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Matthias Rose

Molecular and biochemical characterization of the hexokinase from the starch-utilizing yeast *Schwanniomyces occidentalis*

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Abstract Hexose-phosphorylating enzymes from the starch-utilizing yeast Schwanniomyces occidentalis were purified and two isoenzymes separated. The substrate pattern characterized one of these as a hexokinase phosphorylating glucose and fructose and the other as a glucokinase unable to phosphorylate fructose. The purified Schw. occidentalis hexokinase had a K_M value of 0.98 mM for glucose and 9.3 mM for fructose. The hexokinase gene was cloned by cross hybridization with a probe from the Saccharomyces cerevisiae HXK2 gene. Deletion of Schw. occidentalis hexokinase by gene replacement yielded a mutant unable to grow on fructose as sole carbon source. but still growing on glucose. Deletion mutants of Schw. occidentalis hexokinase prevented glucose repression of invertase and maltase. Growth deficiences and the defect of glucose repression of a S. cerevisiae hexokinase null mutant could be restored by heterologous expression of the Schw. occidentalis hexokinase. Moreover, the results clearly showed the existence of a separate glucokinase in Schw. occidentalis.

Key words Schwanniomyces occidentalis · Yeast Hexokinase · Glucokinase

Introduction

Characterization of *Saccharomyces cerevisiae* hexosephosphorylating enzymes led to the discovery of three distinct enzymes (Maitra 1970, 1975; Colowick 1973). Due to their catalytic activity on the phosphorylation of fructose, mannose and glucose, two of these enzymes are classified as hexokinase PI and PII (A and B, respectively). Under physiological conditions the third enzyme, gluco-

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kinase, was unable to phosphorylate fructose (Maitra 1970). In other yeasts several hexose-phosphorylating enzymes have been described (see references in McCann et al. 1987). They differ in their kinetic parameters such as substrate affinities and maximal velocities. *S. cerevisiae* hexose-phosphorylating enzymes have a molecular weight of approximately 50 kDa (Colowick 1973). Biochemical data from several mammalian hexokinases revealed subunit molecular weights of 50 kDa and 100 kDa (Colowick 1973). According to sequence data, a tandem duplication is responsible for the higher-molecular-weight hexokinases I–III (A–C, respectively) in mammalian cells (Schwab and Wilson 1989, 1991; Thelen and Wilson 1991). The 50-kDa hexokinase IV (D) is also known as glucokinase (Magnusson and Shelton 1989).

Glucose repression is a complex regulatory network which controls the expression of enzymes involved in the utilization of disaccharides and galactose, gluconeogenesis, the tricarboxylic acid cycle, the respiratory chain, and several amino-acid synthesis pathways (reviewed by Entian and Barnett 1992; Gancedo 1992). A selection system for the isolation of S. cerevisiae glucose-repression mutants has been described by Zimmermann and Scheel (1977). In one of the isolated mutants (hex1) the constitutive expression of maltase and invertase was correlated with a reduced hexose-phosphorylating activity (Entian et al. 1977; Entian and Zimmermann 1980). Allelism of *hex1* and the mutation *hxk2* of the structural gene for hexokinase PII was demonstrated (Entian 1980). The structural genes of hexokinase PI (HXK1), PII (HXK2) and glucokinase (GLK1) have been isolated and characterized (Entian et al. 1984; Fröhlich et al. 1984, 1985; Kopetzki et al. 1985; Albig and Entian 1988). Gene-deletion experiments in S. cerevisiae have shown that a hxk2 (hexokinase PII) deletion correlates with glucose-repression failure, i.e., constitutive invertase synthesis (Ma and Botstein 1986; Rose et al. 1991). Single deletions in the genes encoding hexokinase PI or glucokinase had no effect on glucose repression. Calculation of the amount of hexose-phosphorylating activity contributed by the isoenzymes revealed a predominant role for hexokinase PII. Continuous

Institut für Mikrobiologie, Johann-Wolfgang-Goethe-Universität Frankfurt, Marie-Curie-Strasse 9, Gebäude N250, D-60439 Frankfurt/Main, Germany

reduction of hexokinase PII activity also led to an inversely correlated defect in glucose repression. Additionally the glucose-repression defect in hexokinase PII mutants could be compensated by over-expression of hexokinase PI. This was not achieved by over-expression glucokinase, even when the glucose-phosphorylating activity was several times higher than in wild-type strains (Rose et al. 1991). These results indicated a specific function of hexokinases in glucose repression.

Schwanniomyces occidentalis is one of the fastest growing starch-utilizing yeasts (McCann and Barnett 1984). A mutant with a pleiotropic defect in the glucose repression of different enzyme systems metabolizing sugars has been isolated (McCann et al. 1987). Apart from the high levels of invertase for sucrose metabolism and glucoamylase for starch breakdown, the mutant showed a failure in fructose phosphorylation caused by a loss of hexokinase activity. The selection system leading to the isolation of this *Schw. occidentalis* mutant was similar to that used for the selection of glucose-repression mutants in *S. cerevisiae* (Zimmermann and Scheel 1977). A similar mutant has been described in the related *Schwanniomyces castellii* strain CBS 2863 (Boze et et al. 1989).

In an attempt to study the hexose-phosphorylating enzymes of *Schw. occidentalis*, McCann et al. (1987) were not able to separate isoenzymes. Under conditions allowing chromatographic separation of the three *S. cerevisiae* isoenzymes, the authors obtained a single protein fraction which represented both glucose- and fructose-phosphorylation activity. They concluded that *Schw. occidentalis* harbors a hexose-phosphorylating enzyme containing two domains. One of the these is responsible for hexokinase activity, i.e., phosphorylation of glucose and fructose, whereas the other is responsible for glucokinase activity. The mutant strain defective in glucose repression should only possess an active glucokinase domain.

