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## Import of peroxisomal matrix proteins in the yeast *Hansenula polymorpha*

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## Summary

Archaea, prokaryotes and eukaryotes form the three kingdoms of life. The smallest unit of life, which can exist independently, is a cell. Archaea and prokaryotes have a relatively very simple architecture. The cytoplasm (cellular space), containing all metabolites, proteins and genetic material (DNA), is surrounded by a membrane (thin double-layer of lipids) and a protective envelope. Eukaryotes have a more complex subcellular organization and have developed complex compartments that are separated from the cellular fluid (cytosol) by additional membranes to form highly specialized areas providing optimal conditions to carry out specific cellular reactions. These structures are called organelles.

One of the compartments, termed nucleus, contains most of the DNA that is surrounded by the nuclear envelope. Other well known classes of organelles are the endoplasmic reticulum (among others involved in synthesis of proteins that are destined for the cell exterior), the vacuole (the machinery for recycling of waste cellular materials) and the mitochondria (involved in energy production). Mitochondria distinguish themselves from the other organelles in that they are surrounded by two membranes. These organelles contain mitochondrial DNA as well as their own protein synthesis machinery. A similar structure is observed for plant chloroplasts that can use light (photons) as energy source to carry out specific reactions (photosynthesis). These double-membrane structures are thought to originate from early aerobic prokaryotes that have entered the eukaryote cell in early evolution (endosymbiont theory).

A group of organelles that has been discovered comparatively recently are the microbodies. Microbodies, including peroxisomes, glycosomes, glyoxysomes and hydrogenosomes, are essential organelles that can carry out a wide range of functions. Their function depends on the organism in which they occur, the cell type and/or the developmental stage of the organism. Our group studies peroxisomes, organelles that are ubiquitously present. Peroxisomes are surrounded by a single membrane and do not contain DNA or a protein synthesis machinery. Therefore all peroxisomal proteins are nuclear encoded and after their synthesis in the cytosol imported into the peroxisome. They are involved, among others, in seed germination and photorespiration in plants, penicillin biosynthesis in certain fungi, the metabolism of various unusual carbon and nitrogen sources in yeast and in the first steps of the oxidation of very long chain fatty acids in man. The importance of peroxisomes in human

cells is probably best illustrated by the existence of several inherited human diseases that are caused by a defect in the biogenesis/function of these organelles. Peroxisome diseases often are associated with strong physical abnormalities and may lead to an early death (e.g. Zellweger syndrome).

Yeast was adopted as attractive model organism to study the molecular mechanisms of peroxisome biogenesis and functions. This simple unicellular representative of eukaryotes resembles in many aspects cells of higher evolved multi-cellular organisms like animals and plants. Therefore the study of many biological processes in yeast contributes to the understanding of these processes in higher eukaryotes, even in man. Furthermore, yeast strains that are disturbed in peroxisome biogenesis or function are still viable and can grow at cultivation conditions that do not depend on peroxisome function.

The present study has focused on peroxisomal matrix protein import in the yeast *Hansenula polymorpha*. This yeast species can be found in nature on decaying plant material and in the soil. In *H. polymorpha* the size and number of peroxisomes can be readily manipulated by applying suitable growth conditions. During growth on glucose medium the cells contain one small peroxisome. However, after shifting the cells to media supplemented with methanol as the sole carbon and energy source peroxisome multiplication occurs. In wild type cells peroxisomes multiply by pre-fission of existing organelles.

Many studies have been performed with the aim to unravel the principles of peroxisomal matrix protein import. The sorting to and recognition of matrix proteins is now relatively well understood; however, the machinery that translocates these proteins across the peroxisomal membrane to reach their final destiny is still a complete enigma. In chapter I an overview of the current knowledge of protein translocation systems of various membrane systems within the cell is presented, including also few hypotheses to explain peroxisome protein import. Peroxisomal matrix protein import distinguishes itself from other protein transport systems in that it can accommodate large multimeric cofactor bound proteins. Cofactors are special molecules required by a protein to obtain biological activity (e.g. flavin or heme). Binding of cofactors most likely occurs in the cytosol before import of the proteins into the peroxisome. However, cofactor binding requires partial folding of the protein and in some cases also the formation of oligomeric structures (assembly of a few monomer protein units into a higher ordered complex). These complex structures can be imported into the peroxisome. The recognition of most peroxisomal matrix proteins and their transport to the organelle is

dependent on one of the two known peroxisomal targeting signals (PTS) present in the mature proteins. The majority of matrix proteins possess the peroxisomal targeting signal PTS1. The consensus PTS1 consists of the three amino acids -serine-lysine-leucine (-SKL), or variants thereof, that is located at the extreme carboxy-terminus (the end of the protein that is synthesized last). The PTS1 is recognized by the carboxyterminal part of the cytosolic receptor protein Pex5p, which transports the PTS1-containing proteins to the peroxisomes.

The PTS1 is remarkable, because protein targeting to other organelles like mitochondria or the endoplasmic reticulum exclusively depends on organellar targeting sequences located at the part of the protein that is synthesized first (amino-terminus). In most cases these sequences are removed after transport across the membrane by a specific enzyme (a protein that catalyzes biochemical reactions), named processing peptidase.

