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Novel alleles of yeast hexokinase PII with distinct effects on catalytic activity and catabolite repression of *SUC2*

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In the yeast *Saccharomyces cerevisiae*, glucose or fructose represses the expression of a large number of genes. The phosphorylation of glucose or fructose is catalysed by hexokinase PI (Hxk1), hexokinase PII (Hxk2) and a specific glucokinase (Glk1). The authors have shown previously that either Hxk1 or Hxk2 is sufficient for a rapid, sugar-induced disappearance of catabolite-repressible mRNAs (short-term catabolite repression). Hxk2 is specifically required and sufficient for long-term glucose repression and either Hxk1 or Hxk2 is sufficient for long-term repression by fructose. Mutants lacking the *TPS1* gene, which encodes trehalose 6-phosphate synthase, can not grow on glucose or fructose. In this study, suppressor mutations of the growth defect of a *tps1*Δ *hxk1*Δ double mutant on fructose were isolated and identified as novel *HXK2* alleles. All six alleles studied have single amino acid substitutions. The mutations affected glucose and fructose phosphorylation to a different extent, indicating that Hxk2 binds glucose and fructose via distinct mechanisms. The mutations conferred different effects on long- and short-term repression. Two of the mutants showed very similar defects in catabolite repression, despite large differences in residual sugar-phosphorylation activity. The data show that the long- and short-term phases of catabolite repression can be dissected using different hexokinase mutations. The lack of correlation between *in vitro* catalytic hexokinase activity, *in vivo* sugar phosphate accumulation and the establishment of catabolite repression suggests that the production of sugar phosphate is not the sole role of hexokinase in repression. Using the set of six *hxk2* mutants it was shown that there is a good correlation between the glucose-induced cAMP signal and *in vivo* hexokinase activity. There was no correlation between the cAMP signal and the short- or long-term repression of *SUC2*, arguing against an involvement of cAMP in either stage of catabolite repression.

Keywords: hexokinase, catabolite repression, sugar phosphorylation, cAMP, yeast

INTRODUCTION

The addition of glucose or fructose to yeast cells growing on a non-fermentable carbon source such as ethanol causes a global switch in metabolism from gluconeogenesis/respiration to glycolysis/fermentation (Zimmermann & Entian, 1997; Gancedo, 1998). Cells performing gluconeogenesis are catabolite derepressed, while fermenting cells are catabolite repressed. The transition from the derepressed to the repressed state is achieved by altering the activity and stability of enzymes and by switching the expression of a large number of genes on or off (Gancedo & Gancedo, 1997). For instance, the half-life of mRNAs encoding enzymes involved in the utilization of sucrose and in respiration

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Abbreviation: YP, yeast extract/peptone.

drops dramatically after glucose addition (Cereghino & Scheffler, 1996). Subsequently, their transcription is shut down by carbon-catabolite repression (Ronne, 1995; Gancedo, 1998). The key components of the catabolite-repression pathway are the Snf1/Cat1 protein kinase and the protein phosphatase type 1, Glc7 (Gancedo, 1998; Hardie *et al.*, 1998). Snf1/Cat1 appears to control transcriptional regulatory proteins such as the Mig1 and Mig2 repressors (Ronne, 1995; Gancedo, 1998).

Beyond the long-known requirement of hexokinase PII activity for glucose repression (Entian, 1980), little is known about the actual sugar-sensing mechanism. Baker's yeast has three glucose- or fructose-phosphorylating enzymes: the hexokinases PI (Hxk1) and PII (Hxk2), which phosphorylate glucose and fructose (Lobo & Maitra, 1977), and glucokinase (Glk1), which is specific for glucose (Maitra & Lobo, 1983). Since only mutation of *HXK2*, but not of *HXK1*, leads to a loss in glucose repression, a unique role had been ascribed to Hxk2 in triggering glucose repression (Entian, 1980; Entian & Fröhlich, 1984; Entian *et al.*, 1984). Subsequent work has shown, however, that both Hxk1 and Hxk2 contribute to glucose and fructose repression (Hohmann, 1987; Rose *et al.*, 1991; de Winde *et al.*, 1996). Moreover, expression of *HXK1* and *GLK1* is glucose repressible (Sierkstra *et al.*, 1992; Herrero *et al.*, 1995; de Winde *et al.*, 1996), providing a simple explanation for the predominant role of Hxk2 in maintaining glucose repression.

Initially, it had been proposed that Hxk2 has a specific role in signalling besides its catalytic function (Entian & Fröhlich, 1984). Subsequently, a good inverse correlation between hexokinase activity and the degree of catabolite repression has been reported (Ma *et al.*, 1989; Rose *et al.*, 1991) suggesting that the level of sugar phosphates might trigger catabolite repression. This would imply the existence of a system that senses the level of such metabolites, but despite extensive genetic analysis of catabolite repression and glycolytic regulation no such sensor has been found (Ronne, 1995; Zimmermann & Entian, 1997; Gancedo, 1998).

Yeast *tps1* mutants are deficient in growth on rapidly fermented sugars like glucose and fructose because of an unrestricted influx of sugar into glycolysis, which leads to a hyperaccumulation of sugar phosphates and depletion of ATP and phosphate (Thevelein & Hohmann, 1995). Apparently these mutants lack a feedback control of glycolysis on hexokinase activity. The precise regulatory mechanism is not well understood (Blázquez *et al.*, 1993; Hohmann *et al.*, 1996; Ernandes *et al.*, 1998). Reduction of hexokinase activity by deletion of *HXK2* restores growth of the *tps1*Δ mutant on glucose, but not on fructose (Hohmann *et al.*, 1993).

In this work we have exploited the growth defect of the *tps1*Δ *hxk1*Δ mutant on fructose (Hohmann *et al.*, 1993; Van Aelst *et al.*, 1993) in a search for novel regulators of glycolysis and sugar-induced signalling. Unexpectedly, all the mutations studied define alleles of *HXK2* with interesting novel properties. For the first time, mutant alleles of *HXK2* are described in which the capacity to phosphorylate glucose or fructose is differentially affected. We also describe two mutant alleles that confer very different *in vitro* and *in vivo* sugar kinase activity while causing very similar defects in catabolite repression.

