DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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HEXOSE KINASES AND GLUCOSE TRANSPORT IN THE YEAST *HANSENULA POLYMORPHA*

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which will be referred to by their Roman numerals in the text.

I. **Karp, H**., Alamäe, T. 1998. Glucose transport in a methylotrophic yeast *Hansenula polymorpha*. FEMS Microbiol. Lett. 166, 267–273

II. Kramarenko, T., **Karp, H**., J, A., Alamäe, T. 2000 Sugar repression in the methylotrophic yeast *Hansenula polymorpha* studied by using hexokinase-negative, glucokinase-negative and double kinase-negative mutants. Folia Microbiol. 45 (6), 521–529

III. Laht, S., **Karp, H**., Kotka, P., Järviste, A., Alamäe, T. 2002. Cloning and characterization of glucokinase from a methylotrophic yeast *Hansenula polymorpha*: different effects on glucose repression in *H. polymorpha* and *Saccharomyces cerevisiae*. Gene 296, 195–203

IV. **Karp, H**., Järviste, A., Kriegel, T.M, Alamäe, T. 2004. Cloning and biochemical characterization of hexokinase from methylotrophic yeast *Hansenula polymorpha*. Curr. Genet. 44, 268–276

My contribution to the articles referred in the current thesis is as follows:

Ref. I – designed and performed the experiments, analysed the experimental data and participated in writing of the paper.
Ref. II – designed and performed the experiments concerning kinetic parameters of hexokinase and glucokinase proteins, analysed the data and participated in writing the paper.
Ref. III – performed the experiments on substrate specificity of glucokinase, processed the data and participated in writing of the paper.
Ref. IV – conceived, designed and performed the experiments, processed the data and wrote the paper.

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	base pair
CCCP	carbonyl cyanide-*m*-chlorophenylhydrazone
DNP	2,4-dinitrophenol
FMD	formate dehydrogenase
Fru	fructose
Glc	glucose
KDa	kilodaltons
MOX	alcohol (methanol) oxidase
ORF	open reading frame
Poly-P	polyphosphate
Tre-6-P	trehalose-6-phosphate

INTRODUCTION

In nature, yeasts inhabit environment rich in sugars, and glucose is their preferred carbon and energy source. Therefore, it is not surprising that glucose is a key effector molecule of several regulatory responses in yeasts: expression of a large number of genes is repressed by glucose, and expression of others is induced (Ronne, 1995; Rolland *et al*, 2002). The aim of the regulation is to induce utilization of most favoured carbon source (glucose), and to exclude utilization of other carbon sources if sufficient amount of glucose is available. The precise mechanism of glucose signaling in yeasts is not clear yet but due to the participation of glucose transporter and hexokinase in the process, it has similarity to glucose sensing in human pancreatic β-cells (Özcan *et al*, 1996, Mayordomo and Sanz, 2001).

Methylotrophic yeasts, especially *Hansenula polymorpha*, have become popular tools for the expression of foreign proteins mainly under the control of powerful methanol-induced promoters of alcohol oxidase (MOX) and formate dehydrogenase (FMD) genes (Gellissen, 2000). These promoters are sugar-repressed. Thus, knowledge on sugar repression in methylotrophic yeasts is certainly needed to optimize production of proteins of interest. It can be illustrated by the fact that cultivation of *H. polymorpha* recombinant strain on glucose and fructose syrups under strict limitation of sugars, resulted in very high amount (13.5 g/l) of excreted heterologous phytase protein if sugar-repressed FMD promoter was used in the expression cassette (Mayer *et al*, 1999).

Besides synthesis of several enzymes (methanol-specific enzymes, maltase etc) formation of intracellular organelles, peroxisomes, is also down-regulated by glucose (Leão and Kiel, 2003). So, methylotrophic yeasts should be considered as suitable objects to study multiple glucose-induced effects in lower eukaryotes.

In *Saccharomyces cerevisiae*, hexokinase PII that binds glucose and phosphorylates it, participates in the initial phase of glucose sensing. Despite the long history (more than 25 years) of glucose repression study in baker's yeast, experimental evidence about interaction of hexokinase and other key regulators (Mig1p) in glucose signaling was reported only recently (Ahuatzi *et al*, 2004).

Inspired by the work performed on hexokinases and glucose repression in baker's yeast, we initiated study on glucose transport and hexose kinases in *H. polymorpha*. This thesis includes the first publication on sugar transport in this yeast. It also analyses isozymic pattern and regulation of hexokinase and glucokinase expression in *H. polymorpha.* The most recent paper included in the theses is about cloning of the hexokinase gene, study of the hexokinase protein and the role of hexokinase in sugar repression.

1. LITERATURE REVIEW

1.1. Hexose kinases of bacteria, vertebrates and yeasts

1.1.1. General information

Hexose kinases are enzymes phosphorylating hexose sugars such as glucose, fructose and mannose. Hexose kinases can be divided into hexokinases and glucokinases to emphasize the differences in their substrate specificity. Hexokinases phosphorylate glucose, fructose and mannose (therefore both, aldo- and ketohexoses) while glucokinases phosphorylate glucose and mannose (aldohexoses).

Hexose kinases of prokaryotes are typically specific. They phosphorylate a single hexose – glucose, fructose or mannose (Cárdenas *et al*, 1998). Corresponding enzymes from eukaryotes (including yeasts) have usually wider spectrum of sugars to be phosphorylated (Cárdenas *et al*, 1998). An ATP-dependent glucokinase from an archaeon *Aeropyrum pernix* is an exception: it can phosphorylate glucose, fructose, mannose, glucosamine and 2-deoxyglucose being thus similar to yeast hexokinases (Hansen *et al*, 2002).

Usually, hexose kinases use ATP as a phosphoryl donor. However, other possibilities exist. For example, glucokinase of a hyperthermophilic archaebacterium *Pyrococcus furiosus* uses ADP (Kengen *et al*, 1995). The first polyphosphate-dependent glucokinase was discovered in *Microlunatus phosphovorus* (Tanaka *et al*, 2003). *Corynebacterium diphtheriae, Mycobacterium tuberculosis* and *Propionibacterium shermanii* have a bifunctional glucokinase, which can utilize both polyphosphate and ATP as a phosphoryl donor (reviewed in Tanaka *et al*, 2003). As suggested therein, ATP-dependent glucokinase has most probably evolved from a polyphosphate-dependent glucokinase via a bifunctional polyphosphate/ATP-glucokinase.

Vertebrates have four isoforms of hexokinase (Cárdenas, 1998; Wilson, 2003). Three of them have a molecular mass of 100 kDa, and one is a 50 kDa enzyme designated as hexokinase IV, hexokinase D or glucokinase. The size of hexokinase IV is therefore similar to yeast hexokinases (Cárdenas *et al*, 1998). 100-kDa hexokinases have appearantly evolved due to tandem gene duplication (reviewed in Kogure at al, 1993 and Cárdenas *et al*, 1998).

Yeasts have typically several glucose-phosphorylating enzymes. Their pattern and regulation differs among species. For example, while most yeast species have one or two hexokinases and a glucokinase (see also data in Table 1), the milk yeast *Kluyveromyces lactis* has a sole hexokinase and no glucokinase (Prior *et al*, 1993). Some hexose kinases are expressed constitutively, for example the hexokinase of *Kluyveromyces lactis* (Prior *et al*, 1993) and the hexokinase and the glucokinase of *Rhodotorula glutinis* (Mazon *et al*, 1975). Expression of some hexose kinases depends on the carbon source. In the most

thorougly studied yeast species *Saccharomyces cerevisiae* (baker's yeast) expression of hexokinase PII is strongly induced by glucose, while the expression of hexokinase PI and glucokinase is promoted by growth on ethanol and galactose (Fernandez *et al*, 1985). In ethanol-grown *S. cerevisiae* expression of the glucokinase gene *GLK1* is about 25-fold higher than that in glucose-grown cells (Herrero *et al*, 1999).

1.1.2. Kinetic properties of yeast hexose kinases

1.1.2.1. Affinity of the enzymes for glucose, fructose and ATP

To characterize kinetic properties of hexose kinases the affinity of the enzymes for different sugar substrates and ATP is usually measured. The K_m values of yeast hexose kinases for glucose, fructose and ATP were retrieved from the literature and are presented in Table 1. Data concerning hexokinase and glucokinase of a filamentous fungus *Aspergillus niger* are also included.

