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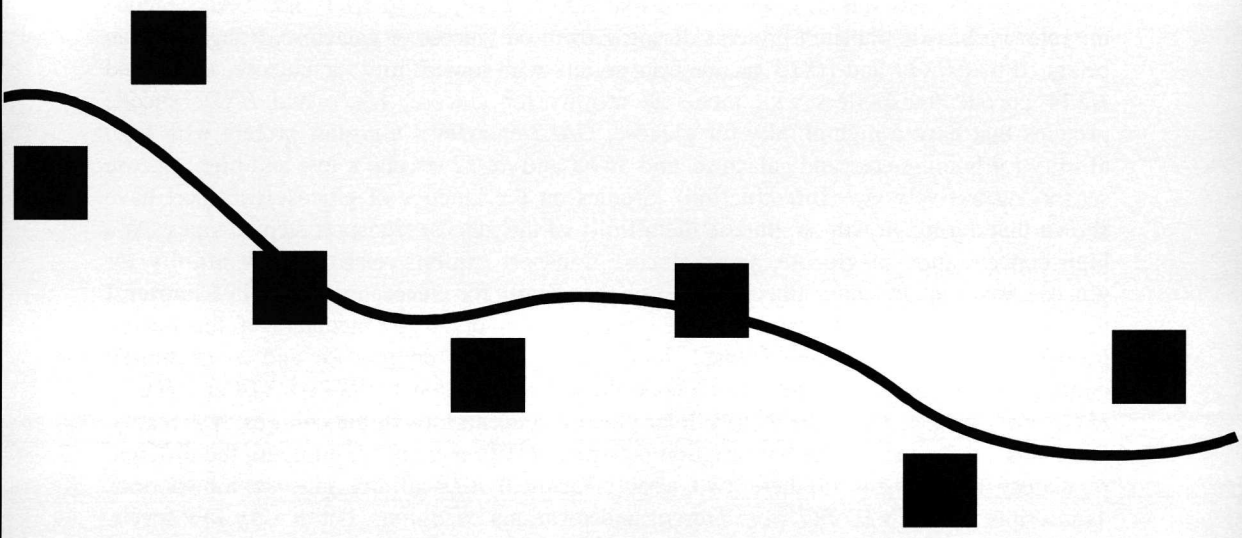
Summary



Physiological functions of hexose transport and hexose phosphorylation in *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* is a unicellular fungus that can grow on a variety of carbon sources. This thesis deals with aspects of the physiological function of the first two steps of the metabolism of hexoses, especially glucose. The first step in hexose metabolism is the transport across the plasma membrane. This process is facilitated by proteins of the hexose transporter family, which are encoded by the *HXT* genes and the *GAL2* gene (**Chapters 2-5**). In the second step of hexose metabolism, the internalized hexose is phosphorylated to hexose-6-phosphate by hexokinase I, hexokinase II, or glucokinase. In addition to its metabolic role in hexose phosphorylation, hexokinase II is involved in glucose repression, and thereby in the regulation of the make-up of the cell machinery (**Chapters 5-8**).

Hexose transport

The entire yeast genome sequence revealed 20 homologues of hexose transporter genes, designated *HXT1-HXT17*, *GAL2*, *SNF3*, and *RGT2*. The abundance of putative hexose transporters strongly raises the question whether the separate transporters have a specific function in the cell.