METHODS

Strains and growth conditions. The yeast strains used were all isogenic to W303-1A (Thomas & Rothstein, 1989). The construction of the deletions of *TPS1* (Hohmann *et al.*, 1993), *HXK1*, *HXK2* and *GLK1* (Rose *et al.*, 1991) have been described previously and the mutant strains in the W303-1A background have been listed elsewhere (de Winde *et al.*, 1996). Strains were grown on standard yeast extract/peptone (YP) media or yeast nitrogen base/ammonium sulphate media supplemented with 2% carbon source, as indicated (Sherman *et al.*, 1983).

Mutant isolation. For the isolation of suppressor mutations, strain YSH 311 (*MAT*a *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2 tps1*Δ::*TRP1 hxk1*Δ::*HIS3*) was grown to saturation in YP plus 2% galactose and approximately 2×10^7 cells per plate were spread onto YP medium plus 2% fructose. Colonies appearing after 3–5 d were spread again on the same medium in order to obtain single colonies. Strains YSH 369 (*MAT*α *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2 tps1*Δ::*LEU2 hxk1*Δ::*HIS3*) and YSH 6.59.-4A (*MAT*α *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2 hxk2*Δ::*LEU2*) were used for genetic analysis. Crossings and tetrad analysis were done according to standard procedures (Sherman *et al.*, 1983).

Sequence analysis. To determine mutations in the different *HXK2* alleles, fragments covering the complete gene were amplified using standard PCR amplification of total genomic DNA. Sequence data were obtained by analysis of three independently cloned PCR fragments and were confirmed by direct sequence determination of the different PCR products (T7 sequenase kits, USB-Amersham).

Blotting techniques. SDS-PAGE was performed on 10% separating gels as described by Laemmli (1970). Immunoblotting was performed according to Towbin *et al.* (1979) using an antiserum raised against commercially available purified hexokinase (Boehringer Mannheim) as the primary antibody at a 1/10000 dilution and peroxidase-labelled goat anti-rabbit IgG as the secondary antibody at a 1/2000 dilution. Northern-blot analysis was performed essentially as described by Crauwels *et al.* (1997).

Biochemical analyses. For determination of specific hexokinase activity, cells were grown in YP medium supplemented with 4% of either glucose or fructose and harvested in the late-exponential growth phase. Crude extracts were prepared in 100 mM potassium phosphate buffer pH 6·5 and the activity was measured as described by Lobo & Maitra (1977). To measure specific invertase activity, cells were grown and treated as for the hexokinase assay and the activity was determined according to Goldstein & Lampen (1975). For cAMP determination, cells were grown on YP medium supplemented with 3% glycerol and 0·1% galactose until late-exponential phase. Incubation with glucose and fructose (100 mM each) and quantification of cAMP followed our established protocol (Thevelein *et al.*, 1987). The same culturing regime was used for cells in which the levels of glycolytic metabolites were determined according to

de Koning & van Dam (1992). For the determination of ethanol production, cells were grown in 100 ml medium in 250 ml Erlenmeyer flasks in a shaker at 300 r.p.m. Samples were taken at different time points and the ethanol in the medium supernatant was determined using the test combination ethanol kit from Boehringer Mannheim.

Sugar transport. The amount of ^{14}C-labelled glucose or fructose taken up within 5 s was measured in exponentially growing cells as described previously (Luyten *et al.*, 1993).

Reproducibility of data. All experiments were performed at least in triplicate from independent cultures. These different experiments gave consistent trends, i.e. the differences between strains were highly reproducible. The absolute values for enzyme activities, metabolite concentration and relative mRNA levels varied between different independent experiments by not more than 30%. The results from representative experiments are shown.

RESULTS

Isolation of *tps1Δhxk1Δ* suppressors

We have shown previously that deletion of the *HXK2* gene suppresses the growth defect of a *tps1Δ* mutant on glucose but not on fructose medium (Hohmann *et al.*, 1993). We have also observed that most of the spontaneous suppressor mutations of a *tps1Δ* mutant isolated on glucose medium are either allelic to *HXK2* or cause a petite phenotype (unpublished observations and Blázquez & Gancedo, 1995). To easily identify petite mutants, we made use of the fact that they do not develop the typical red colour in an *ade2* background like W303-1A. For the isolation of suppressors we used a *tps1Δ hxk1Δ* double mutant, which has a growth defect on glucose and fructose that is indistinguishable from that of the *tps1Δ* single mutant (Hohmann *et al.*, 1993). In a *tps1Δ hxk1Δ* background, additional mutations that strongly diminish the activity of Hxk2 can be recognized easily since the resulting strain should not grow on fructose medium when respiration is blocked by antimycin A (Lobo & Maitra, 1977).

We isolated 128 fructose-positive red mutants from the W303-1A derivative YSH 311 (*MATa tps1Δ::TRP1 hxk1Δ::HIS3*). All but 12 of these mutants failed to grow on fructose medium containing the respiration inhibitor antimycin A and were thus likely to have strongly reduced Hxk2 activity. The remaining 12 mutations segregated 2:2 for suppression of the growth defect of the *tps1Δ::LEU2 hxk1Δ::HIS3* strain, showing that this phenotype was due to a single nuclear mutation in each case. When crossed with an isogenic *hxk2Δ::LEU2* strain, the new mutations cosegregated with the *hxk2Δ* allele in all 8–12 complete tetrads tested for each mutant, suggesting allelism with *HXK2*. Since all putative *tps1Δ hxk1Δ hxk2* spores could grow well on fructose plus antimycin, it appeared that the mutants still retained sufficient hexokinase activity to permit fructose fermentation. Consistent with this, the new *hxk2* mutations were unable to suppress the growth defect of the *tps1Δ* mutant on fructose in the presence of a wild-type *HXK1* gene, probably because hexokinase activity was too high in such strains.

Table 1. Mutations found in the *HXK2* alleles isolated as suppressors of the growth defect of a *tps1Δ hxk1Δ* strain on fructose

Allele	Mutation
hxk2-36	Pro–160 → Ala
hxk2-37	Ala–132 → Pro
hxk2-39	Asp–343 → Glu
hxk2-53	Asp–179 → Gly
hxk2-97	Tyr–346 → Asp
hxk2-129	Glu–456 → Gly

Suppression of the *tps1Δ hxk1Δ* growth defect by the new *hxk2* alleles is most probably due to diminished hexokinase activity, and not to some other property of the mutant Hxk2. When some of the new alleles (nos 39, 53 and 97, see below) were cloned and expressed from a multi-copy plasmid, they conferred hexokinase activity 5–10 times higher than that of an untransformed wild-type and consequently failed to suppress a *tps1Δ* mutant (results not shown). Also the semi-dominant character exibited by all the novel *hxk2* mutations with respect to suppression of a *tps1Δ* mutation (data not shown), which is also apparent for a deletion of *HXK2*, is probably due to reduction of the specific hexokinase activity in the heterozygous diploids. All the heterozygous diploids tested, including the *HXK2/hxk2Δ* strain, had a specific hexokinase activity between 60 and 70% of the wild-type.