Table 1. Hexose kinases of some yeasts and filamentous fungi and their K_m values for glucose, fructose and ATP

Yeast	*Hexose kinase*	*K_mGlc mM*	*K_mFru mM*	*K_mATP mM*	*Reference*
Saccharomyces cerevisiae	hexokinase PI	0.11–0.12	1.1–1.4	0.2	Entian, 1997
	hexokinase PII	0.23–0.25	1.7–1.9	0.11–0.15	
	glucokinase	0.03	–	0.04–0.05	
Schwanniomyces occidentalis	hexokinase	0.55–0.98	7.2–9.3	nd	McCann *et al*, 1987; Rose, 1995
	glucokinase	nd	nd	nd	Rose, 1995
Schizosaccharomyces pombe	hexokinase 1	8.5	1.5	3.2	Petit *et al*, 1996
	hexokinase 2	0.16	1.5	0.4	
Candida tropicalis	hexokinase I	0.34	2.2	nd	Hirai *et al*, 1977
	hexokinase II	nd	nd	nd	
	glucokinase	0.29	–	nd	
Pachysolen tannophilus	hexokinase A	0.36	2.28	nd	Wedlock and Thornton, 1989
	hexokinase B	nd	nd	nd	Wedlock *et al*, 1989
	glucokinase	nd	nd	nd	
Rhodotorula glutinis	hexokinase	0.1–0.2	2.0–3.3	0.5	Mazon *et al*, 1975; Mahlberg *et al*, 1985
	glucokinase	0.1	–	0.6	
Yarrowia lipolytica	hexokinase	0.38	3.56	nd	Petit and Gancedo, 1999
	glucokinase	0.17	–		
Aspergillus niger	hexokinase	0.35	2.0	0.66	Panneman *et al*, 1998
	glucokinase	0.063	120	0.37	Panneman *et al*, 1996

(nd – not determined; "–" – no activity)

As presented in Table 1, the K_m of hexokinases to fructose is about 10 times higher than to glucose. Thus, the affinity of the hexokinase for glucose is much higher than for fructose. Glucokinases of some species (*S. cerevisiae* and *A. niger*) have higher affinity for glucose than their hexokinases, while similar affinities of hexokinases and glucokinase for glucose have been described in *S. occidentalis, R. glutinis* and *C. tropicalis*.

1.1.2.2. The F/G ratio

The ratio of maximum velocities of fructose and glucose phosphorylation, designated as F/G ratio, is a specific property of a hexokinase protein reported already in 1977 by Lobo and Maitra. For example, the F/G ratio of *S. cerevisiae* hexokinase PI is according to different authors 2.5–3.5 and that of the hexokinase PII is 1.0–1.5 (reviewed in Wedlock *et al*, 1989). Though the F/G ratios of hexokinases presented by different authors slightly differ, the F/G ratio of hexokinase PI is much higher than the one of hexokinase PII.

1.1.2.3. The affinity of hexokinase for its substrates: effect of oligomerization

The affinity of *S. cerevisiae* hexokinase PII towards its substrates, ATP and glucose, depends on its oligomerization state. At low enzyme concentration and under glucose derepression conditions the enzyme exists mostly in monomeric form that has a high affinity for glucose and ATP. A high concentration of the enzyme and abundance of glucose in the medium stimulates dimerization of the enzyme that is accompanied by reduction of the affinity towards ATP and glucose (Golbik *et al*, 2001).

Monomerization of the hexokinase is initiated by phosphorylation of the Ser^{14} residue in the protein resulting in strong reduction of the interaction between the two subunits (Behlke *et al*, 1998). Similarly to *S. cerevisiae* hexokinase, the hexokinase of *K. lactis* also shows monomer-dimer transition. As in *S. cerevisiae*, the hexokinase monomer is a high activity/high affinity form of the enzyme for both substrates, glucose and ATP (Bär *et al*, 2003). Thus, intracellular glucose phosphorylation in these two yeasts can be regulated at the level of dimer formation and dissociation.

1.1.2.4. Other sugars phosphorylated by hexokinases

Glucose is the preferred substrate of hexokinases, but most of yeast hexose kinases can also phosphorylate other hexoses, such as fructose (see Table 1), mannose and 2-deoxyglucose (Mazon *et al*, 1975; Petit *et al*, 1998; Petit and

Gancedo, 1999). Phosphorylation of 2-deoxyglucose by hexokinase generates toxic 2-deoxyglucose-phosphate and has therefore been used for the isolation of hexokinase-negative mutants (Zimmermann and Scheel, 1977). Besides these substrates, human glucokinase (Xu *et al*, 1994) and hexokinases from *Rhodotorula glutinis* (Mazon *et al*, 1975), *Aspergillus niger* (Panneman *et al*, 1998) and *Schistosoma mansoni* (Thielens *et al*, 1994) use glucosamine. Hexokinase from *Schistosoma mansoni* uses also galactose (Thielens *et al*, 1994).

1.1.2.5. Effectors of hexokinases

As a rule, hexose kinases of mammals (except hexokinase D) are inhibited by their reaction product glucose-6-phosphate, but yeast hexose kinases are not (Cárdenas *et al*, 1998). The other reaction product ADP inhibits hexose kinases of both, mammals and yeasts (Panneman *et al*, 1996; 1998; Tsai and Chen, 1998).

Inhibition of *S. cerevisiae* hexokinase PII by free ATP depends on its oligomeric state: the monomeric high-affinity form of the enzyme is inhibited by free ATP, while the dimeric form is not (Golbic *et al*, 2000). However, neither the monomeric nor dimeric form of the *K. lactis* single hexokinase shows inhibition by free ATP (Bär *et al*, 2003).

Most interestingly, yeast hexokinases are strongly inhibited by even very low amounts of trehalose-6 phosphate (Tre-6-P). For excample the K_i values of *S. cerevisiae* hexokinases PI and PII for Tre-6-P are 0.2 and 0.04 mM, respectively (Blázques *et al*, 1993). The respective K_i value of the *Yarrowia lipolytica* hexokinase is 0.0036 mM (Petit and Gancedo, 1999). Inhibition by Tre-6-P may have a physiological meaning for *S. cerevisiae* as mutants defective in trehalose-6-phosphate synthase are unable to grow on glucose most probably due to the imbalance of glycolysis and deletion of the hexokinase gene restores their growth on glucose (Hohmann *et al*, 1993). Thus inhibition of hexokinase by Tre-6-P most probably adjusts the sugar flux through the first phase of glycolysis in yeasts (Blázquez *et al*, 1993). Disruption of the *TPS1* gene encoding trehalose-6-P synthase in *H. polymorpha* (Reinders *et al*, 1999) and *Yarrowia lipolytica* (Gancedo and Flores, 2004) had no effect on glucose growth phenotype. So, the loss of TPS1-mediated control can be detrimental for fermentative fungi, such as *S. cerevisiae* as they need strict management of very high glycolytic flux, but not for *H. polymorpha* and *Y. lipolytica* yeasts in which respiratory metabolism prevails.

1.1.3. Molecular study of hexose kinases

The genes encoding hexose kinases of many yeasts and filamentous fungi have been cloned and sequenced. Yeast hexose kinases are about 500 amino acids long being therefore larger than respective bacterial enzymes. Some data on the length and molecular size of yeast hexokinase proteins are presented in Table 2.

Table 2. Some data on yeast and fungal hexokinases and glucokinases extracted from Swiss-Prot and TrEMBL (http://www.expasy.org/sprot/) databasis

Yeast	*Hexose kinase and the accession number*	*Gene designation*	*Nr of AA in the deduced protein*	*Calculated molecular size, KDa*
Saccharomyces cerevisiae	Hexokinase PI; P04806	*HXK1*	485	53.7
	Hexokinase PII; P04807	*HXK2*	485	53.8
	Glucokinase; P17709	*GLK1*	500	55.4
Schizosaccharomyces pombe	Hexokinase 1; Q09756	*hxk1*	484	53.6
	Hexokinase 2; P50521	*hxk2*	455	50.9
Kluyveromyces lactis	Hexokinase; P33284	*RAG5*	485	53.5
Yarrowia lipolytica	Hexokinase; O74996	*HXK1*	534	59.2
Schwanniomyces occidentalis	hexokinase; P50506	*HXK*	478	53.1
Aspergillus niger	Hexokinase; O93964	*hxk*	490	54.1
	Glucokinase; Q92407	*GLKA*	495	54.5

The protein sequences of hexokinases exhibit high similarity, and differ from glucokinase sequences. This characteristic is evident in evolution trees made by different authors (Panneman *et al*, 1998; Petit *et al*, 1996). Identity values between protein sequences of hexokinases and glucokinases are shown in Table 3.