Materials and methods

Strains. Schw. occidentalis strain CBS819 (=ATCC2322) was used as wild-type. The hexokinase-negative S. cerevisiae strain WAY.7-2D/2A was described by Rose et al. (1991). Amplification of plasmids was performed in the Escherichia coli strains RR1 (Ausubel et al. 1989) and XL1-Blue (Stratagene; Heidelberg, Germany).

Media. Yeast strains were grown on YEPD medium containing 1% yeast extract (Life Sciences; Eggenstein, Germany), 2% bacto peptone (Difco; Augsburg, Germany) and 4% glucose. S. cerevisiae transformants were grown on selective medium without uracil, consisting of 0.67% yeast nitrogen base without amino acids (Difco; Augsburg, Germany) and 2% glucose (Ausubel et al. 1989). The Schw. occidentalis hxk mutant was selected according to Zimmermann and Scheel (1977) on medium composed of 1% yeast extract, 2% bacto peptone, 2% soluble starch (MERCK; Darmstadt, Germany) and 150 ppm of 2-deoxyglucose (Sigma; Munich, Germany). E. coli was propagated on LB-medium (Life Sciences; Eggenstein, Germany). For plasmid selection, 40 ppm of Ampicillin was added. The medium was solidified by adding 1.5% Agar (Difco; Augsburg, Germany). All media were sterilized for 20 min at 121 °C. Ampicillin and 2-deoxyglucose were filter-sterilized and added after autoclaving.

Enzyme assays and preparation of crude extracts. The methods used were as described previously (Rose et al. 1991).

Enzyme purification. Hexokinase purification was according to Kopetzki and Entian (1982). The crude extract was applied to a DEAE-Sephacel column $(50 \times 100 \text{ mm}; \text{Pharmacia}, \text{Freiburg}, \text{Germany})$ equilibrated with 25 mM of piperazine/HCl buffer (pH 6.5) and eluted in one step with 12 column volumes of 25 mM piperazine /HCl (pH 3.5). Fractions containing hexokinase activity were pooled and applied to a polybuffer exchanger 94 column (10×380 mm; Pharmacia, Freiburg, Germany). Hexose-phosphorylating enzymes were eluted with 10 column volumes of polybuffer 74 (Pharmacia; Freiburg, Germany), pH 4. Since elution of the Schw. occidentalis hexokinase occurred at the isoelectric point, and the enzyme is extremely unstable at this pH, the tubes in the fraction collector were supplemented with double the expected elution volume containing untitrated 25 mM of piperazine. The pooled fractions were supplemented with ammonium sulphate to 40% saturation and then loaded to a 30×60-mm Phenyl-Sepharose column (Pharmacia; Freiburg, Germany) equilibrated with 25 mM of piperazine/HCl (pH 6.5) 40% saturated with ammonium sulphate. The column was eluted with 25 mM of piperazine/HCl (pH 6.5) supplemented to form a gradient of decreasing ammonium sulphate saturation (40-0%) and an increasing ethylene glycol concentration (0-80%).

Molecular biological techniques. For Southern blotting and hybridization, establishing protocols were followed (Ausubel et al. 1989). Protocols for in vitro DNA recombination were performed according to Ausubel et al. (1989). Cloning of the hexokinase gene was preceded by the identification of a 3.3-kb BamHI DNA fragment which hybridized to S. cerevisiae HXK2 in Southern-blot experiments. Schw. occidentalis DNA was digested with BamHI and a library of 2.5-4-kb DNA fragments inserted into the unique BamHI site of pBR322 was constructed in E. coli. Approximately 2000 clones were investigated by colony hybridization and two clones (pMR100) showed a hybridization signal with the S. cerevisiae HXX2 probe. An additional library was constructed by double digesting chromosomal DNA with EcoRI and XbaI. Fragments of 3-5 kb were ligated into pBR322 linearised with EcoRI and NheI. Colony hybridization identified a plasmid (pMR158) containing the hexokinase gene. Plasmids pMR164 and pMR188 were constructed by insertion of a 2.5-kb BglII fragment harbouring the Schw. occidentalis HXK (see Fig. 4) into BamHI-opened YCp50 (Rose et al. 1987) or YEp24 (Botstein et al. 1979) respectively. For deletion of parts of the Schw. occidentalis HXK gene, pMR158 was digested with EcoRV to release 453 bp of the open reading frame and self-ligated. From the resulting plasmid, a 2-kb BglII fragment containing the hxk deletion was isolated and used for transformation of Schw. occidentalis. DNA was sequenced by the dideoxynucleotide chain-terminating method (Sanger et al. 1977). The DNA sequence was determined using subclones and synthesized oligonucleotide primers. Schw. occidentalis and S. cerevisiae were transformed by the lithium acetate procedure (Ito et al. 1983).

Computing. Sequence data were analysed using the programme packages DNASIS, PROSIS (Hitachi) and the UWGCG package.

Results

Purification of the Schw. occidentalis hexokinase

McCann et al. (1987) purified *Schw. occidentalis* and *S. cerevisiae* hexokinases by using FPLC with an anion exchange column. The *S. cerevisiae* isoenzymes glucokinase, hexokinase PI and hexokinase PII were separated under these conditions. The *Schw. occidentalis* hexose-phosphorylating activity was detected as a single peak. The molecular weight of the *Schw. occidentalis* hexokinase was es-





Fig. 1 Chromatofocussing of *Schw. occidentalis* hexose-phosphorylating enzymes. In the elution profile, the volume activity (phosphorylation of glucose, *filled square*), pH (*open circle*) and absorbance at 280 nm (*asterisk*) were marked. From the twin peak, fractions 1 and 2 were pooled separately and further purified by hydrophobic interaction chromatography

timated at 72 kDa. Genetic data showed the existence of a glucokinase in *Schw. occidentalis* (McCann et al. 1987). In an interpretation of their results McCann et al. hypothesized a bifunctional hexose-phosphorylating enzyme in *Schw. occidentalis*. This enzyme should contain two domains, one of which phosphorylates glucose, and the other of which phosphorylates fructose and glucose. One single protein should contain the functions of glucokinase and hexokinase in *Schw. occidentalis*.

