Import and assembly of peroxisomal proteins in yeast

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Summary

Peroxisomes are single-membrane bound organelles containing a dense matrix of proteins, usually enzymes involved in $H_2O_2$ metabolism and fatty-acid oxidation. Besides these common enzymes, specific enzyme activities depending on cell type and environmental conditions can be demonstrated in peroxisomes. Especially in methylotrophic yeasts like *Hansenula polymorpha*, a diversity of proteins can be found inside the peroxisomes: in addition to catalase, for example alcohol oxidase upon growth on methanol or amine oxidase upon growth on alkylated amines. All these enzymes, which are synthesized in the cytosol, have to be imported selectively into the organelle and assembled into their active enzymatic forms, ranging from octamers in the case of alcohol oxidase to tetramers for catalase and dimers for amine oxidase. This PhD thesis deals with the import and assembly of microbody enzymes, what determines their final intracellular distribution and active oligomeric state inside the organelle.

Chapter 1 gives a general introduction on the import of proteins into peroxisomes, and their morphological relatives, the glyoxysomes, glycosomes and hydrogenosomes, which together with the peroxisomes constitute the microbody family. The chapter defines the different kinds of microbodies, describes their metabolic function and documents past and present ideas on microbody biogenesis. Emphasis of the first chapter is on protein import into microbodies, targeting signals, components of the import machinery and the mechanism of import, including energy requirements.

Our assembly studies on peroxisomal proteins were mainly performed with alcohol oxidase of *Hansenula polymorpha*, a flavoprotein composed of eight identical subunits, each containing a non-covalently bound flavine adenine dinucleotide (FAD) molecule. The role of FAD binding in import, octamerization and activity of the enzyme was investigated via site-directed mutagenesis of the presumed nucleotide-binding domain. Key residues involved in FAD binding, identified via comparative modeling of other flavoproteins with known 3-dimensional structure, were mutated and the fate and characteristics of the mutated proteins, synthesized after gene transfer into homologous and heterologous yeasts, was investigated. Chapter 2 describes our preliminary mutational analysis of alcohol oxidase by expression of an exogenous alcohol oxidase gene behind the phosphoglycerate kinase promoter during growth on glucose, conditions in which the endogenous gene is completely repressed. The alcohol oxidase formed in *Hansenula polymorpha* was imported into peroxisomes and assembled into the active oligomeric form, indicating that all components necessary for import and assembly are also present in peroxisomes of glucose-grown cells. Unfortunately, the fate of alcohol oxidase mutated in the FAD-binding domain could not be established because the mutation reduced the expression of the alcohol oxidase to undetectably low levels. In transformed *Saccharomyces cerevisiae*, the expression level of the mutated alcohol oxidase gene was only slightly decreased compared to the wild-type gene. Import of the mutated protein into peroxisomes, though occurring to a lesser extent than wild-type protein, indicates that import does not require FAD binding. Conclusions on the assembly of alcohol oxidase could not be drawn, because the mutant as well as the wild-type alcohol oxidase failed to octamerize in *S. cerevisiae*.

For our more extensive analysis (Chapter 3) of additional mutants within the FAD-binding domain of *Hansenula polymorpha* alcohol oxidase, we used an expression system of the related methylotrophic yeast *Pichia pastoris*. This system relies on the transformation of an AOX-defective *Pichia pastoris* strain with an integration vector containing the exogenous AOX gene behind the strong *P. pastoris* AOX promoter. In this way, we have expressed AOX genes carrying mutations in conserved (G13, G15, G18 and E39) and non-conserved (E42) amino acids. The three conserved glycines have no direct interaction with the cofactor, but are located in an important loop of the FAD-binding fold. The only amino acid of the FAD-binding fold that interacts directly with the cofactor, via hydrogen bond with the ribose hydroxyl groups of the ADP-moiety of FAD, is a glutamic acid. Although E39 was favored since it is more conserved than E42, both residues were mutated. Mutations in residue 42 had little effect on activity, leaving E39 as the residue interacting with FAD. Altering glutamic acid 42 affected the stability of the enzyme; octamers dissociated easier into monomers. This dissociation occurs in two stages: first the octamers lose activity followed by disassembly into monomers. Mutations in the conserved amino acids...
13, 15, 18 and 39 have dramatic effects. The mutations not only inactivated the protein, but also severely reduced the protein levels, whereas the RNA levels were not grossly affected. We believe that the low protein levels are caused by decreased protein stability. The inactive mutants still formed octamers, suggesting that assembly of alcohol oxidase may occur as follows: first inactive octamers are made which subsequently bind FAD and form the active octameric complex.

Environmental conditions not only determine the protein content of peroxisomes, but also their size and number. An enormous increase in the peroxisomal volume takes place when methylo trophic yeasts are switched from glucose to methanol as the sole source of carbon and energy. We wondered whether these peroxisomes could be used to store heterologously expressed proteins and to protect them from proteolysis. In a first attempt to analyze the import capacity of peroxisomes in methanol-cultured yeasts, we overexpressed alcohol oxidase. In these cells, alcohol oxidase was located both inside the peroxisomes and in the cytosol. The cytosolic protein was composed of monomeric aggregated protein. Because the import of another matrix protein, dihydroxyacetone synthase, was not affected, we assume that the aggregation of alcohol oxidase is the result of its high rate of synthesis. Cytosolic proteins that are probably needed to keep the newly-formed protein in an import-competent conformation, might get saturated with the excessive supply of newly-synthesized alcohol oxidase. Once aggregated, the protein is probably no longer accessible for import.

The last period of my PhD work focussed on topogenesis of peroxisomal proteins. A carboxy-terminal tripeptide, bearing the consensus S/A/C-K/R/H-L tripeptide has been shown to be sufficient for peroxisomal import. Many, especially yeast peroxisomal proteins do not end in such a tripeptide, but contain one or more copies of tripeptide motif at an internal position. Amine oxidase, a peroxisomal protein of *H. polymorpha*, is an example of a peroxisomal protein with an internal SRL located at 9 amino acids from the C terminus. To study whether the import of amine oxidase is mediated via this internal SRL, both the wild-type protein and a mutant in which the SRL signal was deleted, were expressed in *S. cerevisiae* (Chapter 5). Both proteins appeared to be cytosolic, indicating that the import information present in amine oxidase from *H. polymorpha* is not recognized by *S. cerevisiae*. Subsequent studies showed that amine oxidase lacking the internal SRL is still imported into peroxisomes of *H. polymorpha* (Faber et al., in preparation), therefore we had to conclude that the internal SRL in not involved in the peroxisomal targeting. When a SKL or SRL tripeptide was positioned at the C-terminal end of amine oxidase, the proteins accumulated only partially inside the peroxisomes of *S. cerevisiae*. So, it seems that the protein attached to the C-terminal targeting signal modulates the import efficiency. Human catalase is another protein with an internal tripeptide motif. It contains a SHL at 9 amino acids from the C-terminal end. We also expressed this protein in *S. cerevisiae*; it was properly routed into the peroxisomes and also assembled into active tetrameres (Chapter 6). Although we do not yet know whether the internal SHL is involved in import, the data show that the catalase import signals are conserved between eucaryotes: as distant as man and yeast.