Hexokinase proteins contain several conserved regions such as the sugar binding motif and the ATP binding site (Rose, 1995; Zeng *et al*, 1996; Marotta *et al*, 2005). The ATP binding domain present in hexokinases is conserved also in many other functionally diverse ATP-binding proteins, for example in actin and hsp70 protein (Bork *et al*, 1992). The consensus sequence for glucose binding contains a motif Pro-Leu-Gly-Phe-Thr-Phe-Ser-Tyr-Pro, the residues 151–160 in *S. cerevisiae* hexokinase (Entian, 1997). The Ser^{157} of the motif is suspected to play a crucial role in the process of phosphoryl transfer, and it is also the site for autophosphorylation/ inactivation of the hexokinase PII (Heidrich *et al*, 1997). Notably, mutation of respective Ser residue (Ser^{177}) in *Arabidopsis thaliana* hexokinase to Ala yielded in complete loss of catalytic activity of the enzyme (Moore *et al*, 2003). *Kluyveromyces lactis* hexokinase also contains the corresponding Ser residue (Ser^{156}), but inactivation profile by xylose was different from that observed in *S. cerevisiae* – the enzyme maintained high residual activity (Bär *et al*, 2003).

Table 3. Sequence identity values (%) of hexokinase and glucokinase proteins from yeasts and filamentous fungi

	KLHK	SOHK	SCHK1	SCHK2	YLHK	SPHK1	SPHK2	HUGK	SCGK	ANGK
KLHK	100%	68%	70%	72%	62%	46%	33%	32%	36%	36%
SOHK		100%	71%	70%	63%	48%	33%	31%	35%	35%
SCHK1			100%	77%	58%	46%	34%	33%	37%	36%
SCHK2				100%	60%	47%	37%	30%	37%	36%
YLHK					100%	50%	34%	32%	34%	34%
SPHK1						100%	33%	34%	35%	34%
SPHK2							100%	27%	38%	41%
HUGK								100%	28%	31%
SCGK									100%	42%
ANGK										100%

The protein sequences were retrieved from Swiss Prot and compared with program Clustal W (Thompson *et al*, 1994).
KLHK – hexokinase of *Kluyveromyces lactis*, SOHK – hexokinase of *Schwanniomyces occidentalis*, SCHK1 and SCHK2 – hexokinases PI and PII of *Saccharomyces cerevisiae*, YLHK – hexokinase of *Yarrowia lipolytica*, SPHK1 ja SPHK2 – hexokinases 1 and 2 of *Schizosaccharomyces pombe*, HUGK – human glucokinase, SCGK – glucokinase of *S. cerevisiae*, ANGK – glucokinase of *Aspergillus niger*

Expectedly, when the regions responsible for sugar phosphorylation (amino acid residues 102–246) were exchanged between hexokinases PI and PII, the F/G ratio and the affinity constants for glucose also changed characteristically (Rose *et al*, 1991). Moukil *et al* (2000) replaced three glucokinase-specific residues in human glucokinase and managed to get mutant glucokinase, which had the K_m for glucose comparable to the K_m of hexokinase PII. Hochmann *et al* (1999) describe a *S. cerevisiae* HXK2 mutational change of Pro^{160}/Ala, converting the hexokinase from a bifunctional glucose/fructose kinase almost to a glucokinase. The mutation Ser^{231}/Asn in sugar binding region of hexokinase 1 of *Schizosaccharomyces pombe* increases its low affinity to glucose (the native enzyme has Ser residue in a position where all other hexose kinases have Asn) (Petit *et al*, 1998).

Ser^{14} is a crucial residue determining the oligomerization status of the hexokinase PII (Behlke *et al*, 1998). The phosphorylated Ser^{14} is a part of a protein kinase A consensus sequence, which is preceded by a nuclear localization motif mediating the observed transfer of the enzyme to the nucleus (reviewed in Bär *et al*, 2003).

The crystal structure of *S. cerevisiae* hexokinase shows that the polypeptide chain is folded into two domains of unequal size: the large and the small domain. The two domains are separated by a deep cleft containing the active site (Kuser *et al*, 2000). Inspection of the structure shows that most of strictly conserved amino acid residues appear in the active site (Kuser *et al*, 2000). Certain hydrophobic residues belonging to the small domain form a channel that

may act as a tunnel for proton generated in the phosphorylation reaction. Crystallographic structures clearly show that in the closed conformation Ser^{157} interacts with hydroxyl group 3 of the glucose molecul via a carboxyl oxygen (Kuser *et al*, 2000). As noted above, the Ser^{157} is also the site for auto-phosphorylation/inactivation of the hexokinase PII and its importance in the phosphoryl transfer has been shown using biochemical analysis (Heidrich *et al*, 1997). The binding of glucose to the hexokinase protein induces substantial conformational changes (induced fit): loops forming the mouth of the channel close up over the active site, bringing the entrance to the channel into the close proximity with the ligand binding sites. The closed active site conformation is probably completed after additional conformational changes that accompany ATP binding. After the reaction has taken place and the hexokinase cleft opens, the small domain probably will drag ADP away from active site, opening the way to release Glc-6-P (Kuser *et al*, 2000). This is consistent with the model by Kleywegt and Jones (1996; cited in Kuser *et al*, 2000) according to which glucose binding precedes the ATP binding, whereas ADP is released first, followed by the release of glucose-6-phosphate.

1.2. Glucose transport and transporter-like glucose sensors in yeasts

Sugar transport across the plasma membrane is the first and obligatory step of its utilization. Yeasts can use different carbon sources for the growth but evolution has selected mechanisms for the preferential utilization of glucose. So, glucose is likely the major signaling nutrient for *S. cerevisiae* (reviewed in Moreno and Herrero, 2002 and Rolland *et al*, 2002). Sensing of glucose in the medium by yeasts is complicated and both, transporter-like proteins and intra-cellular enzymes (hexokinase) are implicated. Glucose sensing has been studied in *S. cerevisiae* as a model, and yeast glucose sensing system has occurred to be similar to that in plants and human cells (see Rolland *et al*, 2002).

Both, facilitated diffusion and proton-symport transport systems for sugars have been desribed in yeasts. Facilitated diffusion is passive and energy-independent, with glucose moving down the concentration gradient. In energy-consuming proton-symport, a sugar molecule and a proton are cotransported into the cell (Boles and Hollenberg, 1997).

1.2.1. Glucose permeases and transport systems in *S. cerevisiae*

Among about 5600 protein-coding genes in *S. cerevisiae*, at least 271 encode for predicted or established permeases (van Belle and André, 2001). Twenty of them encode proteins similar to glucose (hexose) transporters (*HXT1-17, GAL2, SNF3* and *RGT2*) (Boles and Hollenberg, 1997). As discussed by Wieczorke *et al* (1999), the multitude of hexose transporter proteins in bakers's yeast seems to reflect its adaptation to the variety of environmental conditions to which yeast cells are exposed.

Two kinetically distinct glucose uptake (glucose facilitator) systems have been described in *S. cerevisiae*: a constitutive low-affinity system (K_m=15–20 mM) and a glucose-repressible high-affinity system (K_m1–2 mM) (reviewed by Özcan and Johnston, 1999). These two systems are considered to consist of many different facilitators contributing to kinetic properties of a system (Özcan and Johnston, 1999). This gives to "glucose transporter" and "glucose transport system" different meaning.

The major hexose transporters in *S. cerevisiae* are Hxt1-Hxt4, Hxt6 and Hxt7 (Reifenberger *et al*, 1997, Reifenberger *et al*, 1995). Hxt1 and Hxt3 are low-affinity glucose transporters responsible for transporting glucose into cells growing on high glucose, Hxt6 and Hxt7 are high-affinity glucose transporters (Reifenberger *et al*, 1997), being a subject to glucose-induced proteolytic degradation (catabolite inactivation) (Krampe *et al*, 1998). The nature of Hxt2 and Hxt4 is not that clear (Özcan and Johnston, 1999).

1.2.2. Rgt and Snf proteins as glucose sensors in *S. cerevisiae*

The *RGT2* and *SNF3* genes encode proteins similar to transporter proteins that function as receptors for sensing of extracellular glucose (Özcan *et al*, 1996; Özcan and Johnston, 1999). As transporter-related proteins they possess 12 transmembrane domains, but in addition they have long C-terminal cytosolic extensions (reviewed in Kruckeberg, 1996). Snf3 and Rgt2 are about 70% similar to each other but are less than 30% similar to the other members of the Hxt (hexose transporter) family (Boles and Hollenberg, 1997; Kruckenberg, 1996; Özcan *et al*, 1996). Both genes are expressed at very low levels: about 100- to 300-fold lower than the *HXT1-HXT4* genes (Özcan *et al*, 1996). Consistent with the proposed role as a high-affinity glucose sensor, *SNF3* transcription is repressed at high concentrations of glucose (Marshall-Carlson *et al*, 1990; Neigeborn *et al*, 1986; Özcan and Johnston, 1995). Rgt2 is proposed to function as a low-affinity glucose sensor, and consistent with this role, its expression is independent of glucose concentration (Özcan *et al*, 1996).

Özcan and Johnston (1999) suggest that binding of glucose to the trans-membrane-spanning domain induces a conformational change that is transmitted to the C-terminal signaling domain and affects its interaction with the next component(s) of the signal transduction pathway. They believe that both proteins interact with the same or a similar component of the signal transduction pathway, as the domains that are likely responsible for signaling (the 25-amino-acid repeats) are the same in both proteins.