In the present study, the hexokinase of Schw. occidentalis was purified. The protocol published for the purification of S. cerevisiae hexokinases (Kopetzki and Entian 1982) was used. Yeast cells were disrupted with glass beads and the crude extract separated on a DEAE-Sephacel column. The fractions with the highest hexose-phosphorylating activity were pooled and further purified by chromatofocussing. Glucose-phosphorylating activity eluted in two overlapping peaks (Fig. 1). These peaks were pooled separately. Each pool was supplemented with ammonium sulphate and applied to Phenyl-Sepharose. From the first pool, only one peak eluted at approximately 2.1 mSi/cm. From the second pool, two separate peaks eluted at approximately 3.6 mSi/cm and 2.1 mSi/cm (Fig. 2). Determination of the fructose-over-glucose phosphorylation ratios identified the peak eluting at 3.6 mSi/cm as hexokinase. The protein eluting at 2.1 mSi/cm was identified as glucokinase. The enzyme phosphorylated glucose, but not fructose. From this result, it was concluded that Schw. occidentalis contains two distinct hexose-phosphorylating enzymes, i.e., hexokinase and glucokinase. The purified Schw. occidentalis hexokinase had a K_M value of 0.98 mM for glucose and 9.3 mM for fructose.



Fig. 2 A, B Hydrophobic interaction chromatography of *Schw. occidentalis* hexose-phosphorylating enzymes. Profiles **A** and **B** correspond to pools 1 and 2 obtained from the chromatofocussing, respectively. Indicated are volume activities (substrate glucose: *filled square*; substrate fructose: *filled circle*), conductivity (*open circle*), and absorbance at 280 nm (*asterisk*)

Cloning of the *Schw. occidentalis* hexokinase gene (*HXK*)

Southern hybridization of *Schw. occidentalis* chromosomal DNA with a *S. cerevisiae HXK2* gene probe yielded strong signals, even under stringent washing conditions (Fig. 3). A 3.3-kb *Bam*HI fragment hybridized to the *S. cerevisiae HXK2* probe (Fig. 3). *Schw. occidentalis* chromosomal DNA was digested by restriction endonuclease *Bam*HI and a library of 2.5–4-kb fragments was inserted into pBR322. Positive clones were identified by colony hybridization (pMR100). The DNA sequences flanking both *Bam*HI sites were determined. Comparison of the deduced amino-acid sequences to *S. cerevisiae* hexokinases showed a high degree of identity to one of these flanking sequences. However, this fragment did not cover the entire *Schw. occidentalis HXK* gene (Fig. 4). An additional library containing 3–5-kb *Eco*RI *Xba*I fragments was constructed.



Fig. 3 Southern hybridization of *Schw. occidentalis* DNA with a *S. cerevisiae HXK2* probe. The letters *E, B, P and C* refer to the chromosomal DNA digested with *Eco*RI, *Bam*HI, *Pst*I and *Cla*I, respectively. The sizes of hybridising fragments are labeled in kb



Fig. 4 Restriction map of the *Schw. occidentalis HXK* locus. Only the open reading frame and the flanking DNA was sequenced

Colony hybridization identified a plasmid which contained the complete hexokinase gene (pMR158).

Molecular characteristics of the HXK gene

An open reading frame of 479 codons, encoding a protein of 53.4 kDa, was identified in a 1870-bp DNA sequence (Fig. 5). In the 5' noncoding region a TATA consensus sequence is located at position -99 to -93, relative to the ATG start codon. The codon usage in the *HXK* open reading frame revealed a striking predominance of the codons containing A or T residues in the third position. Only phenylalanine and tyrosine are encoded predominantly by codons containing a C residue in the third position. The overall GC content of the *Schw. occidentalis* open reading frame is only 37.8%, whereas the *S. cerevisiae HXK1* and *HXK2* genes contain 42.0% and 42.5% GC pairs, respectively.

Comparison of *Schw. occidentalis* hexokinase to hexokinases from other organisms

Existing databases contain the sequences of the three S. cerevisiae hexose-phosphorylating isoenzymes as well as those of Kluyveromyces lactis hexokinase, Schistosoma mansoni hexokinase, Plasmodium falciparum hexokinase, and several mammalian hexokinases. An alignment of the yeast genes coding for hexose-phosphorylating enzymes showed several conserved regions in all these genes (Fig. 6). The motif LGFTFSYP is conserved in all yeast hexose-phosphorylating enzymes. In the deduced Schw. occidentalis hexokinase this motif is located at amino-acid positions 152 to 159. The motif contains the essential serine residue of the sugar-binding domain (Schwab and Wilson 1988). The region of amino-acid residues 60 to 100 is characterized by a high degree of conservation. The element AxDGxGxGAA is conserved in the C-terminus of all yeast hexokinases. Studies on mammalian hexokinases identified an aspartate (position 210), a threonine (position 214), and two glutamate residues (positions 268 and 301) (Schwab and Wilson 1988). These amino acids are conserved amongst all yeast hexose-phosphorylating enzymes (Fig. 6).

