Molecular aspects of peroxisome degradation in Hansenula polymorpha

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SUMMARY

From an evolutionary point of view, all organisms are divided into three kingdoms: the kingdom of archaea (unicellular organisms), the kingdom of prokaryotes (bacteria), and the kingdom of eukaryotes. Plants, animals, and fungi (and so yeast as well) belong to the last of the three. Many types of complex biochemical reactions, which together form the basis of life, take place within the cells of all organisms. To avoid disturbances among the interactions, and to improve the efficiency of the reactions, eukaryotic cells are divided into compartments, the so-called organelles. The work described in the present thesis focuses on two of these: the vacuole (or lysosome) and the peroxisome.

It is in the vacuole that the material taken from outside the cell is being degraded. And the material within the cell (including whole organelles) is, when necessary, also degraded there. In this way, the cell obtains building blocks for growth and repair. To perform this function, a mechanism for vacuolar transportation of the enzymes and material to be degraded must exist. Such transportation must be well-regulated, so that the functional components of the vacuole—enzymes that actually perform the degradation work—arrive at their proper destination, for they must be active only within the vacuole, and not in other cellular compartments.

The peroxisome is defined as an organelle in which enzymes that produce and process hydrogen peroxide (H_2O_2) reside. Due to their simultaneous presence within the peroxisome, the produced H_2O_2 is immediately used by another enzyme, and not spread throughout the cytoplasm, where it would cause damage. Like vacuoles, peroxisomes also have transportation routes for the delivery of the appropriate enzymes and substrates.

SUMMARY

Although simple in build–essentially a bag with enzymes–, peroxisomes can function in a large variety of metabolic pathways. In man, they are involved in, for instance, the β-oxidation of the so-called very long chain fatty acids (VLCFAs). The importance of their proper functioning is probably best seen negatively, in the diseases induced by malfunctioning peroxisomes, such as Zellweger syndrome and adrenoleukodystrophy, which lead to physical deformation and mental retardation, usually resulting in early death. In the fungus *Penicillium chrysogenum*, peroxisomes are involved in the synthesis of β-lactam (penicillin-related) antibiotics–a fact that makes the research into peroxisomes interesting for the industry. Another motive for the research lies in the fundamental scientific curiosity as to the basis of regulation and transportation processes within the cell.

Besides for the biogenesis, the specific degradation of peroxisomes is also important for the proper functioning of the cell. In the present thesis, research into the degradation of peroxisomes, known as pexophagy, is described. Superfluous peroxisomes have to be transported to the vacuole for degradation. Keeping these organelles in circumstances under which they are not necessary for growth would cost energy that could otherwise be used for more important tasks in the cell. The degradation process occurs also when peroxisomes are damaged–yet another important function, since peroxisomes with incomplete membranes could spread the produced H_2O_2 throughout the cell, causing damage and leading to its rapid ageing.

To study peroxisome degradation, we used *Hansenula polymorpha*–a yeast species isolated from the flow fields of a cane sugar distillery in Brazil–as model organism. *H. polymorpha* is a ubiquitous yeast species, occurring naturally in spoiled orange juice, in the gut of certain insects, and in the soil. Its large peroxisomes can be induced and degraded on demand, by varying the growth circumstances of the cells, which makes these processes easy to observe. In *H. polymorpha,*

peroxisomes are induced when the cells grow on methanol-containing media, under which conditions the organelles contain the key enzymes for methanol metabolism (alcohol oxidase [AO], for instance). When, next, these cells are transferred to glucose- or ethanol-containing media, the peroxisomes, being no longer necessary for growth, are degraded. The process of pexophagy takes place as follows: first, there has to occur a signal that starts peroxisome degradation; next, a layer of membranes is formed tightly around a single peroxisome; and, finally, the outer membrane of the layer fuses with the vacuolar membrane. In this way, the peroxisome is delivered to the enzymes within the vacuole and degraded.

For all these processes to take place, many specialized proteins are necessary. Which proteins these are and how they function, was one of the questions of the present research. Each protein depends for its synthesis on a gene which is, in eukaryotes, present within the nucleus of the cell. To isolate genes that are involved in pexophagy, a random pool of mutants (organisms with a random gene damaged) was created. Next, cells that were disturbed in pexophagy were taken from the pool, and it was determined which gene in these mutants was damaged. Finally, the function of the protein that was transcribed from this gene was analyzed by finding out (1) which other processes were disturbed and (2) its localization in the cell.

In Chapter One, an overview is given of the current state of affairs within the research into pexophagy, including ideas about possible future steps. We emphasize the specificity of the process compared to other, non-selective processes, and the most likely origin of the sequestering membranes.

Chapter Two describes a technique that can be used for the isolation of those clones from a random pool of mutants which are disturbed in peroxisome degradation. It can also be used for the selection of complemented mutants; these are mutants that have been

given a new, undamaged copy of the damaged gene by the researcher. The technique is based on the detection of the presence of peroxisomal AO in cells, in circumstances under which peroxisomes are usually degraded. When cells that still contain AO are subjected to allyl alcohol, they die, because AO converts allyl alcohol into acrolein, a substance extremely toxic to the cell. WT or complemented cells, on the other hand, survive such treatment, since all AO is, in their case, degraded. A pitfall of this technique, when used for complementation studies, is that often a group of genes (denominated *HYE1*, *-2* and *-3*) that gives a false positive result gets isolated. A probable explanation of this phenomenon is that the Hye proteins detoxify the formed acrolein into less harmful substances. We suggest a way to overcome the problem—namely, by performing an extra assay on the selected colonies. This is an accepted, widely used plate assay, based on a color reaction for AO. Although a laborious procedure, in this case it is not necessary to apply it to the whole group, as the analysis of only a small number of mutants will suffice. In Chapters Three and Four, two genes—isolated by the described techniques—are identified as being involved in pexophagy.