Analysis of novel *HXK2* alleles

All further analyses were done in strains that carried a wild-type *TPS1* gene. The 12 mutants fell into six distinct groups with respect to the specific hexokinase activity and their ability to mediate catabolite repression of invertase activity (data not shown). One typical mutant from each group was analysed further. The *HXK2* gene from each of the mutant strains carried one nucleotide change leading to a substitution of one amino acid (Table 1). The cloned alleles 39, 53 and 97 were retransformed into yeast on multi-copy plasmids. None of these alleles was able to confer the same high hexokinase activity as found in a strain transformed with the same plasmid carrying the wild-type *HXK2* gene (data not shown).

Table 2 shows the specific hexokinase activity conferred by the six selected *HXK2* alleles and that of control strains. Deletion of *HXK1* and/or *GLK1* caused only a marginal reduction in the specific hexokinase activity, consistent with the predominant expression of *HXK2* during growth on glucose. On fructose medium, *HXK1* is also expressed to a moderate extent (de Winde *et al.*, 1996). All six *HXK2* mutant alleles caused diminished specific hexokinase activity, although to a very different extent. Interestingly, fructose phosphorylation was more severely affected than glucose phosphorylation in mutants *hxk1Δ hxk2-36 glk1Δ* and *hxk1Δ hxk2-53 glk1Δ*. Note that mutant *hxk1Δ hxk2-39 glk1Δ* conferred

Table 2. Hexokinase and invertase activities in strains containing specific hexokinase mutations

Strain (relevant genotype)	Growth on glucose				Growth on fructose			
	Hxk activity (fructose)*	Hxk activity (glucose)*	F/G ratio†	Invertase activity*	Hxk activity (fructose)*	Hxk activity (glucose)*	F/G ratio†	Invertase activity*
HXK1 HXK2 GLK1	660	660	1·00	70	690	630	1·10	80
hxk1Δ HXK2 GLK1	630	660	1·05	70	620	640	0·97	80
HXK1 hxk2Δ GLK1	490	210	2·33	1200	310	120	2·58	300
hxk1Δ hxk2Δ GLK1	10	150	0·07	7000	<5	80	–	1100
HXK1 HXK2 glk1Δ	680	670	1·01	80	710	630	1·13	80
hxk1Δ HXK2 glk1Δ	600	600	1·00	80	600	590	1·02	80
HXK1 hxk2Δ glk1Δ	520	160	3·25	1250	240	50	4·8	200
hxk1Δ hxk2Δ glk1Δ	<5	<5	–	400	<5	<5	–	1400
hxk1Δ hxk2-36 glk1Δ	50	270	0·19	90	50	280	0·18	110
hxk1Δ hxk2-37 glk1Δ	20	20	1·00	170	5	5	1·00	150
hxk1Δ hxk2-39 glk1Δ	110	100	1·10	1300	100	70	1·43	480
hxk1Δ hxk2-53 glk1Δ	150	250	0·60	100	170	280	0·61	100
hxk1Δ hxk2-97 glk1Δ	170	170	1·00	130	190	180	1·05	110
hxk1Δ hxk2-129 glk1Δ	410	410	1·00	1500	340	300	1·13	680

* μmol min^{-1} (mg protein)$^{-1}$. † Activity with fructose divided by activity with glucose.