In previous studies it was shown that *HXT1-HXT4*, *HXT6-HXT7* and *GAL2* encode the relevant hexose transport proteins during growth on glucose or galactose. Roughly it can be stated that *HXT1* and *HXT3* encode transporters with low-affinity for glucose, *HXT2* and *HXT4* encode transporters with moderate affinity for glucose, *HXT6* and *HXT7* encode proteins that have a high-affinity for glucose, *GAL2* encodes a transport protein with high affinity for both glucose and galactose, and *SNF3* and *RGT2* encode a low and high glucose sensor, respectively (see **Introduction**). Studies on the kinetics of glucose transport have shown that during growth on glucose the affinity of the glucose transport step changes. At a high concentration of glucose, yeast glucose transport exhibits relatively low affinity for glucose, while approaching glucose depletion the affinity for glucose increases. In **Chapter 2** the kinetics of glucose transport and the transcription of all 20 members of the hexose transporter gene family were studied during batch growth on glucose and in chemostat cultures under various nutrient-limited conditions. Transcription of *HXT1-HXT4* and *HXT6-HXT7* was correlated with the extracellular glucose concentration in the cultures. Noticeably, there was a difference in the transcription pattern of *HXT6* and *HXT7*, implying the different regulatory mechanisms of these two almost identical high-affinity glucose transporters. Transcripts of *HXT8-HXT17* were present under various conditions, but at very low levels, suggesting a minor role in hexose transport for the proteins encoded by these genes. *GAL2* mRNA was only detected in galactose-limited cultures, showing the requirements of *GAL2* induction by galactose. *SNF3* and *RGT2*, two members of the *HXT* family that encode glucose sensors, were transcribed at low levels that did not clearly correlate with the extracellular glucose concentration. This suggests a glucose sensing mechanism which is not regulated by the external glucose concentration *per se*. Kinetics of glucose transport were roughly in agreement with the previously identified kinetic parameters of the hexose and galactose proteins present as predicted from the *HXT* genes transcribed. In addition, in aerobic glucose-limited cultures at the lower dilution rates the residual glucose concentration did not change significantly with changes in the dilution rate. Only when approaching the critical dilution rate, the residual glucose concentration increased. The glucose transport capacity calculated from zero *trans*-influx experiments and the residual glucose concentration

exceeded the measured *in situ* glucose consumption rate at the lower dilution rates, which suggests the existence of a constraint on the glucose transport activity (for instance a pool of intracellular glucose). At high dilution rates, however, the calculated glucose transport capacity was too low to account for the *in situ* glucose consumption rate. Discrepancies in (the determination of) low-affinity glucose transport seem to be involved.

During the studies described in **Chapter 2** various conditions were identified where the previously uncharacterized hexose transporter homologue *HXT5* was transcribed. Transcript levels of *HXT5* were abundant after glucose exhaustion during batch growth on glucose. Furthermore, *HXT5* mRNA was present in slowly growing cells, i.e. at low dilution rates in glucose-limited cultures and during (slow) growth on carbon sources other than glucose (in ethanol-, fructose- and galactose-limited cultures). Under ethanol-limited culture conditions *HXT5* was almost the only, but certainly the most prominently transcribed *HXT*-gene. Remarkably, glucose was present in the culture supernatant of ethanol-, fructose- and galactose-limited cultures. These results suggest that *HXT5* encodes first a 'reserve' hexose transporter since it was present when no glucose was present, possibly to be able to transport glucose rapidly when it becomes available, or secondly functions as a 'reverse' transporter, since excreted glucose was measured during growth on carbon sources other than glucose where *HXT5* transcript levels were relatively high. The expression pattern of *HXT5* and its possible role in the physiology of yeast were studied in more detail in **Chapter 3**. From Northern blot analysis on *HXT5* transcription and from studies with a strain in which *HXT5* was tagged with the green fluorescent protein (GFP) it became apparent that *HXT5* is expressed and present as a protein during conditions of relatively slow growth, sporulation and during growth on non-fermentable carbon sources.

The kinetic parameters of the protein encoded by *HXT5* were determined in strain RE605 that possesses neither *HXT1-HXT4* nor *HXT6-HXT7*, which encode the major hexose transporters. It was found that *HXT5* encodes a functional hexose transporter, that has moderate affinity for glucose (K_m = 10 mM), moderate/low-affinity for fructose (K_m = 40 mM) and poor affinity for mannose. The RE700 strain, which lacks *HXT1-HXT7* completely, did not show any hexose uptake on a 5s time-scale.

In spite of the clear evidence that Hxt5p is a hexose transporter that is present when glucose is scarce or absent, no obvious phenotype was found of an *HXT5* deletion. However, when stationary phase cells, where Hxt5p is abundant in wild-type cells, were shifted to fresh glucose medium, a longer lag in growth initiation was observed in the *hxt5* deletion strain, than in the wild-type strain. In addition, this shift seemed to initiate pseudohyphal growth in the *hxt5* deletion strain. However, the exact role of the *HXT5* gene product remains unclear.

In the past, glucose transport has often been described as rate-limiting step of glycolysis in yeast. Through Metabolic Control Analysis (MCA) the extent to which the transport of glucose across the plasma membrane controls the glycolytic flux has been determined in cells of *S. bayanus* at one particular condition: the moment of glucose depletion during batch growth on glucose (**Chapter 4**).