Chapter II describes the re-investigation of the import of alcohol oxidase (AO), an octameric flavo-enzyme (consists of 8 subunits, each containing the cofactor FAD) that contains an authentic PTS1 signal (ARF). The enzyme catalyzes the first step of methanol metabolism in *H. polymorpha*. Import of AO was originally described to depend on its PTS1 and the PTS1 receptor Pex5p, because in a strain which had lost the ability to produce Pex5p, AO is mislocalized to the cytosol and fails to enter peroxisomes. However, removal of the PTS1 did not affect peroxisome import. In fact, even deletions of up to 16 amino acids at the carboxy-terminus did not affect import of AO into peroxisomes. This result clearly demonstrated that import of AO is not dependent on its PTS1. Also, AO import did not depend on the C-terminal half of Pex5p that contains the binding sites for the PTS1 sequence, but required the function of the amino-terminal part of the Pex5p molecule. This result indicates that AO protein must contain a novel PTS that is still unknown.

AO monomers bind their FAD cofactor in the cytosol prior to import. This binding event is essential, as AO protein that lacks the cofactor FAD fails to import into peroxisomes. This observation suggests that cofactor binding is an important prerequisite in the import pathway and takes place prior the Pex5p recognition process.

As a logical continuation of the above study, we aimed to delineate the region within the first part of Pex5p of *H. polymorpha* that is involved in AO binding and hence, in import of AO into peroxisomes (chapter III). In this work, we took advantage of the recent finding that Pex5p from *Penicillium chrysogenum* (a filamentous fungus that can produce penicillin) is able to mediate import of PTS1 proteins in *H. polymorpha* mutants that are unable to synthesize their own Pex5p. However, the *P. chrysogenum* Pex5p was not capable to

transport AO to peroxisomes. Various strains have been constructed that produce different Pex5p hybrid proteins composed of parts of *P. chrysogenum* and *H. polymorpha* Pex5p. We could clearly show that the amino-terminal part of *H. polymorpha* Pex5p can mediate AO import, whereas the corresponding region of *P. chrysogenum* Pex5p failed to do so. However, none of the hybrid proteins containing fusions of both Pex5p species within the first part of Pex5p could fully function in AO import. This led us to conclude that the AO binding site in Pex5p was not a discrete amino acid stretch. In previous work we showed that the AO sorting signal is dependent on a certain degree of (FAD binding dependent) folding of the protein and therefore must be composed of spatially separated amino acids in the partially folded AO monomer. We speculate that the complementary Pex5p binding structure also is composed of amino acids that are distributed over the N-terminal part of Pex5p and is only formed and accessible in properly folded *H. polymorpha* Pex5p.

Furthermore, the localization of other PTS1 matrix proteins (dihydroxyacetone synthase [DHAS] and catalase [CAT]) was analyzed in the strains producing the hybrid Pex5 proteins. In all cases the peroxisomal marker protein GFP.SK1 (green fluorescent protein fused to the PTS1 -SK1) was normally localized to the peroxisomes indicating that the PTS1 sorting, translocation and Pex5p recycling machinery normally functioned. However, for CAT and DHAS other subcellular locations were observed, that varied from completely cytosolic to a dual localization in peroxisomes and the cytosol. This surprising result is consistent with the view that efficient import of these proteins requires additional information but solely the PTS1 tripeptide. It is tempting to suggest that the import efficiency of CAT and DHAS is enhanced by the PTS1 context. As both CAT and DHAS are imported as oligomers, this context may depend on cofactor binding dependent folding of the proteins prior to import to expose all required Pex5p binding elements.

Chapter IV describes the import of the peroxisomal matrix protein catalase. Catalase decomposes the toxic compound hydrogen peroxide, generated from methanol, into water and oxygen. Import of this tetrameric protein depends on the PTS1 pathway. A mutant strain producing catalase that lacks its PTS1 (-SKI) shows mislocalization of CAT to the cytosol. Therefore a second PTS, similar to that observed for AO does not exist in the catalase protein. However, we were able to show that cofactor binding plays a role in increasing the efficiency of binding between catalase and its receptor Pex5p. Analysis of catalase mutants disturbed in binding their cofactor heme revealed that the efficiency of catalase import differs dependent on the amino acid composition of the PTS1. In a mutant strain, disturbed in heme binding to

catalase, a dual location of the protein in the cytosol and peroxisomes was observed. Hence, the endogenous PTS1 (-SKI) was not sufficient for import all heme lacking catalase into peroxisomes. However, this import failure was restored when the -SKI sequence was replaced by the stronger PTS1 sequence, -SKL. These data provided for the first time evidence that the cofactor heme is bound to catalase in the cytosol, because until now heme binding was suggested to take place in peroxisomes, and that this heme binding event contributes to efficient CAT sorting.

The biosynthesis of heme involves a complex pathway, the final step of which takes place in mitochondria, where a ferrous iron ion is incorporated into the cofactor molecule. Mitochondrial frataxin, a protein described in chapter V, is supposed to function in this process. It also has been implicated in the formation of iron-sulfur clusters. In both processes frataxin may serve as an iron donating protein. The gene encoding frataxin was identified via functional complementation of an available *H. polymorpha* mutant affected in growth on methanol. Mutations in the human gene FRDA (encoding frataxin) are the cause of a degenerative disease, named Friedreich's ataxia (FRDA) after Nicholas Friedreich, who reported this ataxia already in 1863. In the mutant strain of *H. polymorpha* analyzed in this study, we did not observe impairments in iron-sulfur cluster biosynthesis. However, a slight decrease in heme biosynthesis was observed. The mutant frataxin protein contained a single amino acid substitution (glycine, amino acid position 132 is substituted by serine) that affects the function of frataxin. Analysis of catalase activity in the mutant strain grown on glucose medium showed a clear decrease in activity, most likely due to the observed decrease in heme biosynthesis, the cofactor of the catalase protein. Furthermore, we observed increased accumulation of reactive oxygen species (ROS), a typical feature of cells defective in frataxin.