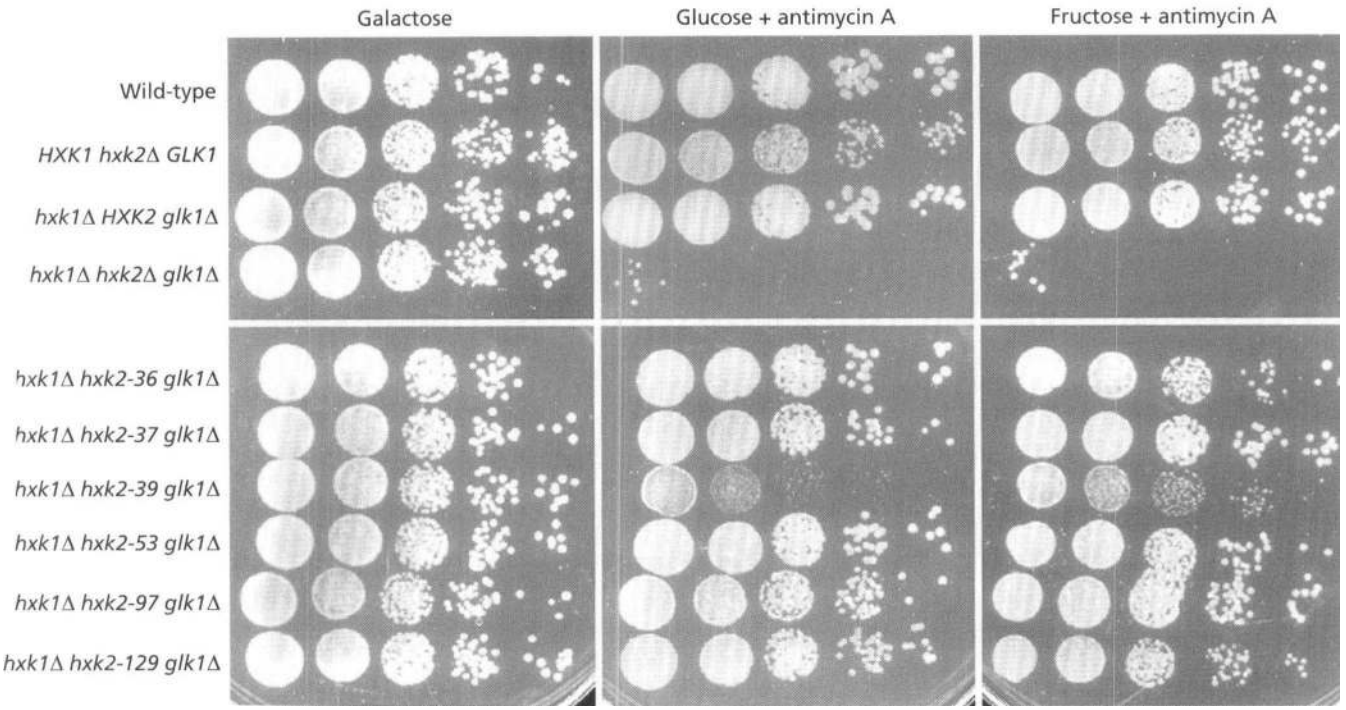

Fig. 1. Growth properties of strains carrying different hexokinase mutations. Strains were pregrown in complete (YP) medium with 2% galactose; 10 μl serial 1:10 dilutions were spotted onto YP medium containing 2% galactose, 2% glucose and 2% fructose. The plates with glucose and fructose contained 1 p.p.m. of the respiration inhibitor antimycin A.

less than 20% of the hexokinase activity of the strain expressing wild-type *HXK2* while mutant *hxk1Δ hxk2-129 glk1Δ* showed the highest residual hexokinase activity (approx. 70%).

The growth properties of each mutant were tested by spotting serial dilutions of galactose-grown cells on medium containing glucose or fructose as the carbon source (Fig. 1). All strains grew normally on medium

Table 3. Enzymic properties of the mutant Hxk2 proteins

Strain (relevant genotype)	Glucose		Fructose		ATP		K_m sugar/K_m ATP	
	K_m*	V_{max}†	K_m*	V_{max}†	K_m*	V_{max}†	Glc/ATP	Fru/ATP
hxk1Δ HXK2 glk1Δ	0·16	215	0·61	250	0·10	220	1·6	6·1
hxk1Δ hxk2-36 glk1Δ	0·23	145	5·10	235	2·11	255	0·1	2·4
hxk1Δ hxk2-37 glk1Δ	0·31	110	2·46	170	0·37	190	0·8	6·6
hxk1Δ hxk2-39 glk1Δ	0·31	175	1·00	165	1·10	240	0·3	0·9
hxk1Δ hxk2-53 glk1Δ	0·47	255	2·01	410	0·43	370	1·1	4·7
hxk1Δ hxk2-97 glk1Δ	0·53	175	2·20	245	0·46	190	1·2	4·8
hxk1Δ hxk2-129 glk1Δ	0·54	175	2·46	95	0·01	130	54·0	246·0

* mM. † mmol min^{-1}.

without the respiration blocker antimycin A (not shown). In the presence of antimycin A, only strain *hxk1Δ hxk2-39 glk1Δ* had a clear growth problem. The *in vivo* hexokinase activity of mutant *hxk1Δ hxk2-36 glk1Δ* with fructose and *hxk1Δ hxk2-37 glk1Δ* with both substrates is probably higher than that monitored *in vitro*, since these strains grew normally (Fig. 1).

Neither the hexokinase protein level as measured by Western blot analysis nor the *HXK2* mRNA level as monitored by Northern blot analysis was altered in the *hxk2* mutants as compared to the wild-type *HXK2* allele (data not shown).

Properties of the *hxk2* products

The kinetic properties of the mutant Hxk2s were determined in crude extracts prepared from strains in a *hxk1Δ glk1Δ* background (Table 3). Substrate affinity was affected to a different extent in the six mutants tested: Hxk2-36 and Hxk2-37 showed a reduced affinity for fructose and for ATP, Hxk2-39 had reduced affinity for ATP, and Hxk2-53 and Hxk2-97 had slightly reduced affinity for all three substrates. Hxk2-129 had a somewhat reduced affinity for the sugars but, remarkably, a tenfold higher affinity than the wild-type enzyme for ATP. The relative affinities for glucose/ATP of Hxk2-36 and for both glucose/ATP and fructose/ATP of Hxk2-39 and Hxk2-129 were strongly altered. Only mutants Hxk2-37 and Hxk2-129 showed a markedly reduced V_{max} with glucose or fructose as substrate, respectively, while Hxk2-53 had an increased V_{max}. Competitive inhibition of the mutant hexokinases by trehalose 6-phosphate (Blázquez *et al.*, 1993) was not significantly altered compared to that of wild-type Hxk2 (data not shown).

Glycolytic metabolism in the novel *hxk2* mutants

To estimate the *in vivo* activity conferred by the mutant *hxk2* alleles, we determined the rate of accumulation of glucose 6-phosphate and fructose 6-phosphate after the addition of glucose or fructose. Because of the rapid initial accumulation of sugar phosphates in *tps1Δ* mutants after sugar addition, we presume that sugar uptake is not limiting at this stage (Van Aelst *et al.*, 1993). The strains were pregrown in glycerol medium, which in wild-type cells allows expression of all three sugar-kinase genes (*HXK1*, *HXK2* and *GLK1*). This explains why, after glucose addition, sugar phosphate levels were lower in the *hxk1Δ glk1Δ* mutant than in the wild-type (Fig. 2).

Sugar phosphate accumulation was affected in all mutants to a different extent. In mutant *hxk1Δ hxk2-36 glk1Δ*, the high glucose 6-phosphate peak after addition of glucose and the relatively low fructose 6-phosphate accumulation after fructose addition were consistent with the specific hexokinase activity determined *in vitro*. In contrast, mutant *hxk1Δ hxk2-37 glk1Δ* showed an intermediate glucose 6-phosphate and a high fructose 6-phosphate peak, indicating that the *in vivo* activity of Hxk2-37 is much higher than that measured *in vitro*, as already inferred from the growth properties. The *hxk1Δ hxk2-39 glk1Δ* strain showed the most severe reduction in sugar phosphate accumulation both after glucose and fructose addition of all mutants studied.

The behaviour of mutant *hxk1Δ hxk2-129 glk1Δ* was very different from that of all the other strains. The sugar phosphate levels 30 s after sugar addition were high, in agreement with the relatively high *in vitro* hexokinase activity. However, after this first time point, sugar phosphate levels did not drop in this mutant but rather were even higher than that of the wild-type after 300 s. Also, mutant *hxk1Δ hxk2-53 glk1Δ* showed aberrant kinetics of sugar phosphate accumulation. With both sugar substrates the product level increased slowly, reaching the highest value only at the second or third time point.