The amino-acid sequences of the Schw. occidentalis hexokinase and the S. cerevisiae hexokinases PI and PII are 71% and 69% identical, respectively. The K. lactis hexokinase is identical to the Schw. occidentalis hexokinase in 68% of its amino-acid residues. Yeast hexokinases are only approximately 40% identical to S. cerevisiae glucokinase. The degree of identify of all yeast hexose-phosphorylating enzymes to the C-terminal domain of rat-brain hexokinase (type I), which retains the catalytical active domain, is roughly 36 to 37% (Table 1). Pairwise comparisons show a close relationship of the yeast hexokinases. Hexose-phosphorylating enzyme sequences from the data banks were used for multiple alignment. All known yeast hexose-phosphorylating enzymes were included in this phylogenetic study. The rat hexokinases were choosen as representatives of the mammalian hexokinases. S. mansoni and P. falciparum hexokinases were also included. A phylogenetic tree was constructed from the multiple alignment data (Fig. 7). The yeast hexokinases form a group of closely related enzymes. Another group of related enzymes in this study consists of the rat hexokinases and the S. mansoni hexokinase. S. cerevisiae glucokinase and P. falciparum hexokinase are not related to any other hexose-phos-

Table 1 Degree of identity by pairwise comparisons of different hexose-phosphorylating enzymes. The percentages of identity were calculated by the programme GAP. The labels SoHXK, KIHXK, ScHXK1, ScHXK2, ScGLK1 and RbHXK mark *Schw. occidentalis* hexokinase, *K. lactis* hexokinase (Prior et al. 1993), *S. cerevisiae* hexokinase PII (Fröhlich et al. 1985), *S. cerevisiae* glucokinase (Albig et al. 1988), and the C-terminal domain of the rat-brain hexokinase (Schwab and Wilson 1988, 1989), respectively

	SoHXK	KIHXK	ScHXK1	ScHXK2	ScGLK1	RbHXK
SoHXK	100%	68%	71%	69%	39%	36%
KIKXK		100%	71%	73%	40%	37%
ScHXK1			100%	76%	41%	37%
ScHXK2				100%	40%	37%
ScGLK1					100%	38%
RbHXK						100%

Fig. 5 DNA sequence and predicted amino-acid sequence of the *Schw. occidentalis* hexokinase

-181 TGAGC -171 -161 -151 -121 -91 -141 -131 -111 -101CCGCGCTTCG ATATGCTGAC AGTGTCCGAA TTTCTTTGTG ACATCACACC ATCAACGAAG TAGAACCAAG TAGTAATTTC GTATATAAAT -81 -71 -61 -51 -41 -31 -21 -11 ATTTANATTT ACCAAACATT TGTCTAAAGA AAAATTTTAA TTCAATTATA TTAAAATTTC TAATAGATT TATTACACAT TAAGCACAAA ATG GTT CAC TTA GGT CCA AAA CCT CCA CAA CAT AGA AAA GGA TCC TTC TTG GAT GTT CCT GAA TAT TTG TTG AAG MET Val His Leu Gly Pro Lys Pro Pro Gln His Arg Lys Gly Ser Phe Leu Asp Val Pro Glu Tyr Leu Leu Lys 150 GAA TTG ACA GAA CTC GAA GGA TTA TTA ACA GTT TCA GGT GAA ACA TTA AGG AAG ATT ACT GAT CAC TTT ATT TCA Glu Leu Thr Glu Leu Glu Gly Leu Leu Thr Val Ser Gly Glu Thr Leu Arg Lys Ile Thr Asp His Phe Ile Ser 225 GAA TTG GAA AAA GGT TTA TCT AAA CAA GGG GGA AAT APT CCT ATG ATT CCA GGA TGG GTT ATG GAC TTC CCA ACA Glu Leu Glu Lys Gly Leu Ser Lys Gln Gly Gly Asn Ile Pro MET Ile Pro Gly Trp Val MET Asp Phe Pro Thr 300 GGA AAA GAA ATG GGT GAT TAC TTG GCT ATT GAT TTA GGT GGT ACT AAT TTG AGA GTT GTT TTA GTT AAG TTA GGT Gly Lys Glu MET Gly Asp Tyr Leu Ala Ile Asp Leu Gly Gly Thr Asn Leu Arg Val Val Leu Val Lys Leu Gly 375 GGT AAC AGG GAC TITT GAC ACT CAA TOO AAG TITO GOA TIG COA GAA AAC ATG AGA ACT GOO AAG TOT GAA GAG Gly Asn Arg Asp Phe Asp Thr Thr Gln Ser Lys Phe Ala Leu Pro Glu Asn MET Arg Thr Ala Lys Ser Glu Glu 450 TTA TEG GAA TIT ATT GET GAG TET TTA CAA AAG TTE GIG GAA GAA TAT CGA AAT GET GIT ETG TEA AAT TTA Leu Trp Glu Phe Ile Ala Glu Cys Leu Gln Lys Phe Val Glu Glu Glu Glu Phe Arg Asn Gly Val Leu Ser Asn Leu 525 CCA TTA GGT TTC ACC TTT TCA TAC CCA GCA TCT CAA GGT TCT ATC AAT GAA GGG TAT TTG CAA AGA TGG ACC AAA Pro Leu Gly Phe Thr Phe Ser Tyr Pro Ala Ser Gln Gly Ser Ile Asn Glu Gly Tyr Leu Gln Arg Trp Thr Lys 600 675 CCA ATT GAA GTT GTT GCG TTA ATC AAT GAC ACC ACA GGT ACT TTA GTT GCT TCT ATG TAC ACC GAT CCA GAA GCT Pro Ile Glu Val Val Ala Leu Ile Asn Asp Thr Thr Gly Thr Leu Val Ala Ser MET Tyr Thr Asp Pro Glu Ala 750 ANA ATG GGT TTA TTT TCC GGT ACT GGT TGT AAT GGT GCT TAC TAC GAT GTT GTC GAT AAC ATT CCA AAA TTA GAA Lys MET Gly Leu Phe Ser Gly Thr Gly Cys Asn Gly Ala Tyr Tyr Asp Val Val Asp Asn Ile Pro Lys Leu Glu 825 GGA AAG GTT CCA GAT GAC ATT AAA AGC TCT TCC CCA ATG GCC ATC AAC TGT GAA TAC GGT GCT TTC GAT AAT GAG Gly Lys Val Pro Asp Asp Ile Lys Ser Ser Pro MET Ala Ile Asn Cys Glu Tyr Gly Ala Phe Asp Asn Glu 900 CAT ATC ATT TTG CCT AGA ACT AAA TAC GAT ATC CAA ATC GAT GAA GAA TCA CCA AGA CCA GGA CAA CAG GCT TTC His Ile Ile Leu Pro Arg Thr Lys Tyr Asp Ile Gln Ile Asp Glu Glu Ser Pro Arg Pro Gly Gln Gln Ala Phe 975 GAA AAG ATG ATC TCT GGT TAC TAC TTA GGT GAA GTT TTA AGA TTG ATT TTA CTT GAT TTA ACC TCT AAA CAA TTA Glu Lys MET Ile Ser Gly Tyr Tyr Leu Gly Glu Val Leu Arg Leu Ile Leu Asp Leu Thr Ser Lys Gln Leu 1050 ATT TTC AAA GAC CAA GAT TTG TCT AAA TTA CAA GTT CCA TTC ATT TTA GAT ACC TCA ATC CCA GCT AGA ATT GAA Ile Phe Lys Asp Gln Asp Leu Ser Lys Leu Gln Val Pro Phe Ile Leu Asp Thr Ser Ile Pro Ala Arg Ile Glu 1125 GAA GAT CCG TTT GAA AAC TTA TCT GAT GTC CAA GAA TTA TTT CAA GAA ATT TTA GGT ATT CAA ACT ACT TCT CCA Glu Asp Pro Phe Glu Asn Leu Ser Asp Val Gln Glu Leu Phe Gln Glu Ile Leu Gly Ile Gln Thr Thr Ser Pro 1200 GAA AGA AAA ATC ATC CGT CGT CTA GCG GAA TTG ATC GGT GAA AGA TCA GCC AGA TTA TCA ATT TGT GGT ATT GCT Glu Arg Lys Ile Ile Arg Arg Leu Ala Glu Leu Ile Gly Glu Arg Ser Ala Arg Leu Ser Ile Cys Gly Ile Ala 1275 GCT ATT TGC AAG AAG AGA GGC TAC AAA ACC GCT CAT TGT GCC GCT GAT GGT TCA GTC TAC AAC AAA TAC CCA GGT Ala Ile Cys Lys Lys Arg Gly Tyr Lys Thr Ala His Cys Ala Ala Asp Gly Ser Val Tyr Asn Lys Tyr Pro Gly 1350 TTC AAA GAA AGA GCT GCT AAA GGT TTG AGA GAT ATC TTT CAA TGG GAA TCT GAA GAA GAT CCA ATT GTC ATT GTG Phe Lys Glu Arg Ala Ala Lys Gly Leu Arg Asp Ile Phe Gln Trp Glu Ser Glu Glu Asp Pro Ile Val Ile Val 1425 CCT GCA GAA GAT GGT TTA GGT GCA GGT GCC GCT ATC ATT GCT GCA TTG ACT GAA AAA AGA TTA AAG GAT GGA TTA Pro Ala Glu Asp Gly Leu Gly Ala Gly Ala Ala Ile Ile Ala Ala Leu Thr Glu Lys Arg Leu Lys Asp Gly Leu 1450 1460 1470 1480 1490 1500 1440 1510 AGA AAACTAATGA TTACGATTGA AATTCTAAAA ATGCTTTCGT TCAATTTCTG GATGTCAATT TACTAGCTGG CCG TTG GTA TGA Pro Leu Val 1520 1530 1540 1550 1560 1570 1580 1590 1600 TTTATTACTT CATTGETATTA ACTTTTTTAC TTCTTTATAT CTAATCCTTG TAAGAATGETT TCGACGATAA AGTTTTATAA AATTATCGAA 1610 1620 1630 1640 1650 1660 1670 1680 TGACTTTAAA TATAAATGAT TTCTATTTIG CATATTGATA GTAGAGTACA GATATAGAAA TTAGCTTTA CCATAAGACA AGCTT