1.2.3. Studies of sugar transport in other yeasts and fungi

Most aerobic yeasts have two kinetically different glucose transport systems: a high-affinity proton-symport and a low-affinity facilitated diffusion. These two systems have been described for example for yeasts *Kluyveromyces marxianus* (Gasnier, 1987), *Candida wickerhamii* (Spencer-Martinis and Van Uden, 1985), *Pichia ohmeri* (Verma *et al*, 1987), *Candida utilis* (Peinado *et al*, 1988), *Pichia pinus* (Alamäe and Simisker, 1994) and *Candida albicans* (Cho *et al*, 1994). The K_m values of two transport systems for *Pichia ohmeri* were 0.05–0.15 mM and 1–5 mM (Verma *et al*, 1987), for *Candida intermedia* 0.16 mM and 2.0 mM (Loureiro-Dias, 1987), and for *Pichia pinus* 0.1 mM and 4.6 mM (Alamäe and Simisker, 1994). Usually the low-affinity system is constitutively expressed and the high-affinity system is repressed at high glucose concentrations in the medium.

As for genetic studies, *K. lactis* has one high-affinity transporter for glucose encoded by *HGT1* (Billard *et al*, 1996), but several genes may be responsible for the low-affinity glucose transport (Weslowski-Louvel *et al*, 1992). Similarly to *S. cerevisiae*, *K. lactis* also has a proton symporter for the transport of fructose (Diezemann and Boles, 2003). In the fission yeast *Schizosaccharomyces pombe* a family of six hexose transporter genes (*Ght1-Ght6*) has been identified (Heiland *et al*, 2000). In *Pichia stipitis* three genes encoding glucose transporters (*SUT1*, *SUT2* and *SUT3*) have been identified, which probably constitute only a subfamily of glucose transporters (Weierstall *et al*, 1999). Wei *et al* (2004) found at least 17 putative hexose transporters in the genome of *Aspergillus nidulans.*

Glucose sensor homologues of Snf3 and Rgt2 of *S. cerevisiae* have been described in *K. lactis* and *H. polymorpha*. In *K. lactis rag4* mutants, glucose repression of several genes is abolished. Rag4, like Snf3 and Rgt2 has a characteristic pattern of transmembrane domains, a long C-terminal cytoplasmic tail and it can most probably mediate signaling of both high and low concentrations of glucose (Betina *et al*, 2001). The amino acid sequence of *H. polymorha* hexose transporter analogue Gcr1p shares a highest similarity with a core region of Snf3p of *S. cerevisiae*. The Gcr1p is supposed to have a regulatory role in repression pathway, along with involvement in hexose transport in *H. polymorpha* (Stasyk *et al*, 2004).

1.3. Participation of hexokinase in sugar sensing

1.3.1. Sugar sensing mechanisms

For yeasts and many other organisms glucose is the preferred carbon source and presence of a high concentration of glucose in the medium represses transcription of genes responsible for the utilization of alternative carbon sources – organic acids, alcohols and disaccharides. The phenomenon is called glucose repression (Ronne, 1995; Gancedo, 1998). Therefore, yeasts must sense presence and concentration of glucose in the medium. Sensing of glucose in the medium by baker's yeast is mediated by hexokinase (see below). Moreover, hexokinases are implicated in sugar sensing also in plants and human tissues (Harrington, 2003). The mechanisms of hexokinase-dependent sugar sensing differ among organisms, and details of the signaling cascades are not clear yet.
In human pancreatic β-cells level of a glucose-derived metabolite is sensed and glucokinase acts as an indirect glucose sensor (reviewed by Rutter, 2001; Schuit *et al*, 2001). The affinity of glucokinase for glucose is low and therefore intracellular phosphorylation of glucose by glucokinase is the rate-limiting step of glucose metabolism (Xu *et al*, 1994). If the level of glucose inside the pancreatic β-cells reaches a threshold, its catabolism is initiated by phosphorylation by glucokinase resulting in increased intracellular ATP level. The increased ATP has effect on ion channels that mediate insulin secretion (reviewed by Rutter, 2001; Schuit *et al*, 2001). A key position of glucokinase in glucose sensing can be illustrated by the fact that mutations in the glucokinase cause a specific form of diabetes (Rutter, 2001 and references therein).

However, in some organisms hexokinase-mediated sugar signaling does not involve sensing of a metabolite but is suggested to rely on a specific structural feature of the hexokinase protein. The alternative sensing cascades include conformational changes and phosphorylation of proteins as well as interactions with other proteins such as kinases, phosphatases and/or membrane proteins (reviewed in Koch *et al*, 2000). The model of hexokinase-ependent glucose sensing in baker's yeast will be briefly described in 1.3.2.

1.3.2. Hexokinase and sugar repression signaling in *S. cerevisiae*

Sugar repression signaling in yeasts has been most thoroughly investigated in the model yeast *Saccharomyces cerevisiae.* The studies on the expression of *MAL*, *SUC* and *GAL* genes that are regulated by glucose repression show that hexokinase PII is crucial for glucose repression. Point and deletion mutants of this enzyme show reduced glucose repression of maltase, invertase and galactokinase (Entian, 1997; Moreno and Herrero, 2002). Hexokinase PII is also

involved in glucose-induced repression of hexokinase PI and glucokinase (Rodríguez *et al*, 2001). Physiology of hexokinase PII-negative strains also shows dramatic changes: they have fully oxidative metabolism at high glucose concentration in early exponential batch cultures, initial absence of ethanol production, postponed and shortened diauxic shift and higher biomass yields (Diderich *et al*, 2001). So, hexokinase-negative mutants are "blind" – they cannot properly respond to the presence of high glucose concentrations in the medium. Interestingly, overexpression of hexokinase PI but not of glucokinase substitues for isoenzyme PII in glucose repression (Rose *et al*, 1991). Fructose repression in *S. cerevisiae* can be contributed by both hexokinases (De Winde *et al*, 1996; Hohmann *et al*, 1999). These findings suggest that the establishment of sugar repression needs i) the ability and capacity of a hexose kinase to phosphorylate a sugar, and ii) a specific domain present in hexokinases PI and PII, and absent in glucokinase. Consistent with this hypothesis, the inability of the *S. cerevisiae* glucokinase to transmit the glucose repression signal can be explained by its low degree of homology to hexokinase (only 28%; Albig and Entian, 1988). Hexose kinase activity and sugar signaling ability of the hexokinase 2 protein are most probably mediated through separated domains of the protein because glucose repression is not linearly relieved with decreased kinase activity (Hohmann *et al*, 1999; Kraakman *et al*, 1999) and mutant alleles with low catalytic activity are still fully functional in glucose signaling (Mayordomo and Sanz, 2001). Interestingly, the presence of a specific regulatory domain of the hexokinase protein was suggested already in 1984 by Entian and Fröhlich.

As reported by Kraakman *et al* (1999), primary signal for glucose repression in *S. cerevisiae* can be a conformational change in hexokinase PII protein generated in the phosphoryl transfer reaction and the onset of catalysis is needed for the contact of hexokinase with other proteins mediating the signal transfer. Conformational change in the hexokinase protein (induced fit) after binding of glucose has been clearly demonstrated (Kuser *et al*, 2000).

Consistent with dual roles in signaling and catalysis, Hexokinase PII (Hxk2) is found in both the nucleus and cytoplasm (Randez-Gil *et al*, 1998). Localization of hexokinase 2 is determined by glucose: if cells grow on glucose, hexokinase PII moves to the nucleus and interacts with the transcriptional repressor Mig1 to generate a nuclear repressor complex (Ahuatzi *et al*, 2004). Mig1 protein is identified as the main transcriptional repressor binding to several promotors of glucose-repressed genes (Ronne, 1995). The activity of Mig1 is regulated by Snf1-dependent phosphorylation (Ahuatzi *et al*, 2004; Papamichos-Chronakis *et al*, 2004) and by some authors also by subcellular localization (reviewed in Ahuatzi *et al*, 2004).

The model of glucose repression pathway in *S. cerevisiae* by Ahuatzi *et al* (2004) is summed up in Figure 1.