High-affinity sugar uptake by derepressed cells within the first 5 s after the addition of radioactive glucose or fructose is dependent on sugar kinases (Bisson & Fraenkel, 1983; Smits *et al.*, 1996). All the mutants showed high-affinity glucose or fructose uptake under these conditions, consistent with the presence of active hexokinase. However, high-affinity glucose and fructose

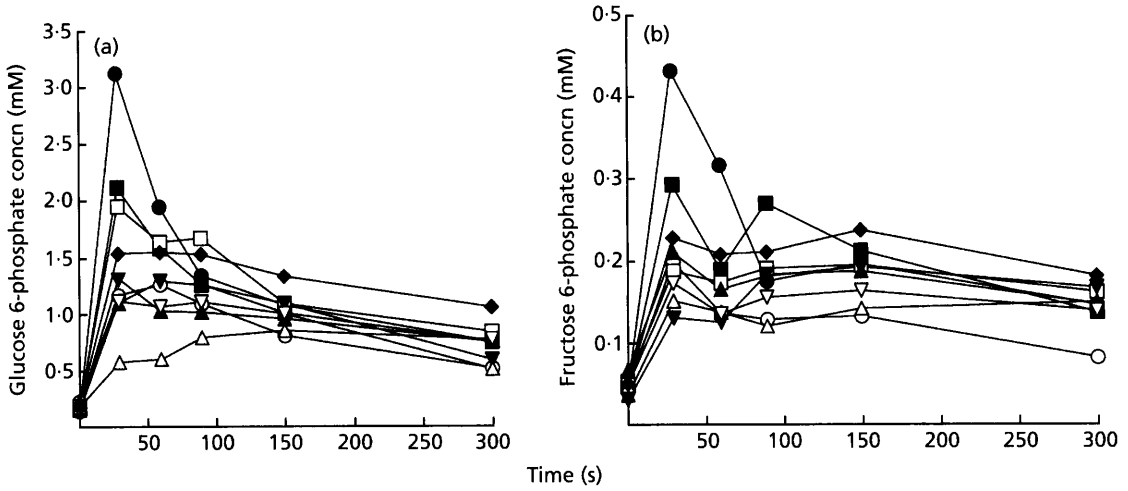

Fig. 2. Accumulation of the product of the hexokinase reaction after addition of 100 mM substrate to glycerol-grown cells *in vivo*. (a) Glucose 6-phosphate after glucose addition. (b) Fructose 6-phosphate after fructose addition. ●, Wild-type; ○, *HXK1 hxk2Δ GLK1*; ■, *hxk1Δ HXK2 glk1Δ*; □, *hxk1Δ hxk2-36 glk1Δ*; ▲, *hxk1Δ hxk2-37 glk1Δ*; △, *hxk1Δ hxk2-39 glk1Δ*; ▼, *hxk1Δ hxk2-53 glk1Δ*; ▽, *hxk1Δ hxk2-97 glk1Δ*; ◆, *hxk1Δ hxk2-129 glk1Δ*.

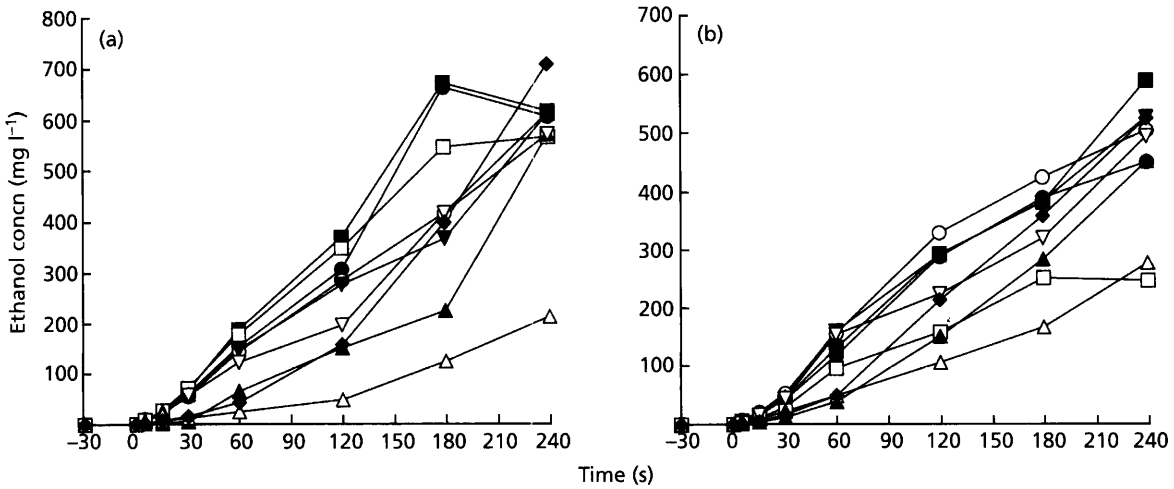

Fig. 3. Ethanol production after the addition of 100 mM glucose (a) or fructose (b) to glycerol-grown cells. ●, Wild-type; ○, *HXK1 hxk2Δ GLK1*; ■, *hxk1Δ HXK2 glk1Δ*; □, *hxk1Δ hxk2-36 glk1Δ*; ▲, *hxk1Δ hxk2-37 glk1Δ*; △, *hxk1Δ hxk2-39 glk1Δ*; ▼, *hxk1Δ hxk2-53 glk1Δ*; ▽, *hxk1Δ hxk2-97 glk1Δ*; ◆, *hxk1Δ hxk2-129 glk1Δ*.

uptake were reduced in mutant *hxk1Δ hxk2-39 glk1Δ* as well as – to a lesser extent – in mutants *hxk1Δ hxk2-53 glk1Δ* and *hxk1Δ hxk2-97 glk1Δ*, consistent with the lower sugar phosphate accumulation of these three mutants (data not shown).