phorylating enzyme included in the phylogenetic tree (Fig. 7).

stein 1983). This technique was also applied to disrupt a *Schw. occidentalis* gene (Dohmen et al. 1990).

Disruption of the Schw. occidentalis HXK gene

In *S. cerevisiae* chromosomal DNA sequences were replaced by in vitro-constructed DNA fragments. After transformation, these fragments were integrated at the target sequence by the homologous recombination system (RothA plasmid containing a deletion in the Schw. occidentalis hexokinase open reading frame was constructed. This plasmid contained only a deletion of the open reading frame. The deleted sequence was not substituted by a transformation marker for Schw. occidentalis. A restriction fragment containing the deletion in the hexokinase open reading frame was transformed into Schw. occidentalis. Hexokinase disruption mutants were selected by plating Fig. 6 Multiple alignment of amino-acid sequences of yeast hexose-phosphorylating enzymes using of the program CLUSTAL. The labels SOHXK, KLHXK, SCHXK1, SCHXK2 and SCGLK1 refer to Schwanniomyces occidentalis hexokinase, Kluyveromyces lactis hexokinase (Prior et al. 1993), Saccharomyces cerevisiae hexokinase PI (Kopetzki et al. 1985), Saccharomyces cerevisiae hexokinase PII (Fröhlich et al. 1985), and Saccharomyces cerevisiae glucokinase (Albig et al. 1988), respectively