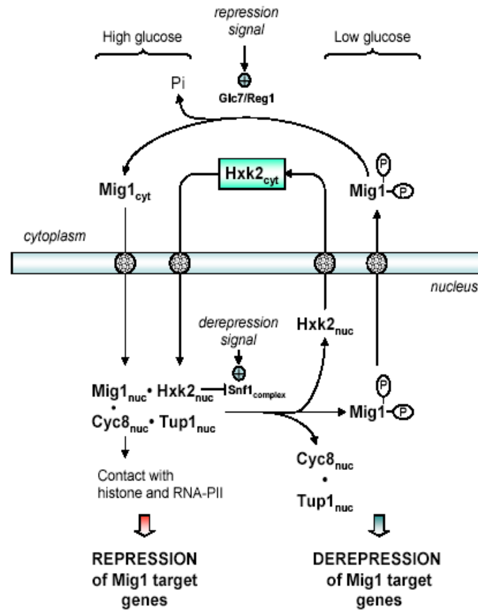

Figure 1. A model explaining the involvement of Mig1 in the nucleocytoplasmic translocation of Hxk2 (Ahuatzi *et al*, 2004); P – phosphate groups.
The addition of glucose inactivates Snf1 kinase, dephosphorylates Mig1 (by Reg1-Glc7 phosphatase complex) and induces Mig1 and Hxk2 nuclear import. The binding of hexokinase to the MIG1 element recruits the Tup1-Cyc8 (Ssn6p) complex that represses gene transcription by direct binding to their promotor region during growth on glucose. Upon glucose removal Mig1 is rapidly phosphorylated by SNF1 protein kinase complex, the Mig1 and Cyc8-Tup1 interaction abolishes and Mig1 with Hxk2 are translocated into the cytoplasm.

1.3.3. Studies on glucose repression signaling in other yeasts and filametous fungi

Among yeasts other than *S. cerevisiae*, a key role of hexokinase in glucose repression has also been reported for *Schwanniomyces occidentalis* (Rose, 1995), *Pachysolen tannophilus* (Wedlock and Thornton, 1989) and *Kluyveromyces lactis* (Goffrini *et al*, 1995). Mutants of these organisms deficient in hexokinase show reduced glucose repression. However, hexokinase-negative mutants of a filamentous fungus *Aspergillus nidulans* retain glucose repression (Ruijter *et al*, 1996). Recently, Filippi *et al* (2003) showed that in *A. nidulans*, both hexokinase and glucokinase exhibit glucose-signaling ability.

Methylotrophic yeasts are suitable organisms for the study of glucose repression mechanisms as formation of methanol-specific enzymes and organelles (microbodies) that is strongly induced during growth on methanol, is severely repressed by glucose (Sibirny *et al*, 1988; van Dijk *et al*, 2000). Formation of

methanol-specific enzymes is also repressed by ethanol, but glucose and ethanol repression are regulated independently (Sibirny, 1997).

For the study of glucose repression in methylotrophic yeasts several regulatory mutants with glucose-nonrepressible phenotype have been described. In *Candida boidinii* mutants described by Sakai *et al* (1987) and in analogous mutants of *Pichia pinus* characterized by Alamäe and Simisker (1994), glucose repression defect was linked to changes in glucose transport system. The *Pichia pinus* mutants described by Sibirny *et al* (1988) had a low activity of phosphofructokinase. Glucose nonrepressible mutants of *Hansenula polymorpha* described by Roggenkamp (1988) and Alamäe and Liiv (1998) were both suggested to be deficient in a transcriptional repressor. Phenotypically similar regulatory mutants of *H. polymorpha* have been described by Parpinello *et al* (1998) and Stasyk *et al* (2004) showing metabolically unnecessary induction of methanol utilization metabolism and formation of peroxisomes during growth on glucose. Mutant described by Stasyk *et al* (2004) was shown to be deficient in a hexose transporter homologue gene *GCR1*.

H. polymorpha is an exception among methylotrophic yeasts as it can grow on disaccharides, maltose and sucrose (Alamäe and Liiv, 1998). Utilization of disaccharides is glucose-repressed in yeasts, and is thoroughly studied in a *S. cerevisiae* model. Therefore, it is possible to compare glucose repression of *MAL* genes between a methylotrophic yeast and *S. cerevisiae.* Repression of maltose and sucrose utilization by glucose was shown in *H. polymorpha* by my co-workers (Alamäe and Liiv, 1998). The maltase gene *HPMAL1* and the respective protein of *H. polymorpha* were characterized also in our group (Liiv *et al*, 2001). Most interestingly, expression of the *HPMAL1* was glucose-repressed in *S. cerevisiae* and in the promoter region of *HPMAL1* the putative binding sites for *S. cerevisiae* repressor protein Mig1p were detected (Alamäe *et al*, 2003). So, baker's yeast and *H. polymorpha* may have similar repressor proteins executing glucose repression.

These theses present the first study on hexose kinases of methylotrophic yeasts and their role in sugar repression.

2. RESULTS AND DISCUSSION

Methylotrophic yeasts have been studied mainly from the aspects of 1) their use in biotechnology – expression of different proteins using very strong methanol-induced promoters (Gellissen, 2000) and 2) biogenesis of peroxisomes (Leão and Kiel, 2003). Our group was the first one that started detailed assay of metabolism of sugars (including glucose, fructose, maltose and sucrose) in a methylotrophic yeast *Hansenula polymorpha*. Together with my colleagues, I have studied transport of glucose and intracellular phosphorylation of sugars by hexokinase and glucokinase in *H. polymorpha*.

Glucose is the preferred carbon source for yeasts. If glucose is present, utilization of other carbon sources is down-regulated – glucose repression comes into play. Studies on bakers's yeast have shown that for the sensing of glucose in the medium both membrane-located transporter-like sensors and cytosolic hexokinase are needed (Özcan *et al*, 1996).

2.1. Glucose transport in *H. polymorpha* (I)

2.1.1. Kinetic characteristics of glucose transport systems

Glucose transport studies were performed measuring incorporation of labelled glucose at different concentrations into yeast cells. Yeasts were grown under various conditions to promote expression of different transport systems. To evaluate the presence of different transport systems, the data were plotted according to Eadie-Hofstee. Two kinetically different glucose transport systems were revealed. The affinities (K_m) of low- and high-affinity systems for glucose were 1.75 mM and 0.05–0.06 mM, respectively. The Eadie-Hofstee curves obtained for glucose-repressed, glucose-derepressed and ethanol-grown cells were all monophasic: only the low-affinity system was operating in glucose-repressed cells and only the high-affinity system was detected in glucose-derepressed and ethanol-grown cells. Monophasic curves typical for glucose-repressed and glucose-derepressed cells are presented in Fig 1A and D (I). The Eadie-Hofstee curve of glucose transport for ethanol-grown cells was very similar to that shown in Fig 1D (I). The V_{max} value of the low-affinity system was about 2–3 times higher than that of the high-affinity system (I, Table 1). The K_m values of the high- and low-affinity glucose transport systems in *H. polymorpha* were in good agreement with literature data on glucose transport systems in other yeasts.

2.1.2. Expression of the high-affinity glucose transport system in yeasts growing on different carbon sources

To study carbon source dependent expression of the high-affinity glucose transport system, the velocity of 0.05 mM glucose uptake was measured. At this low concentration glucose uptake through the low-affinity system was negligible and could therefore be ignored. Analogous approach has earlier been used for the study of high-affinity glucose uptake in *S. cerevisae* (Bisson and Fraenkel, 1984) and *Candida utilis* (Peinado *et al*, 1988).

According to the Table 2 (I), the high-affinity system was highly expressed in *H. polymorpha* cells grown on low-glucose medium (0.1%), on ethanol and methanol, and its presence was strongly reduced if the cells were grown on either 2% glucose or 2% fructose. Thus, the expression pattern of the high-affinity glucose transport system in *H. polymorpha* was subject to glucose repression similarly to that in *C. utilis* (Peinado *et al*, 1988) and *S. cerevisiae* (Bisson and Fraenkel, 1984).

2.1.3. Kinetic rearrangements of glucose transport in response to altered growth conditions

Smooth transition of a low-affinity system to a high-affinity system was observed when glucose-repressed cells were transferred to medium containing 0.05% glucose (I, Fig 1A-D). While a high-affinity transport system for glucose was only emerging in cells after 30 minutes of glucose derepression (I, Fig 1B), it became dominating after 90 minutes of derepression (I, Fig 1C) and was the only glucose transport system detected after 180 minutes of derepression (I, Fig 1D). Since a protein synthesis inhibitor geneticin prevented the formation of the high-affinity system (I, Fig 2A), synthesis of new carrier proteins is probably needed for kinetic rearrangement of the transport system.

Development of the low-affinity glucose transport system was followed by transferring of ethanol-grown (derepressed) cells to the medium containing 2% glucose. Uptake of 2 mM glucose was measured 30, 90 and 180 minutes after the transfer (I, Fig 2B). The capacity of glucose transport initially decreased, and then began to increase. The initial decrease of uptakes can probably be described as glucose-induced inactivation of the high-affinity glucose transport system initially present in ethanol-grown cells. The following increase in uptake rate we attribute to the synthesis of a new glucose transport system since it was prevented by geneticin. As fully glucose-repressed cells had only a low-affinity glucose transport system we assume that the system emerging during the transfer of ethanol-grown cells to high-glucose medium is a low-affinity glucose transport system.