Experimental determination of a flux control coefficient requires that the activity of the component of interest be modulated in some manner. To this end, the activity of glucose transport was modified in two ways: i) by inhibition of the glucose transport step by maltose, a competitive inhibitor of glucose transport both in the yeast *S. cerevisiae* and *S. bayanus*, ii)

by modulation of the concentration of the substrate of the glucose transport step, extracellular glucose. The response of the glycolytic flux to such modulations has been determined experimentally. Furthermore the elasticity of the transport step to extracellular glucose has been derived. Subsequently, the flux control coefficient was derived from the ratio of the response and elasticity coefficients. The results demonstrated that in cells of *S. bayanus* grown on glucose and harvested at the point of glucose exhaustion, the transport of glucose across the plasma membrane has a high control on the glycolytic flux.

Regulation of metabolism by hexokinase II

Glucose repression is a regulatory mechanism that is responsible for the repression of genes encoding enzymes that are involved in oxidative metabolism and in the metabolism of carbon sources other than glucose. As a consequence glucose is the preferred carbon and free energy source of *S. cerevisiae*, and glucose metabolism is fermentative in the presence of oxygen. Besides its importance for hexose metabolism, hexokinase II plays a prominent role in the mechanism of glucose repression.

As was shown in the past and in **Chapter 2** high-affinity glucose transport is repressed at high concentrations of glucose by the regulatory mechanism of glucose repression involving hexokinase II. In **Chapter 5** the effect of the expression of different hexokinases on the kinetics of glucose transport and the transcript levels of the major hexose transporters *HXT1-HXT7,* was determined. It was confirmed that a null mutation of the *HXK2* gene prevents repression of high-affinity glucose transport at abundant glucose. Moreover, it was found that the high-affinity component in the *hxk2* null strain is associated with an elevated transcription of the high-affinity transporter genes *HXT2* and *HXT7*, and a diminished transcription of the low-affinity transporter genes *HXT1* and *HXT3* compared to the wild-type. Additional deletion of *HXT7* in the *hxk2* null strain revealed that the high-affinity component could mostly be ascribed to the expression of *HXT7*; however, a previously unidentified very high-affinity component (K_m = 0.24 mM) was due to other factors, possibly *HXT2*.

Hexokinases from the 'non-conventional' yeasts *Schizosaccharomyces pombe* or *Yarrowia lipolytica* could restore repression of high-affinity glucose transport in a triple hexokinase deletion strain (*hxk1 hxk2 glk1*). This implies that the function of hexokinase II in glucose repression has been conserved during (this little bit of) evolution.

During the studies described in **Chapter 5** it became evident that the absence of hexokinase II strongly influences the physiology of *S. cerevisiae* (**Chapters 6-8**).

Chapter 6 describes the effects of the deletion of *HXK2* on the distribution of metabolic fluxes, the enzyme activities, and the intracellular metabolite concentrations, during batch growth on glucose. In aerobic batch cultures on glucose the *hxk2* null strain displayed severely changed properties such as increased mitochondrial activities and altered enzyme activities around pyruvate, as compared to the wild-type. This resulted in fully oxidative growth during early exponential growth, or in other words: an initial absence of fermentation (ethanol production), a postponed and shortened diauxic shift, and a higher biomass yield.

The effects of the deletion of hexokinase II on *S. cerevisiae* in continuous cultures are described in **Chapter 7**. In glucose-limited aerobic chemostat cultures of a wild-type *S. cerevisiae* CEN.PK113-7D and a derived *HXK2*-null strain, metabolic fluxes were identical

at lower dilution rates. Interestingly, the critical dilution rate (ethanol production) and maximal growth rate in the *hxk2* null strain were higher than for the wild-type. After a glucose pulse to the steady-state aerobic glucose limited chemostat cultures, metabolic fluxes were similar in the two strains. In contrast, the concentrations of some intracellular metabolites were clearly different. This implies that hexokinase II is not involved in the control of the glycolytic flux under these conditions, but the different set of kinetic parameters for the glucose-phosphorylating step, results in different metabolite concentrations at identical fluxes.

In **Chapter 8** it is shown that an *hxk2* null mutant strain is able to co-consume carbon sources, i.e. glucose in combination with sucrose, ethanol or galactose. By contrast in wild-type *S. cerevisiae*, enzymes involved in the initial metabolism of carbon sources other than glucose are repressed, and the depletion of glucose precedes consumption of other carbon sources present.