SOHXK MVHLGPKPPQHRKGSFLDVPEYLLKELTELEGLLTVSGETLRKITDHFISELEKGLSK-OGGN------KL'HXK MVRLGPKKPPARKGSMADVPANLMEQIHGLETLFTVSSEKMRSIVKHFISELDKGLSK-KGGN-----SCHXK1 MVHLGPKKPQARKGSMADVPKELMDEIHQLEDMFTVDSETLRKVVKHFIDELNKGLTK-KGGN------SCHXK2 MVHLGPKKPQARKGSMADVPKELMQQIENFEKNFTVPTETLQAVTKHFISELEKGLSK-KGGN------SCGLK1 MSFDDLHKATER-----AVIQAVDQICDDFEVTPEKLDELTAYFIE0MEKGLAPPKEGHTLASDKG Mv lgpkkp Rkgs dvp l е tV É hFI EL KGL k SOHXK I PMI PGWVMDF PTGK EMGDYLAIDLGGTNI.RVVLVKLGGNRDFDTTOSK FAL PFNMRTAKS---FFT MFF KLHXK IPMIPGWVVEYPTGKETGDFLALDLGGTNLRVVLVKLGGNHDFDTTQNKYRLPDHLRTGTS---EQLWSF SCHXK1 IPMIPGWVMEFPTGKESGNYLAIDLGGTNLRVVLVKLSGNHTFDTTOSKYKLPHDMRTTKH--OEELWSF SCHXK2 IPMIPGWVMDFPTGKESGDFLAIDLGGTNLRVVLVKLGGDRTFDTTQSKYRLPDAMRTTON--PDELWEF SCGLK1 iPMIPawV PtGKE G LA DLGGTN1Tvv1VkL G FdttO K 1P Lw F IAECLQKFVEEEFRNGVLS-----NLPLGFTFSYPASQGSINEGYLQRWTKGFDIEGVEGHDVVPMLQAA SOHXK KUHXK IAKCLKEFVDEWYPDGVSE----PLPLGFTFSYPASQKKINSGVLQRWTKGFDIEGVEGHDVVPMLQEQ IADSLKDFMVEQELLNTKD----TLPLGFTFSYPASQNKINEGILQRWTKGFDIPNVEGHDVVPLLQNE SCHXK1 IADSLKAFIDEQFPQGISE----PIPLGFTFSFPASQNKINEGILQRWTKGFDIPNIENHDVVPMLQKQ SCHXK2 ${\tt LARRTLAFM} KKYHPDELAKGKDAKPMKLGFTFSYPVDQTSLNSGTLIRWTKGFRIADTVGKDVVQLYQEQ$ SCGLK1 e hDVVp 10 iA l F e pLGFTFSYPasQ iN G LqRWTKGFdI SOHXK IEKRKVP-IEVVALINDTTGTLVASMYTD----PEAKMGLFSGTGCNGAYYDVVDNIPKLEGK KLHXK IEKLNIP-INVVRLINDTTGTLVASLYTD----POTKMGIIIGTGVNGAYYDVVSGIEKLEGL ISKRELP-IEIVALINDTVGTLIASYYTD-----PETKMGVIFGTGVNGAFYDVVSDIEKLEGK SCHXK1 ITKRNIH-IEVVALINDTTGTLVASYYTD-----PETKMGVIFGTGVNGAYYDVCSDIEKLQGK SCHXK2 SCGLK1 LSAQGMPMIKVVALTNDTVGTYLSHCYTSDNTDSMTSGEISEPVIGCIFGTGTNGCYMEEINKITKLPQE i k T V LINDT GTL as YTd p kmG GTG NGa vdv ТКЬ SOHXK VPDDI--KSSSPMAINCEYGAFDNEHIILPRTKYDIQIDEE-SPRPGQQAFEKMISGYYLGEVLRLILLD KLHXK LPEDI--GPDSPMAINCEYGSFDNEHLVLPRTKYDVIIDEE-SPRPGOOAFEKMTSGYYLGEIMRLVLLD LADDI--PSNSPMAINCEYGSFDNEHLVLPRTKYDVAVDEQ-SPRPGQQAFEKMTSGYYLGELLRLVLLE SCHXK1 SCHXK2 $\label{eq:lsddi-ppsapmainceygsfdnehvvlprtkyditidee-sprpgqtfekmssgyylgeilrlalmdditidee-sprpgqtfekmsgyylgeilrlalmditidee-sprpgqtfekmsgyylgeilrlalmditidee-sprpgqtfekmsgyylgeilrlalmditi$ SCGLK1 LRDKLIKEGKTHMIINVEWGSFDNELKHLPTTKYDVVIDQKLSTNPGFHLFEKRVSGMFLGEVLRNILVD pMaINcEyg FDNEh LPrTKYD De SprPGqq FEKm SGyyLGE R1 L di SOHXK LTSKQLIFKD----QDLSK-LQVPFILDTSIPARIEEDPFENLSDVQELFRRNFRYSKTTSPERKIIRRL KUHXK LYDSGFIFKD----ODISK-LKEAYVMDTSYPSKIEDDPFENLEDTDDLFKTNLNI-ETTVVERKLIRKL LNEKGLMLKD----QDLSK-LKQPYIMDTSYPARIEDDPFVFLEDTDDIFQKDFGV-KTTLPERKLIRRL SCHXK1 MYKOGFIFKN----ODLSK-FDKPFVMDTSYPARIEEDPFENLEDTDDLFONEFGI-NTTVOERKLIRRL SCHXK2 SCGLK1 LHSQGLLLQQYRSKEQLPRHLTTPFQLSSEVLSHIEIDDSTGLRETELSLLQSLRLP-TTPTERVQ1QKL dtsp IE Dpf Ld k ad sk f TT ERk Ir L SOHXK AELIGERSARLSICGIAAIC-----KKRGYKTAHCAADGSVYNKYPGFKERAAKGLRDIFOWES---EE KLHXK AELVGTRAARLTVCGVSAIC-----DKRGYKTAHIAADGSVFNRYPGYKEKAAQALKDIYNWDVEKMED CELTGTRAARLAVCGIAAIC-----QKRGYKTGHIAADGSVYNKYPGFKEAAAKGLRDIYGWTGDASKD SCHXK1 SELIGARAARLSVCGTAAIC-----OKRGYKTGHIAADGSVSTRYPGEKEKAANALKDIYGWTOPHLDD SCHXK2 SCGLK1 VRAISRRSAYLAAVPLAAILIKTNALNKRYHGEVEIGCDGSVVEYYPGFRSMLRHALA---LSPLGAEGE elgRArL cg AIc KRgykt h aaDGSV YPG ke aa L di w SOHXK DPIVIVPAEDGLGAGAAIIAALTEKRLKDGLPL----V KLHXK HPIQLVAAEDGSGVGAAIIACLTQKRLAAGKSVGIKGE SCHXK1 - PITIVPAEDGSGAGAAVIAALSEKRIAEGKSLGIIGA SCHXK2 YPIKVVPAEDGSGPGAAVIAALAQKRIAEGKSVGIIGA SCGLK1 RKVHLKIAKDGSGVGAALCALVA----pi v AeDG G GAA iA l kr g

out the transformants on a medium composed of starch and 2-deoxyglucose.

