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Alcohol oxidase in Hansenula polymorpha

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A limited number of yeast species is capable of utilizing methanol as the sole carbon and energy source for growth. In these yeasts, the first step in methanol metabolism is catalyzed by the enzyme alcohol oxidase (AO). This enzyme oxidizes methanol, using oxygen as electron acceptor, into formaldehyde and hydrogen peroxide. In its active form AO consists of eight identical subunits, each of which contains one flavin adenine dinucleotide (FAD) molecule as the prosthetic group. In vivo this form is confined to the matrix of special cell organelles, called peroxisomes. These organelles are massively induced during growth of cells on methanol. Depending on the growth conditions the matrix is partly or completely crystalline due to crystallization of AO protein. Like all peroxisomal enzymes studied so far, AO is encoded by a nuclear gene and synthesized on free polysomes in the cytosol. After its synthesis, the monomeric protein is translocated into peroxisomes, where assembly into the active octameric enzyme occurs.

The molecular mechanisms involved in assembly, activation and inactivation of AO were largely unknown at the outset of the investigations. The wealth of information on AO of *Hansenula polymorpha*, which also included the nucleotide sequence of the gene, regulation of expression and kinetic properties, made AO of this organism a suitable candidate for studies on the mechanisms of AO assembly, activation and inactivation.

In Chapter 1 the current knowledge on properties of the peroxisomal enzyme AO is reviewed, including its synthesis, import into peroxisomes, assembly and crystallization.

Chapter 2 describes experiments in which the stage of FAD binding during AO assembly was studied. The experiments were based on the finding that in vivo FAD dissociates from AO during incubation of cells with KCN, causing complete inactivation of the octameric enzyme. No reactivation of the apo-octameric enzyme was observed after removal of KCN and incubation of the cells in fresh methanol-containing media. Similar results were obtained when the media were supplemented with excess FAD. These findings suggested

that FAD binding most probably occurs to the monomeric form prior to octamerization. The recovery of AO activity in the cells was fully accounted for by synthesis of new AO protein. Evidence was obtained that a heterogeneity exists among the peroxisomes present in the cell; protein import was confined to small and developing organelles, whereas the large organelles containing inactive AO, were preferably proteolytically degraded.

Properties of AO when expressed in baker's yeast (*Saccharomyces cerevisiae*) are described in chapter 3. Wild type baker's yeast does not contain AO and is not capable of utilizing methanol as a sole carbon and energy source. In baker's yeast, transformed with the AO gene of *H. polymorpha*, the AO protein expressed was partly imported into peroxisomes, whereas most of the protein resided in the cytosol and was present in large protein aggregates. Assembly of AO into active enzyme and binding of FAD to the AO protein was not observed in the transformed baker's yeast, irrespective of its subcellular localization. The same gene was expressed in fusion products of protoplasts prepared from the transformed strain and wild type *H. polymorpha* cells, under conditions in which the nuclear gene of *H. polymorpha* was completely repressed. In these fusion products AO activity was detected, which was cytochemically demonstrated to be confined to peroxisomes of *H. polymorpha*. These observations suggested that FAD binding and assembly are not spontaneous processes but require one or more "assembly factors", present in peroxisomes of *H. polymorpha*, but absent in the organelles of *S. cerevisiae*. Peroxisome-deficient (PER) mutants of *H. polymorpha* were isolated from a collection of strains, which were deficient in the utilization of methanol. Two mutants completely lacked recognizable peroxisomal structures. Genetic analysis revealed that the deficiency to utilize methanol and the absence of peroxisomes in a PER mutant can be due to a mutation in a single gene (chapter 4).

In a PER mutant high expression of AO was obtained during growth of cells in glucose-limited continuous cultures in the presence of choline as the sole nitrogen source (chapter 5). By (immuno-) cytochemistry it was shown that AO was localized in the cytosol

and the nucleus. At high expression rates, large crystalloids were formed; generally one cytosolic crystalloid was observed per cell, occasionally they were also encountered inside the nuclei. The molecular architecture of the crystalloids was similar to those described for AO crystalloids in peroxisomes of wild type cells. Biochemical analysis revealed that the protein assembled into active octameric enzyme as efficiently as in wild type cells, suggesting that putative "assembly factors" are also located and active in the cytosol of the mutant.

Chapter 6 describes studies on methanol-metabolism in a PER mutant. In glucose-limited continuous cultures using methanol as a second substrate in the feed, all peroxisomal enzymes involved in methanol-metabolism were expressed and active at comparable levels in PER and wild type cells. Similarly, the cytosolic enzymes of the dissimilatory pathway, formaldehyde dehydrogenase and formate dehydrogenase, were induced to the same extent in both strains. The addition of low concentrations of methanol to the feed of glucose-limited continuous cultures, resulted in a minor increase in cell yield of the PER cultures. However, when higher methanol concentrations were used, the cell densities gradually decreased and finally dropped below the original value of the culture prior to the addition of methanol. This was in contrast to the results obtained with wild type cultures in which a linear increase in biomass with increasing amounts of methanol was observed. These findings suggested that in PER cells at enhanced methanol supply rates the metabolism of methanol may require energy and/or causes toxic defects. The enzyme activities of two enzymes, indirectly involved in methanol metabolism, were significantly different in PER and wild type cells. Furthermore residual methanol and formaldehyde, excreted by the cells, were detected in the PER cultures. The absence of intact peroxisomes resulted most probably into two major drawbacks, namely i) the generation of hydrogen peroxide in the cytosol and ii) the inability of the cells to control formaldehyde fluxes over the dissimilatory and assimilatory pathways. As a result H_2O_2 and formaldehyde are most probably metabolized in PER cells via pathways which are energetically less advantageous or even energy

consuming, when compared to the normal pathways in wild type cells.

In PER and wild type cells also different mechanisms of AO inactivation were observed (chapter 7). In wild type cells selective inactivation of AO occurs after transfer of cells to glucose- or ethanol-containing media, which involves proteolytic turnover of whole peroxisomes, including all matrix enzymes. When PER cells, containing large cytosolic AO crystalloids, were transferred to glucose-excess conditions, the subsequent decrease in AO activity could fully be accounted for by dilution of the enzyme due to growth; proteolytic degradation of AO was not observed. Similar results were obtained with a second PER mutant, impaired in the import of peroxisomal enzymes. This mutant is characterized by the presence of small peroxisomes together with a large cytosolic AO crystalloid. Upon the shift of cells to glucose excess conditions part of the small organelles present in these cells were degraded by mechanisms similar to those described for wild type cells. Proteolytic degradation of cytosolic AO crystalloids was not observed. These findings suggested that proteolytic degradation of AO is dependent on its localization inside peroxisomes.

Transfer of cells to methanol-excess conditions resulted in modification inactivation as has been described for wild type cells. Modification inactivation was also observed when PER cells were transferred to ethanol-containing media, conditions which in wild type cells lead to proteolytic degradation of peroxisomes, including the matrix proteins. Accordingly, phosphorylation / dephosphorylation as a possible mechanism of AO modification was studied (chapter 8). As a first step the presence of the key enzymes involved in this mechanism was investigated. From both biochemical and cytochemical experiments evidence was obtained that both protein kinase and phosphohydrolase activities are associated with peroxisomes of *H. polymorpha*. However, using either in vivo or in vitro experiments, phosphorylation of AO could not unequivocally be demonstrated.