To estimate how the novel *HXK2* mutations affect glycolytic flux, we measured ethanol production in the six selected mutants during growth on glucose (Fig. 3). The wild-type, the *hxk2Δ* mutant and the *hxk1Δ glk1Δ* strain showed the same ethanol production rate. Also, the profile of the mutants *hxk1Δ hxk2-53 glk1Δ* and *hxk1Δ hxk2-97 glk1Δ* was almost indistinguishable from that of the wild-type on both sugars. The ethanol-production rate of mutant *hxk1Δ hxk2-36 glk1Δ* was only diminished in fructose medium, consistent with its hexokinase activity being more affected with fructose as substrate. Mutants *hxk1Δ hxk2-37 glk1Δ* and *hxk1Δ hxk2-129 glk1Δ* showed a longer lag phase before their ethanol-production rate approached that of the wild-type. The ethanol production rate of mutant *hxk1Δ hxk2-39 glk1Δ* was strongly reduced on both glucose and fructose, consistent with the low sugar phosphate accumulation.

Establishment of catabolite repression of the *SUC2* gene for invertase

The *SUC2* gene for invertase is usually used as a marker for catabolite repression, since its expression only

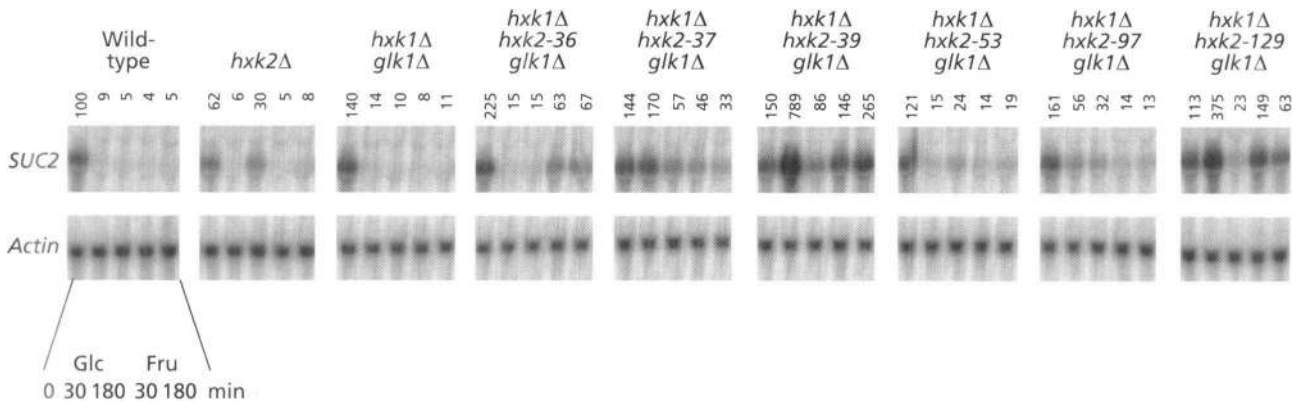

Fig. 4. *SUC2* mRNA levels after the addition of 100 mM glucose or fructose to raffinose-grown cells. Levels (numbers above lanes) were quantified with respect to actin mRNA, which is constant under these growth conditions. The wild-type mRNA level at time zero was set to 100.

responds to the glucose and fructose levels in the growth medium. The specific activity of invertase shows strong derepression on glucose medium in the absence of *HXK2* and even more in the absence of *HXK2* and *HXK1* (Table 2). On fructose medium, deletion of *HXK2* causes only slight derepression, as previously observed (Hohmann, 1987; de Winde *et al.*, 1996). The mutant alleles *hxk2-36*, *hxk2-53* and *hxk2-97* mediated virtually normal repression of invertase activity. Mutants *hxk1Δ hxk2-39 glk1Δ* and *hxk1Δ hxk2-129 glk1Δ* showed derepression of the invertase activity in glucose medium very much like the *hxk2Δ* mutant. This is particularly remarkable for allele *hxk2-129*, since it showed the highest specific-hexokinase activity of all six mutants investigated. In addition, mutants *hxk1Δ hxk2-39 glk1Δ* and *hxk1Δ hxk2-129 glk1Δ* had higher invertase activity than the *hxk2Δ* mutant in fructose medium.

We monitored by Northern-blot analysis short- and long term catabolite repression of *SUC2* mRNA by taking samples 30 and 180 min after the addition of glucose or fructose to raffinose-grown cells from the six *hxk2* mutants in a *hxk1Δ glk1Δ* background and the control strains. The *hxk1Δ glk1Δ* mutant showed catabolite repression exactly like the wild-type (Fig. 4). The *hxk2Δ* mutant showed short-term glucose repression but long-term repression in glucose medium was severely affected. Fructose repression in the *hxk2Δ* mutant was comparable to that of the wild-type, as reported previously (de Winde *et al.*, 1996). Each of the six *hxk1Δ glk1Δ hxk2* mutant strains exhibited a unique pattern of *SUC2* expression with respect to short- and long-term catabolite repression. Mutant *hxk1Δ hxk2-53 glk1Δ* behaved almost like the wild-type. Mutant *hxk1Δ hxk2-36 glk1Δ*, which had specifically low fructose-phosphorylating activity *in vitro*, did not completely repress the *SUC2* mRNA level after fructose addition but showed normal glucose-repression. Thus, this is the first *hxk2* mutant specifically affected in fructose repression. Mutant *hxk1Δ hxk2-97 glk1*, on the other hand, showed only partial short- and long-term glucose repression but normal fructose repression. Mutant *hxk1Δ hxk2-37 glk1Δ* behaved in a similar way, although fructose repression was only partially affected, despite very low *in vivo* fructose kinase activity.

Mutants *hxk1Δ hxk2-39 glk1Δ* and *hxk1Δ hxk2-129 glk1Δ* showed a similar pattern of *SUC2* expression, despite a very different hexokinase activity and sugar catabolism. Both mutants showed respectively a five- and threefold enhanced instead of decreased *SUC2* mRNA level 30 min after glucose addition. Long-term glucose repression, however, was almost normal in mutant *hxk1Δ hxk2-129 glk1Δ* and a twofold repression could be seen also in mutant *hxk1Δ hxk2-39 glk1Δ*. Neither mutant showed short-term fructose repression. Mutant *hxk1Δ hxk2-39 glk1Δ* did not show long-term fructose repression, while there was a minor, twofold fructose repression of the *SUC2* mRNA level in mutant *hxk1Δ hxk2-129 glk1Δ*.