This selection system was used by McCann et al. (1987) to isolate glucose-repression mutants of *Schw. occidentalis*. In this selection they identified a hexokinase mutant which had a pleiotropic defect in the glucose repression of sugar-utilizing enzymes (McCann et al. 1987). Starch utilization in *Schw. occidentalis* is glucose repressible. Glucose-repression mutants show a high starch-hydrolysing activity (McCann et al. 1987). Glucose released from starch competes with 2-deoxyglucose for transport into the cell. Glucose-repression mutants survive on this medium; wild-type cells die because of the cytotoxicity of the 2-deoxyglucose taken up.

A Schw. occidentalis hexokinase deletion mutant was selected on a starch/2-deoxyglucose medium. This mutant had lost almost all fructose-phosphorylating activity. The glucose-phosphorylating activity was reduced by 50%. Activities of maltase and invertase increased in the hexokinase mutant (Table 2).

Expression of the Schw. occidentalis hexokinase in S. cerevisiae

The data of McCann et al. (1987) showed the participation of *Schw. occidentalis* hexokinase in glucose repression. These data were confirmed by the *hxk* deletion mutant. De-



Fig. 7 Phylogenetic tree of hexokinases. Included are all sequenced hexokinases apart from the mammalian hexokinases which were represented by the rat hexokinases I to IV (Magnusson and Shelton 1989; Schwab and Wilson 1989, 1991; Thelen and Wilson 1991). From the tandem-duplicated mammalian hexokinases I to III, only the C-terminal half representing the catalytic domain was included in the comparisons. The origins of the yeast sequences are quoted in the legend of Fig. 6. The sequences of *P. falciparum* and *S. mansoni* hexokinases were published by Olaffson et al. (1992) and Shoemaker et al. (1992), respectively. The evolutionary relatedness of the hexokinases is proportional to the length of the *horizontal bars*. The figure was prepared by the programme TREE

Table 2 Specific activities of the *Schw. occidentalis* wild-type and hxk mutant strain (nmol/min×mg)

Strain	Hexokinase		Maltase	Invertase
	Glucose	Fructose		
Wild type	151	197	1	99 2042
nak mutam	70	0	15	2012

tailed studies on the effect of hexose phosphorylation on glucose repression were performed in S. cerevisiae (Rose et al. 1991). This test system was used for heterologous expression of the Schw. occidentalis hexokinase. A fragment containing the complete HXK gene was cloned into plasmids YEp24 (Botstein et al. 1979) and YCp50 (Rose et al. 1987), respectively. The fragment contained the original promoter, which allowed expression of the Schw. occidentalis hexokinase in S. cerevisiae (Table 3). The hexokinase-deficient strain WAY.7-2D/2A was transformed by a plasmid carrying the Schw. occidentalis HXK gene. Only low hexokinase activities were measured in transformants carrying the YCp50 derivative. Compared to the untransformed S. cerevisiae strain, maltase and invertase activities were decreased in the transformant. Higher hexokinase activity was measured after transformation of strain WAY.7-2D/2A with the derivative of the episomal plasmid YEp24. Maltase activity in this transformant was decreased by a factor of ten compared to the untransformed strain. The invertase activity was decreased by a factor of seven. The glucose-repression defect of a hexokinase-negative S. cerevisiae strain was at least in part compensated by the expression of Schw. occidentalis hexokinase.

Discussion

The hexokinase of *Schw. occidentalis* was purified by using an established protocol for the purification of *S. cerevisiae* hexokinases. During the purification, differences in stability became obvious. *Schw. occidentalis* hexokinase was unstable under the conditions used to elute the enzyme from the chromatofocussing column. Proteins elute from the chromatofocussing column at the isoelectric point. A severe decrease in enzyme activity could be overcome by changing the pH immediately after elution from the chromatofocussing column. This was achieved by filling the tubes in the fraction collector with double the expected fraction volume of untitrated piperazine buffer. Even under these conditions, the pooled fractions had to be applied immediately on the hydrophobic interaction chromatography column to obtain a high yield of enzyme activity.

The elution of glucokinase from the chromatofocussing column was never recognized when *S. cerevisiae* hexosephosphorylating enzymes were purified under the same conditions (data not shown). *Schw. occidentalis* glucoki-

Table 3 Specific activities of a hexokinase-negative *S. cerevisiae* strain expressing *Schw. occidentalis* hexokinase (nmol/min×mg)

Strain	Plasmid	Hexokinase		Maltase	Invertase
		Glucose	Fructose		
WAY.7-2D/2A	<u> </u>	124	7	655	1410
WAY.7-2D/2A	pMR164(YCp50)	244	145	167	890
WAY.7-2D/2A	pMR188(YEp24)	482	892	69	204

nase was more stable under the purification conditions than the *S. cerevisiae* enzyme. The data gave no direct hint as to whether the *S. cerevisiae* glucokinase was lost during the DEAE anion exchange or at the chromatofocussing step.

The complete separation of Schw. occidentalis glucokinase and hexokinase was achieved by additional hydrophobic interaction chromatography. This demonstrated the existence of distinct isoenzymes in Schw. occidentalis. The K_M values determined for the purified hexokinase were higher than the values previously published (McCann et al. 1987). For their experiments McCann et al. used mixtures of hexokinase and glucokinase enzymes. They determined the overall values in the hxk mutant strain which contained only glucokinase, and that of the wild-type strain which contained both glucokinase and hexokinase. They calculated the K_M for glucose from both determinations. For phosphorylation of glucose by Schw. occidentalis hexokinase I obtained a K_M of 0.98 mM. This value differs by a factor of approximately two from that reported by McCann et al. The difference in the K_M values for fructose is, however, not that severe. These K_M values were about one order of magnitude higher compared to those determined for S. cerevisiae hexokinases PI and PII (see McCann et al. 1987; Rose et al. 1991).