2.1.4. Substrate specificity of glucose transport systems

Competition experiments (I, Table 3) showed that the two glucose transport systems in *H. polymorpha* exhibited different substrate specificities. The high-affinity system was specific for glucose, and only 2-deoxyglucose efficiently competed with glucose for the entry. At the same time, several sugars such as fructose, maltose, mannose and xylose acted as glucose competitors in the case of the low-affinity system. Glucose, mannose and fructose share a common facilitator in *S. cerevisiae* (Bisson and Fraenkel, 1983). Xylose has also been shown to share a common carrier with glucose in yeasts (Lucas and Van Uden, 1986).

Inhibition of the low-affinity system by maltose was unexpected since the other disaccharide used, sucrose, showed no inhibition. For *S. cerevisiae* (Loureiro-Dias and Peinado, 1984) and *Torulaspora delbrueckii* (Alves-Araujo *et al*, 2004) a high-affinity proton symport specific for maltose has been described in maltose grown cells (Loureiro-Dias and Peinado, 1984). Sucrose is hydrolyzed in *S. cerevisae* outside the cell to glucose and fructose by invertase, and the resulting monosaccharides enter the cell via glucose facilitators (Boles and Hollenberg, 1997). However, the alpha-glucoside transporter of *S. cerevisiae* transports various alpha-glucosides, including sucrose and maltose (Stambuk *et al*, 2000 and references therein). The alpha-glucoside transporter Sut1 using both sucrose and maltose as substrates has also been characterized in *Schizosaccharomyces pombe* (Reinders and Ward, 2001). In *H. polymorpha* (Alamäe and Liiv, 1998; Liiv *et al*, 2001) maltose and sucrose are both hydrolysed inside the cell by maltase, and probably both disaccharides have to be transported into the cell prior their intracellular splitting. The gene *HPMAL2* for a putative maltose transporter has recently been isolated and sequenced by our group (Viigand *et al*, submitted). Disruption of the *HPMAL2* in the genome and study of the disruptants will show whether the gene encodes a functional protein, and whether maltose and sucrose use the same permease for the entry into the cell.

2.1.5. Effect of metabolic inhibitors on glucose transport systems: suggestions on the nature of transport systems

To obtain information on the nature of the two different glucose transport systems, sensitivity of glucose transport to the protonophore CCCP and the inhibitor of ATP formation 2,4-dinitrophenol (DNP) was studied. The high-affinity system was highly sensitive to the presence of both CCCP and DNP (I, Table 4). Even 0.05 mM CCCP caused almost complete inhibition of glucose transport. The low-affinity system was much less sensitive to these inhibitors.

The high-affinity system was also clearly more sensitive to pH than the low-affinity one (data not shown).

According to our experiments, the high-affinity glucose transport in *H. polymorpha* is a proton symport. The nature of the low-affinity transport system is not that clear. Though partly inhibited by CCCP and DNP, and moderately sensitive to pH, the low-affinity transport system may be facilitated diffusion. In this case the sensitivity might be due to the presence of a small amount of high-affinity transport component in these cells that was not detected by kinetic analysis.

2.2. The pattern of hexose kinases in *H. polymorpha* (II)

Yeasts have specific pattern of hexose kinases (see literature review chapter 1). For example, the baker's yeast has three hexose kinases, and only one of them (hexokinase PII) is implicated in glucose repression signaling. To study the role of hexokinase in glucose repression in *H. polymorpha*, the number of glucose–phosphorylating enzymes had to be elucidated first. Two glucose-phosphorylating enzymes, a hexokinase phosphorylating both glucose and fructose, and a glucose-specific glucokinase were electrophoretically separated (II, Fig 1). Having only one hexokinase and one glucokinase makes *H. polymorpha* an attractive object to study their functions in the cell as presence of several hexose kinases makes the situation more complicated.

2.3. Functional properties of hexokinase and glucokinase of *H. polymorpha* and their participation in glucose repression (II, III, IV)

2.3.1. Purification of hexokinase protein (IV)

Hexokinase of *H. polymorpha* was purified from crude cell extract using a two-step ion exchange chromatographic procedure followed by analytical gel filtration as a final step (IV, Table 1). The first ion exchange chromatography on a HiLoad 26/10 Q Sepharose HP column resulted in a perfect separation of hexokinase and glucokinase proteins, as judged by fructose phosphorylating activity of the eluted fractions. As shown previously (II, III), fructose phosphorylation relies specifically on hexokinase and glucokinase cannot phosphorylate fructose. SDS electrophoresis of the purified enzymes revealed one major protein band, which migrated with an electrophoretic mobility ranging between those of the 46 kDa and 79 kDa standard proteins (data not shown).

As a result of purification procedure, the recovery of hexokinase was 7% (IV, Table 1) and the final preparation was 106-fold purification of the enzyme. The specific catalytic activity of the final preparation was *ca* 100 units mg^{-1} protein.

2.3.2. Functional properties of hexose kinases

Properties of glucokinase were studied using crude extracts of specific strains as enzyme preparations. In reference II the hexokinase-negative mutants (containing only glucokinase) were used. In reference III the double kinase-negative strain A31–10 transformed with the plasmid pRSH3Bcu harbouring the *H. polymorpha* glucokinase gene *HPGLK1* was used. Properties of hexokinase were mostly studied using the purified enzyme (IV). For the measurement of the K_m of hexokinase for glucose and fructose the crude extract of glucokinase-negative mutants was also used (II). The K_m values of the hexokinase for these two sugars obtained using either the crude extract of mutants or the purified protein were coincidental.

2.3.2.1. Substrate specificity of H. polymorpha hexokinase and glucokinase (II, III, IV)

In order to identify sugars that may act as a substrate for *H. polymorpha* hexose kinases, the rate of ADP formation from ATP was measured in the presence of different hexoses and pentoses (see methods in III and IV). *H. polymorpha* hexokinase could use D-fructose, D-glucose, 2-deoxy-D-glucose, D-mannose and D-glucosamine, while D-xylose, D-galactose and methyl-α-D-glucoside were not used as substrates (IV). *H. polymorpha* glucokinase could use D-glucose, D-mannose and 2-deoxy-D-glucose but could not phosphorylate D-fructose, D-xylose and D-galactose (III). To measure the relative activity of *H. polymorpha* hexose kinases, production of ADP from ATP was measured with different concentrations of some sugars (III, Table 1 and IV, Table 2).

Our data showed that substrate specificity of *H. polymorpha* hexose kinases is similar to that of respective kinases in *Aspergillus niger* (Panneman *et al*, 1996; 1998) and *Rhodotorula glutinis* (Mazon *et al*, 1975).

Comparing the K_m values of two hexose kinases of *H. polymorpha,* one can see that glucokinase has higher affinity to glucose than hexokinase. K_m values for ATP of *H. polymorpha* glucokinase and hexokinase are similar. Respective data are presented in Table 4.

Table 4. K_m values of hexokinase and glucokinase of *H. polymorpha* (II, III, IV)

	K_m Glc mM	K_m Fru mM	K_m ATP mM	F/G
Hexokinase	0.26	1.1	0.32	2.8
Glucokinase	0.05	–	0.28	nd

(nd – not determined; "–" – no activity)

Comparing our data with those obtained with other yeasts, K_m values of *H. polymorpha* hexokinase for glucose and fructose are quite similar to those described for the hexokinases from *Saccharomyces cerevisiae* (Entian, 1997), *Rhodotorula glutinis* (Mazon *et al*, 1975), *Candida tropicalis* (Hirai *et al*, 1977) and *Yarrowia lipolytica* (Petit and Gancedo, 1999). K_m of *H. polymorpha* glucokinase for glucose is close to that of *A. niger* glucokinase (Panneman *et al*, 1996) and *S. cerevisiae* glucokinase (Maitra, 1970). The affinity data of yeast hexokinases and glucokinases are presented in Table 1 of the present thesis.

The K_m of *H. polymorpha* glucokinase for ATP is lower than the respective value (0.05 mM) of *S. cerevisiae* glucokinase (Maitra, 1970), and close to the K_m for ATP (0.21 mM) of *Y. lipolytica* glucokinase (Petit and Gancedo, 1999).

High F/G ratio (about 2.8) of *H. polymorpha* hexokinase suggests its functional similarity to hexokinase PI from *S. cerevisiae* (Gancedo *et al*, 1977), hexokinase B from *Pachysolen tannophilus* (Wedlock *et al*, 1989) and hexokinase from *R. glutinis* (Mazon *et al*, 1975).