With respect to long- and short-term repression, the *hxk2Δ* and the *hxk1Δ hxk2-129 glk1Δ* strains represent two extremes: the *hxk2Δ* mutant has normal short-term disappearance of *SUC2* mRNA but lacks long-term repression. The *hxk1Δ hxk2-129 glk1Δ* strain lacks the short-term effect – which is rather converted into induction – but has normal long-term glucose repression.

cAMP signalling in the *hxk2* mutants

The addition of glucose or fructose to derepressed cells triggers a rapid, transient increase in the intracellular cAMP level (Thevelein & Beullens, 1985). This effect has been shown to be dependent on sugar phosphorylation (Beullens *et al.*, 1988). We monitored the intracellular level of cAMP in mutants expressing *HXK2* or the new *hxk2* mutant alleles as their only source of hexokinase activity after the addition of glucose or fructose (Fig. 5). All mutants were affected in the magnitude of the glucose-induced cAMP signal. Mutant *hxk1Δ hxk2-39 glk1Δ* hardly showed any signal at all. In

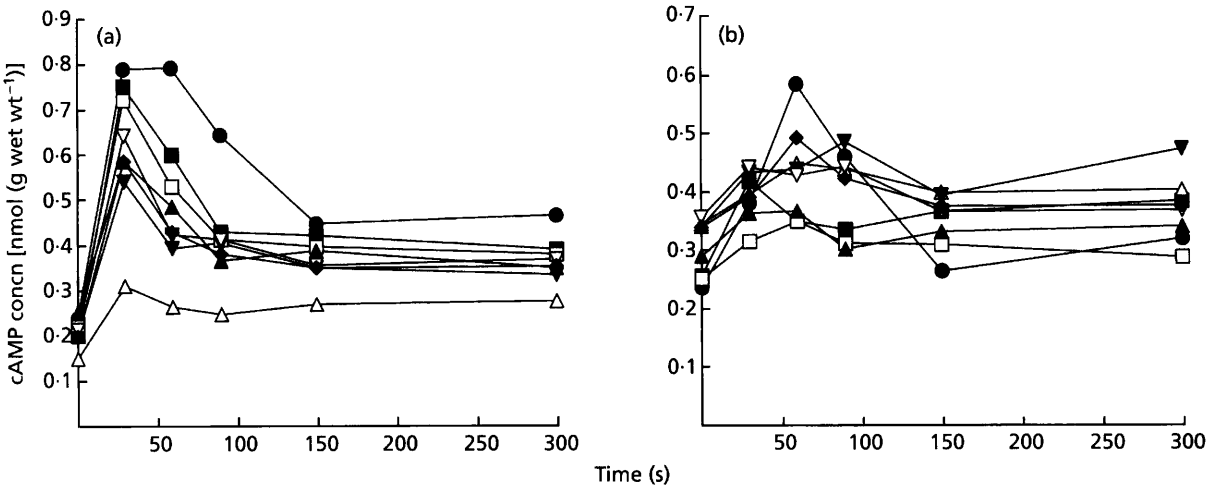

Fig. 5. cAMP content after the addition of 100 mM glucose (a) or 100 mM fructose (b) to glycerol-grown cells. ●, Wild-type; ■, *hxk1Δ HXK2 glk1Δ*; □, *hxk1Δ hxk2-36 glk1Δ*; ▲, *hxk1Δ hxk2-37 glk1Δ*; △, *hxk1Δ hxk2-39 glk1Δ*; ▼, *hxk1Δ hxk2-53 glk1Δ*; ▽, *hxk1Δ hxk2-97 glk1Δ*; ◆, *hxk1Δ hxk2-129 glk1Δ*.

none of the other mutants was the highest value less than two-thirds of that of the control strain expressing only wild-type *HXK2*. The pattern observed for the mutants is very similar to the glucose 6-phosphate peak (Figs 2a and 5a). Fructose does not induce an equally clear cAMP spike like glucose. The new *hxk2* mutants showed, to different degrees, a broader signal with fructose than the wild-type strain.

DISCUSSION

By screening for suppression of the growth defect on fructose of a *tps1Δ hxk1Δ* double mutant we have isolated novel alleles of the *HXK2* gene, encoding the major hexokinase isoform Hxk2. In fact, all respiration-proficient suppressor mutants analysed were *HXK2* mutations. This is remarkable because the screen was initially designed to prevent the selection of *hxk2* mutations, which are predominant when screening for *tps1* suppressors on glucose medium (our unpublished data and Blázquez & Gancedo, 1995). We had chosen to screen for suppressors in a *tps1Δ hxk1Δ* mutant on fructose since a *hxk1 hxk2* double-deletion mutant can not grow on fructose. However, there was still a strong bias towards the selection of *hxk2* mutants, which displayed residual catalytic activity and interesting properties. These novel *hxk2* mutants could be used as a tool in structure–function analysis of Hxk2 in the future.

The detailed analysis of the mutants identified in this work leads to five major conclusions:

1. Different mutations in Hxk2 can affect the catalytic activity with the two sugar substrates glucose and fructose to different extents, suggesting that the structural requirements for binding of glucose and fructose can be separated.

2. We have confirmed that the establishment of catabolite repression is at least a two-step process, with a short-term disappearance of the repressible mRNA and a long-term sustained effect. These two phases can be dissected using different alleles of *HXK2*.

3. We have isolated for the first time a *HXK2* mutation that has lost its ability to mediate fructose repression while glucose repression is still normal.

4. Not all *HXK2* mutants conform to the previously established inverse correlation between the catalytic activity of Hxk2 and its ability to mediate catabolite repression. This suggests that the role of hexokinases in catabolite repression may be more complex than simply catalytic.

5. There is a good correlation between sugar phosphate and cAMP accumulation after sugar addition to derepressed yeast cells. There is, however, no correlation between cAMP accumulation and the ability of the strain to trigger short- or long-term catabolite repression, arguing against a role for cAMP in either process.

Implications for Hxk2 structure and function

We attempted to correlate the nature and position of the affected amino acids in the published three-dimensional structure of yeast hexokinase (Anderson *et al.*, 1978; Bennett & Steitz, 1980) and the highly similar mammalian glucokinase (Gidh-Jain *et al.*, 1993) with the effects on substrate recognition and catalytic activity.