The Schw. occidentalis HXK gene was cloned by hybridization to a S. cerevisiae HXK2 probe. The DNA sequence revealed an open reading frame of 479 codons. The deduced protein has a molecular weight of 53.4 kDa. Compared to these data, the S. cerevisiae hexokinase PI (53.7 kDa; Kopetzki et al. 1985) and hexokinase PII (53.9 kDa; Fröhlich et al. 1985) are only slightly larger. The Schw. occidentalis hexokinase showed a high degree of identical and conserved amino-acid residues when compared to hexokinases from other organisms (Fig. 6). The sugar-binding consensus (amino-acid residues 152 to 159; Schwab and Wilson 1988) is conserved in all these hexokinases. The amino-acid residues 60-100 and a AxDGxGxGAA motif near the C-terminus are conserved in the Schw. occidentalis hexokinase. These domains might be involved in nucleotide binding (Hol 1985; Schwab and Wilson 1988). Several of the amino acids in positions 160 to 200 are also conserved. These amino acids might additionally contribute to the catalytic active structure of the enzyme. A reciprocal exchange of S. cerevisiae hexokinase PI and PII amino-acid residues 101 to 246 altered the substrate affinities and velocities of these isoenzymes (Rose et al. 1991). It was concluded from these results that the essential amino-acid residues involved in the catalysis of hexose phosphorylation are located in a well-conserved part (residues 100-250) of the enzyme.

Amino-acid sequence comparisons revealed an approximately 70% identity of the *Schw. occidentalis* hexokinases to all other known yeast hexokinases (Table 1). Even *S. cerevisiae* hexokinase PI and PII are only 76% identical in their amino-acid sequences. The *S. cerevisiae* glucokinase (Albig and Entian 1988) and the catalytic domain of therat-brain hexokinase (Schwab and Wilson 1988) share less than 40% amino acids with the *Schw. occidentalis* hexokinase. For detecting evlutionary relationships of hexokinases, a phylogenetic tree was prepared (Fig. 7). A close relationship was obtained among the Schw. occidentalis, K. lactis and S. cerevisiae hexokinases. The S. cerevisiae hexokinases are more closely related to each other than to any other yeast hexokinase. A duplication of the hexokinase gene in S. cerevisiae probably occurred after separation from a common ancestral hexokinase shared by Schw. occidentalis, K. lactis and S. cerevisiae. Among these three yeasts, only S. cerevisiae possesses two hexokinase isoenzymes, whereas K. lactis (Prior et al. 1993) and Schw. occidentalis both have only one hexokinase. For the Schw. occidentalis hexokinase the evolutionary distance to both S. cerevisiae hexokinases is equal. The rat hexokinases show a close relationship. S. mansoni hexokinase is also included in this group. S. cerevisiae glucokinase shares only a very low degree of relationship to the mammalian hexokinases and to the yeast hexokinases, respectively. The evolution of the yeast glucokinase has diverged very early from a common ancestral enzyme.

Disruption of the Schw. occidentalis HXK gene resulted in a mutant with a lack in the glucose repression of maltase, invertase and starch-metabolizing enzymes. The mutant was unable to grow on fructose as carbon source. The fructose-phosphorylating activity in this mutant was near the point of detection, whereas glucose phosphorylation was reduced by 50%. This again displayed the existence of a glucokinase and a single hexokinase in Schw. occidentalis. The amount of glucose-phosphorylating activity covered by the hexokinase is approximately 50%. The other 50% is covered by glucokinase. In S. cerevisiae 85% of the glucose-phosphorylating activity is contributed by hexokinases (Rose et al. 1991). In contrast to the aforementioned yeasts Schw. occidentalis and S. cerevisiae, K. lactis contains no glucokinase (Prior et al. 1993).

Schw. occidentalis hxk mutants displayed a pleiotropic lack of glucose repression (Table 2; McCann et al. 1987). An active hexokinase is necessary in Schw. occidentalis for triggering glucose repression. This is consistent with the data obtained from S. cerevisiae (Rose et al. 1991). The Schw. occidentalis hexokinase was expressed in a hexokinase-negative S. cerevisiae strain. This heterologous expression was achieved from the original promoter of the Schw. occidentalis hexokinase gene. Transformed S. cerevisiae strains were complemented for their lack of growth on fructose as a sole carbon source. Enzymatic activities measured in the transformants revealed a degree of expression of Schw. occidentalis hexokinase in relation to its copy number. Maltase and invertase activities decreased in the transformants compared to the untransformed S. cerevisiae strain. The expression of Schw. occidentalis hexokinase in a hexokinase-negative S. cerevisiae strain complemented the lack in glucose repression. S. cerevisiae strains which expressed different hexokinase activities indicated the importance of hexokinase in glucose repression. A low hexokinase activity resulted in a failure of glucose repression. The reduced expression of hexokinase PII, or the over-expression of hexokinase PI resulted in consistent activities essential for intact glucose repression. A complementation

of the lack in glucose repression of hexokinase-negative strains was not achieved by over-expression of glucokinase (Rose et al. 1991). Even the *Schw. occidentalis* hexokinase was able to restore glucose repression in *S. cerevisiae*.

Expression of the K. lactis RAG5 encoding its sole hexokinase in a hexokinase-negative S. cerevisiae strain had no effect on glucose repression (Prior et al. 1993). In K. lactis there might be a slightly different function for hexokinase, which is probably the only hexose-phosphorylating enzyme. Little is known about glucose repression in K. lactis. Some data are available on β -galactosidase, but a dependence on the genetic background of this strain has been reported (Breunig 1989). The mechanism of glucose repression might be different in K. lactis.

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