2.3.2.2. Physiological inhibitors of H. polymorpha hexokinase and effect of pH on enzyme activity (IV)

Hexokinases of yeasts and filamentous fungi are inhibited at physiological concentrations of ADP (Panneman *et al*, 1998). According to our study, *H. polymorpha* hexokinase was inhibited by ADP in a competitive manner with respect to ATP (K_i=1.6mM; IV, Fig 3B). Consistent with the data on yeast hexokinases (Entian, 1997; Cárdenas *et al*, 1998), *H. polymorpha* hexokinase was not inhibited by glucose-6-phosphate (data not shown), that is a second product of hexokinase reaction. Similar results are obtained for the hexokinases from *Aspergillus niger* (K_i=1.1 mM; Panneman *et al*, 1998) and *Schizosaccharomyces pombe* (K_i=1.45 mM; Tsai and Chen, 1998).

Our experiments show strong inhibition of the *H. polymorpha* hexokinase by trehalose-6-phosphate (K_i=12 µM) and sensitivity of inhibition to competition by glucose (IV, Fig 3A). Reinders *et al* (1999) have reported a strong inhibition of glucose phosphorylation in *H. polymorpha* by 1 mM trehalose-6-phosphate. Comparison of the K_i values indicates that *H. polymorpha* hexokinase is more sensitive to trehalose-6-phosphate inhibition than hexokinase PII (K_i=40 µM) and hexokinase PI (K_i=200 µM) of *S. cerevisiae* (Blázques *et al*, 1993), but still

less sensitive than *Y. lipolytica* hexokinase (K_i=3.6 μM; Petit and Cancedo, 1999). Glucokinase of *S. cerevisiae* is not inhibited by trehalose-6-phosphate (Blázques *et al*, 1993). As reported in reference IV, glucokinase of *H. polymorpha* also shows no sensitivity to trehalose-6-phosphate.

Glucose phosphorylating activity of *H. polymorpha* hexokinase was routinely measured at pH 7.5 in Tris buffer. By testing hexokinase activity at different pH values we found the highest activity at pH 8.5. The activity of hexokinase at pH 7.5 was 91% of the maximum (data not shown). This is good accordance with data on other hexokinases. Hexokinase from *A. niger* displays maximal activity between pH 7.5 and 8.5 (Panneman *et al*, 1998) and the hexokinase of *S. cerevisae* shows highest activity at pH 8.2 (Ogawa *et al*, 2001).

2.3.3. Participation of hexose kinases in sugar repression (II, III, IV)

2.3.3.1 Glucose and fructose phosphorylation in H. polymorpha grown on different carbon sources (II)

Glucose and fructose phosphorylating activities were measured in cell-free extracts grown on different carbon sources. The specific activities were clearly dependent on the carbon source in the growth medium (II, Table II): the activities were high when the cells were grown on glycolytic substrates and much lower if a gluconeogenetic substrate as ethanol, methanol or glycerol was used as a carbon source. Low activity of glucose phosphorylation in methanol-grown *H. polymorpha* and its up-regulation in glucose-grown cells has been shown before (Parpinello *et al*, 1998).

The ratio of fructose phosphorylating activity to glucose phosphorylating activity (F/G ratio) was high (close to 2.0) if the cells were grown on glucose and fructose, and much lower if gluconeogenetic substrates were used as a carbon source (II, Table II). As glucokinase does not phosphorylate fructose, the F/G value in the cell extract reflects the relative amounts of hexokinase and glucokinase proteins in the cell. Taking into account glucose- and fructose phosphorylating activities in extracts and F/G ratio of hexokinase (2.8), phosphorylation of glucose due to glucokinase was calculated. It turned out that during growth on glucose and fructose, hexokinase accounted for 67–72% of the total glucose phosphorylating activity of the cell whereas in the cells grown on ethanol, methanol or glycerol glucose phosphorylating activity due to hexokinase was only 28–33%. High expression of glucokinase in ethanol- or glycerol-grown cells and its repression in glucose- and fructose-grown cells has also been shown for *S. cerevisiae* (Herrero *et al*, 1995).

Reporter gene study in our group has also shown that expression from the *HPHXK1*gene promoter is up-regulated if *H. polymorpha* cells are exposed to sugars (Viigand *et al*, submitted).

2.3.3.2. Participation of hexokinase and glucokinase in sugar repression (II, III, IV)

In *H. polymorpha* both, glucose and fructose repress the synthesis of alcohol oxidase and catalase (II). As shown by Toomas Kramarenko (II), hexokinase-negative mutants of *H. polymorpha* retain glucose repression of these enzymes while repression by fructose is abolished, suggesting a specific requirement for the hexokinase protein in fructose repression. In order to prove it by gene complementation, hexokinase negative mutant A3 (II) was transformed with the plasmid pYT3HPHXK1 carrying *H. polymorpha* hexokinase gene and glucose and fructose repression of alcohol oxidase and catalase was studied in the transformants. Wild-type strain LR9, glucokinase-negative mutant A31-7 and mutant A3 transformed with the empty vector pYT3 were used as references. Table 3 (IV) confirms that *HPHXK1* complements fructose phosphorylation deficiency of the hexokinase-negative mutant and shows repressed level of catalase and absence of alcohol oxidase activity in all studied strains grown on either glucose or fructose.

To study the induction of alcohol oxidase and catalase by methanol in the presence of glucose and fructose, the strains were grown on specific media. The results are shown in Table 4 (IV). In all studied strains alcohol oxidase and catalase were highly induced by methanol, while glucose prevented the induction of both enzymes. The behaviour of the hexokinase-negative mutant transformed with the empty vector pYT3 confirms that *H. polymorpha* glucokinase can mediate glucose repression. From this aspect, *H. polymorpha* differs from *S. cerevisiae* and *Schwanniomyces occidentalis,* in which the presence of hexokinase in the cell is specifically needed, and glucokinase cannot substitute the function of hexokinase (Ma and Botstein, 1986; Rose *et al*, 1991; Walsh *et al*, 1991; Rose, 1995). As discussed in reference III, glucose-signaling property of *H. polymorpha* glucokinase may be advantageous to this yeast. Fructose repressed the synthesis only in the strains that possess hexokinase – the only fructose phosphorylating enzyme in *H. polymorpha*. The results of this study together with earlier findings obtained by our group indicate that in *H. polymorpha* phosphorylation of glucose or fructose is required for the establishment of repression by the respective sugar.

It can be assumed that sugar-repression signaling process in *H. polymorpha* and in the best studied yeast model *S. cerevisiae* (see literature review chapter 2) exhibit significant differences. The earlier and present results of our working group show that:

1) glucokinase that phosphorylates glucose but not fructose can mediate glucose repression but not fructose repression;
2) hexokinase that phosphorylates both glucose and fructose can mediate repression by both sugars;
3) the degree of identity of *H. polymorpha* hexokinase and glucokinase is only 38% (see 2.4.), but they can both mediate glucose repression.

Therefore it can be hypothesized that some metabolite produced due to glucose or fructose phosphorylation by respective hexose kinase might act as a messenger in sugar-induced signal transduction in *H. polymorpha.*

2.4. Characterization of the *H. polymorpha* hexokinase gene and the protein deduced from the genomic sequence (IV)

H. polymorpha hexokinase gene *HPHXK1* was cloned by complementation of the glucose growth deficiency of the *H. polymorpha* double kinase negative mutant A31–10 with a genomic library. Sequencing revealed an ORF of 1,452 bp that encoded a protein consisting of 483 amino acids with the calculated molecular mass of 54.2 kDa. Functional subclone of the *HPHXK1* in pYT3 (pYT3HPHXK1) contained the hexokinase ORF (1452 bp), 688 bp of the promoter region and 429 bp of the 3′noncoding region. The growth of hexokinase-negative mutants A3 and A25–19 on fructose was corrected by introduction of the plasmid pYT3HPHXK1 (ref IV, Fig 1). This suggests that the hexokinase ORF in the insert of pYT3HPHXK1 is linked to portions of the upstream and downstream regions, which are sufficient for the full expression of the hexokinase. Inspection of these DNA regions revealed a putative TATA-element (TATAAA), starting at position –63. Downstream of the *HPHXK1* ORF, we found a TACATA element 29 nucleotides from the T nucleotide of the stop codon TAA and a AATAAA element positioned 3 nucleotides downstream of this. According to Guo and Sherman (1996) and Van Helden *et al* (2000), these motifs might function in poly(A) signaling.

The deduced amino acid sequence showed a high degree of homology with several yeast hexokinases. The highest degree of identity (56%) was revealed with the hexokinase from *S. occidentalis.* Identity of *H. polymorpha* hexokinase with glucokinases was significantly lower: 38% with *H. polymorpha* glucokinase and 39% with *S. cerevisiae* glucokinase. Sequence alignment of the *H. polymorpha* hexokinase with those of the hexokinases from *S. cerevisiae*, *K. lactis* and *S. occidentalis* revealed several evenly distributed conserved regions (IV, Fig 2A). Residues considered to be involved in the binding of glucose and the phosphoryl, ribose and adenine moieties of the nucleotide substrate according to Zeng *et al* (1996) were also detected in the *H. polymorpha* hexokinase protein (IV, Fig 2A). The N-terminal part of the *H. poly-*

morpha hexokinase, however, is rather different from the N-termini of other yeast hexokinases and aligns much better with the N-termini of the glucokinases from *S. cerevisiae* and human pancreas (IV, Fig 2B).