It is becoming increasingly clear that there is a big overlap in the genes involved in pexophagy and other transportation routes to the vacuole, e.g. autophagy and the Cvt (cytoplasm-to-vacuole targeting) route. Autophagy is a process which occurs when cells are experiencing a shortage of nitrogen. They react to the situation by delivering parts of the cytoplasm and organelles to the vacuole for degradation and recycling. In this way, the cell obtains energy and building blocks needed to survive the period of famine. The Cvt route is a process for the transportation of aminopeptidase1 and α-mannosidase1 to the vacuole. These are two of the enzymes involved in the degradation processes that take place within this compartment. We have, so far, not determined whether *H. polymorpha* does possess a Cvt route,

but it has been studied extensively in *Saccharomyces cerevisiae* (baker's yeast).

An example of overlapping vacuolar transportation processes is the gene *PDD7*, described in Chapter Three. This gene codes for Pdd7p protein, which is involved in autophagy as well as in pexophagy. When the gene is damaged, both processes are disturbed. And the *Sc* homologue of this gene, *ATG1*, is involved not only in autophagy and pexophagy but also in the Cvt pathway.

Another example of partial overlap is *ATG11*, the gene described in Chapter Four. *ATG11* is important for peroxisome degradation, but it is not involved in autophagy. Cells lacking nitrogen degrade parts of their cytosol, but surprisingly, peroxisomes are excluded from this process. It appears that a special mechanism involving Atg11p, responsible for specific peroxisome degradation, not only after addition of glucose, but also during autophagy, exists. The *Sc* homologue of Atg11p has been described as being involved in the Cvt route. All these processes (peroxisome degradation, autophagy and the Cvt pathway) appear to partly make use of the same mechanism, though unique constituents for each pathway also exist.

Chapter Five presents the observation that a lowering of temperature in cells induces peroxisome degradation. The degradation is specific for peroxisomes, since microscopic analysis shows the known pattern with individual sequestration and fusion with the vacuole. The reason behind the phenomenon is unclear. The observation has to be taken into account during experiments performed, as is usual, at room temperature, such as microscopy of living cells.

✲

Superficially regarded, *H. polymorpha* is not reminiscent of man. Yet, the interiors of all eukaryotic cells have much in common, and this

principle, known as the 'unity of biochemistry', suggests that understanding the biochemical processes occurring in *H. polymorpha* can teach us much about similar processes occurring in other eukaryotic cells, and, thus, help us understand human diseases, as well as contribute to the research towards the production of antibiotics. In the present thesis, small steps toward such understanding have been made, yet steps that could eventually lead to full elucidation of one of the many processes which together form the basis of life.

Growth factor-induced phenotypic modulation

Isolated smooth muscle cells in culture do not immediately start dividing, even in a medium containing all nutrients required. Before entering the cell cycle, the cells first accommodate their phenotype to their new environment. They lose their contractile properties and modulate to a proliferative and synthetic phenotype, characterised by decreased contractile protein and M_3 receptor expression, but enhanced expression of PKC and synthetic organelles [1;2]. This process of phenotypic modulation has been postulated to occur *in vivo* during periods of airway inflammation, in view of the increased airway smooth muscle (ASM) mass seen in asthmatics which is partly due to an increase in cell number [3]. A central role for phenotypic modulation in smooth muscle growth is also acknowledged in vascular remodeling. In atherosclerotic lesions, neointima formation is accompanied by modulation of smooth muscle cell phenotype to favour conditions of growth and extracellular matrix deposition [4].

ASM phenotypic modulation can be induced by exposing cells to high concentrations of fetal bovine serum (FBS). The main disadvantage of studying phenotypic plasticity in cell culture is, however, that cell to cell contacts and cell to matrix contacts are disrupted. Even at confluence, cell culture cannot mimic the three-dimensional context of intact muscle. The extracellular matrix in which the muscle cells are embedded has been found of major importance: vascular smooth muscle cells can be retained in their contractile phenotype when cultured in laminin-coated flasks or on matrigel, which contains solubilized basement membrane proteins [5;6]. In addition, human ASM cells cultured on laminin exhibit a contractile phenotype, whereas collagen I and fibronectin favour progression to a proliferative and less contractile phenotype [7].

To study the impact of the natural mix of extracellular matrix components on phenotypic modulation, Tao et al. cultured ASM cells on top of a ethanol-fixed layer of dead ASM cells, reasoning that the complex mix of extracellular matrix proteins synthesized by smooth muscle cells would create a more physiological environment [8]. They found that cells cultured under such conditions did not lose contractile responsiveness to methacholine, suggesting that phenotypic modulation is less prominent in this setting. Also, these cells did not spread to the extent that smooth muscle cells do when plated on glass and they did not acquire stress fibres. It is important to note, however, that the conditions used to create this matrix environment (smooth muscle cells only, stimulation with 10 % FBS) preclude a proper comparison to intact muscle.

Therefore, we used an organ culture approach in our studies, since this model has the advantage of having all endogenous extracellular matrix constituents and cell to cell contacts intact. Using this model, we showed that growth factor-induced phenotypic modulation can be induced in the intact muscle (Chapter 2). In summary, this study demonstrated that treatment of bovine tracheal smooth muscle (BTSM) strips with FBS caused a time-dependent ($t_{½}$ = 2.8 days) decrease in maximal contraction compared to serum-deprived control strips. Contractility was not