PEX7 recycling?
- Is PEX7 insertion into the DTM cargo-dependent?

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