At the same time, the N-terminal part of *H. polymorpha* glucokinase did not show good alignment with hexokinases (not shown).

To evaluate the phylogenic position of the *H. polymorpha* hexokinase, protein sequences of hexokinases and glucokinases were retrieved from electronic databases and aligned using the Clustal W program. The resulting phylogenetic tree (IV, Fig 2C) shows that *H. polymorpha* hexokinase clusters within the hexokinases, but shows only moderate relatedness to the group of hexokinases from *S. cerevisiae*, *K. lactis* and *S. occidentalis*, which are closely related to each other.

CONCLUSIONS

The present work can be summarized as follows:

1. *Hansenula polymorpha* has two kinetically different glucose transport systems: a low-affinity system and a high-affinity system. Expression of the systems is dependent on the carbon source and the high-affinity system is subject to glucose and fructose repression. The two systems differ in substrate specificity with the high-affinity system being more specific towards glucose. The kinetic rearrangement of the glucose transport system in response to altered growth conditions is dependent on *de novo* protein synthesis. Both systems are sensitive to metabolic inhibitors, suggesting active transport mechanisms.
2. *H. polymorpha* has two distinct hexose kinases: a hexokinase and a glucokinase. Glucose and fructose phosphorylating activity is induced during growth of *H. polymorpha* on a high concentration of sugars. Hexokinase and glucokinase are differentially expressed: in cells grown under glycolytic conditions (on sugars) hexokinase dominates, while glucokinase is predominately expressed if cells are grown on gluconeogenic substrates. As glucokinase has a very high affinity for glucose (K_m 0.050 mM), its domination can be advantageous to the cells growing on energetically poor substrates, allowing fast utilization of even low concentrations of sugars if they suddenly become available.
3. Substrate specificity of hexokinase and glucokinase differs. Hexokinase can use D-fructose, D-glucose, 2-deoxy-D-glucose and D-mannose as substrates. Glucokinase can use all these sugars except D-fructose. Thus, hexokinase is the only enzyme responsible for fructose phosphorylation in *H. polymorpha.* Consistent with enzymological data, transformation of hexokinase-negative mutants with the *H. polymorpha* hexokinase gene *HPHXK1* on a plasmid restores the ability of mutants to grow on fructose.
4. Hexokinase of *H. polymorpha* is inhibited by trehalose-6-phosphate and ADP, but not by glucose-6-phosphate.
5. Sequencing of the *H. polymorpha* hexokinase gene *HPHXK1* revealed 1452 bp ORF encoding a protein of 483 amino acids. *H. polymorpha* hexokinase protein has a high degree of identity to hexokinases from other yeasts and a significantly lower homology to glucokinases.
6. Complementation of the *H. polymorpha* hexokinase-negative mutants with the *H. polymorpha* hexokinase gene on a plasmid restores the ability of the mutants to respond to fructose repression. These results confirm that in *H. polymorpha* phosphorylation of the sugar (glucose or fructose) is required for the establishment of repression by respective sugar.

   Threfore, some metabolite produced due to phosphorylation of glucose by either hexokinase or glucokinase, or due to phosphorylation of fructose by hexokinase, may act as a messenger in sugar-signaling.

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SUMMARY IN ESTONIAN

Heksoosi kinaasid ja glükoosi transport pärmil *Hansenula polymorpha*

Metülotroofseid pärme, eriti *Hansenula polymorpha'*t, on palju kasutatud võõrvalkude tootmiseks väga tugevate metanooliga indutseeritavate ning glükoosiga represseeritavate promootorite kontrolli all. Seetõttu on oluline uurida glükoosi repressiooni mehhanisme neil pärmidel. Glükoosi signaali vahendamises osalevad pagaripärmil, taimedes ning ka pankrease β-rakkudes nii glükoosi transporterid kui ka glükoosi fosforüülivad ensüümid. Metülotroofsel pärmil *H. polymorpha* ei olnud enne antud tööd uuritud ei glükoosi transporti ega heksoosi kinaase. Töö tulemuste alusel võib teha järgmised järeldused:

1. Pärmil *H. polymorpha* on kaks kineetiliselt erinevat glükoosi transportsüsteemi: madalafiinne ja kõrgafiinne süsteem. Transportsüsteemide ekspressioon sõltub süsinikuallikast söötmes. Kõrgafiinse transportsüsteemi ekspressioon on glükoosi ja fruktoosiga represseeritav. Transportsüsteemide substraadispetsiifika on erinev – kõrgafiinne süsteem on glükoosispetsiifilisem. Transportsüsteemide keskkonnatingimustest sõltuvaks ümberkorraldumiseks on vajalik *de novo* valgusüntees. Mõlemad transportsüsteemid on metaboolsetele inhibiitoritele tundlikud.
2. Pärmil *H. polymorpha* on kaks heksoosi kinaasi: heksokinaas ja glükokinaas. Glükoosi ja fruktoosi fosforüülimise aktiivsus on suurim, kui rakud kasvavad kõrge suhkrusisaldusega söötmes. Heksokinaasi ja glükokinaasi osakaal rakus sõltub süsinikuallikast söötmes: glükolüütilistes tingimustes (suhkrutel) kasvanud rakkudes domineerib heksokinaas ning glükoneogeneetilistel substraatidel kasvanud rakkudes domineerib glükokinaas. Kuna glükokinaasi afiinsus glükoosile on kõrge (K_m 0.050 mM), siis võib tema domineerimine energiavaestel substraatidel kasvavates rakkudes olla kasulik, võimaldades keskkonda ilmuva suhkru kasutamist ka siis, kui seda on vähe.
3. *H. polymorpha* heksokinaasi ja glükokinaasi substraadispetsiifilisus on erinev. Heksokinaas suudab fosforüülida D-fruktoosi, D-glükoosi, 2-desoksü-D-glükoosi ja D-mannoosi. Glükokinaas fosforüülib kõiki neid suhkruid v.a D-fruktoos. Seega on heksokinaas ainus fruktoosi fosforüüliv ensüüm pärmis *H. polymorpha*.

   Kooskõlas ensümoloogiliste andmetega taastab heksokinaasnegatiivse mutandi transformeerimine *H. polymorpha* heksokinaasi geeniga *HPHXK1* mutandi kasvu fruktoosil.
4. *H. polymorpha* heksokinaasi inhibeerivad trehaloos-6-fosfaat ja ADP, kuid mitte glükoos-6-fosfaat, mis on tüüpiline pärmide heksokinaasidele.

5. Sekveneeriti *H. polymorpha* heksokinaasi geen *HPHXK1*. Selle 1452 aluspaari pikkune avatud lugemisraam kodeerib 483 aminohappelist valku, mille järjestus sarnaneb teiste pärmide heksokinaaside järjestusega ning sarnasus glükokinaasidega on oluliselt väiksem.
6. *H. polymorpha* heksokinaasnegatiivsete mutantide transformatsioon heksokinaasi geeniga taastas neis fruktoosi repressiooni. Need tulemused kinnitavad, et pärmis *H. polymorpha* on suhkru (glükoosi või fruktoosi) repressiooniks vajalik vastava suhkru fosforüülimine. Seega võib glükoosi tunnetamise vahendajaks olla mõni metaboliit, mis tekib glükoosi fosforüülimisel kas heksokinaasi või glükokinaasi poolt või fruktoosi fosforüülimisel heksokinaasi poolt.

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Karp, H., Alamäe, T. 1998. Glucose transport in a methylotrophic yeast *Hansenula polymorpha*. FEMS Microbiol. Lett. 166, 267–273

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Scientific work

Since 1994 I have been working in the group of Dr. Tiina Alamäe. I have studied sugar metabolism of methylotrophic yeast *H. polymorpha*, concentrating mainly on glucose phosphorylating enzymes and glucose transport. To purify hexokinase, I worked two months of the year 2000 in the laboratory of Medical Department of Dresden Technical University under supervision of Prof. Thomas Kriegel.

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Teadustegevus

Alates 1994. aastast olen töötanud dr. Tiina Alamäe töögrupis. Olen uurinud metülotroofse pärmi *H. polymorpha* metabolismi, keskendudes peamiselt glükoosi fosforüülivatele ensüümidele ja glükoosi transpordile. Heksokinaasi puhastamiseks töötasin kaks kuud aastal 2000 Dresdeni Tehnikaülikooli meditsiiniosakonna laboris Prof. Thomas Kriegeli juhendamisel.