The *hxk2-36* mutation has remarkable consequences since it converts the enzyme from a bifunctional glucose/fructose kinase almost to a glucokinase. The mutation changes Pro-160 to Ala. Pro-160 is the last of a block of highly conserved amino acids in the hexokinase gene family. This ^{152}PLGFTFSFP^{160} motif has previously been proposed to function in sugar binding (Schirch & Wilson, 1987). Site-directed mutagenesis and computer-

assisted modelling of human β-cell glucokinase (Gidh-Jain *et al.*, 1993) and of yeast Hxk2 have identified the neighbouring Ser-158 as an important determinant of sugar-binding affinity, sugar/ATP-binding co-operativity and of the phosphoryl transfer from ATP to the sugar (Xu *et al.*, 1995). Indeed, Ser-158 has recently been mapped as the site of the hexokinase autophosphorylation when the enzyme is inactivated upon binding of D-xylose (Heidrich *et al.*, 1997), stressing the central role of this amino acid in the phosphoryl transfer reaction. Mutation of Pro-160 to Ala may distort the Ser-158 region and hence affect ATP-binding and V_{max}, without significant impact on glucose binding affinity. In contrast, fructose-binding affinity is severely affected in Hxk2-36, with no apparent effect on V_{max}. Mutation of Ser-158 itself likewise causes differential effects on binding affinity, V_{max} and sugar-induced repression kinetics for glucose and fructose (J. H. de Winde and others, unpublished). This discrepancy is indicative of different substrate recognition requirements of Hxk2 for glucose and for fructose. The difference appears to be reflected in the extent of catabolite repression mediated through Hxk2-36; repression is normal with glucose, but partially deficient with fructose (Fig. 4).

In Hxk2-37, Ala-132 is changed into a Pro. This mutation is likely to distort a structurally conserved α-helix within the small lobe of hxk, affecting both sugar- and ATP-binding affinity and hence, V_{max}.

Mutations in Hxk2-39 (Asp-343 to Glu) and Hxk2-97 (Tyr-346 to Asp) are in neighbouring amino acids within the heart of the large lobe, away from the sugar-binding cleft. These mutations have profoundly different effects on enzyme activity, substrate recognition and sugar-induced signalling.

The *hxk2-53* mutation changes Asp-179 into Gly. Asp-179 immediately follows the ^{174}WTKGF^{178} motif, which is highly conserved in the hexokinase family, located near the top of the small lobe. The mutation decreases all substrate affinities to a comparable extent but increases V_{max} for all substrates. Apparently, substrate recognition is not affected since sugar-induced signalling is comparable to wild-type.

Finally, the mutation in *hxk2-129* alters Glu-457 into a Gly. Glu-457 is part of a highly conserved motif ^{457}EDGSGAGAAV^{466} at the C-terminal end of all known members of the hexokinase family. This motif comprises a structurally conserved and important connecting helix of the ATPase domain of diverse ATP-binding proteins (Bork *et al.*, 1992). Insertion of a glycine at the beginning of the connecting helix may tilt this helix, causing the two ATP-binding lobes to come into closer contact (Bork *et al.*, 1992). In line with this, mutation of Glu-456 into Gly causes a 10-fold decrease in the K_m for ATP. Apparently, this structural distortion and increased ATP-binding affinity severely affect catalytic activity and substrate recognition, exemplified by severe decreases of V_{max} and abnormal sugar-induced signalling. Interestingly, the effects on sugar-induced signalling are comparable to those of Hxk2-39, which has a 10-fold increased K_m for ATP. This again separates kinetic properties of hexokinase from the function in signalling.

Short- and long-term repression

We have shown previously that the establishment of the repressed state of catabolite-repressible genes is at least a two-step process consisting of a rapid, initial disappearance of the messengers and a long-term or sustained repression (de Winde *et al.*, 1996). The new data confirm the (at least) two-stage nature of catabolite repression by adding a new mutant phenotype: switching of short-term repression into induction while at the same time long-term repression is still functional, at least partially. This phenotype is most pronounced in mutant *hxk1Δ hxk2-129 glk1Δ* with glucose. Higher *SUC2* mRNA levels 30 min after sugar addition were also observed in mutants 37 (with glucose), 39 (with glucose) and 129 (with fructose), in each case followed by diminished levels after 180 min. Thus, different hexokinase mutations can affect either short- or long-term catabolite repression, or can affect either of the two more than the other. We suggest that short-term and long-term repression of catabolite-repressible genes are mediated by the sugar kinases or products produced by them, but using different signalling pathways.

Glucose and fructose repression

We have shown previously that repression by glucose or fructose has different requirements with respect to the sugar kinase isoform (de Winde *et al.*, 1996). Mutant *hxk1Δ hxk2-36 glk1Δ* is, to our knowledge, the first strain in which fructose repression of *SUC2* is diminished while glucose repression is normal. This mutant is also more affected for fructose than for glucose phosphorylation and therefore this effect is in agreement with the previously established inverse correlation between sugar kinase and *SUC2* repression. Still, this phenotype demonstrates that also mutations in *HXK2* can lead to diminished fructose repression.

Role of sugar kinases in long-term catabolite repression

The actual role of the sugar kinases in long-term catabolite repression has been a matter of controversy for many years. Our data challenge the presently accepted view that the role of the sugar kinases in catabolite repression is directly related to their catalytic activity. This view was based on the good inverse correlation between hexokinase and invertase activity in a collection of random *hxk2* mutants (Ma *et al.*, 1989), *HXK1-HXK2* fusions and *HXK2* promoter truncations (Rose *et al.*, 1991). We do not see an inverse correlation between *in vitro* hexokinase and invertase activities in our mutants. We also do not see such an inverse correlation when we consider the rate of sugar phosphate accumulation as an indicator for *in vivo* hexokinase activity and when *SUC2* expression was monitored more directly by Northern-blot analysis. Alleles

hxk2-39 and *hxk2-129* best demonstrate the lack of correlation. They behave similarly in terms of catabolite repression: short-term glucose induction is seen instead of repression but, at least to some extent, long-term repression and no apparent fructose repression are also seen. These two mutants have very different *in vitro* hexokinase activities, very different sugar phosphate accumulation profiles after sugar addition *in vivo* and different ethanol-production rates. The lack of correlation between *in vitro* and apparent *in vivo* hexokinase activity and long-term repression also becomes apparent when the *hxk2-129* allele is compared to *hxk2-53*. The *hxk1Δ hxk2-129 glk1Δ* mutant has the highest fructose 6-phosphate level among all the mutants and, after a lag phase, a high ethanol-production rate on fructose but lacks fructose repression of *SUC2*. Mutant *hxk1Δ hxk2-53 glk1Δ*, on the other hand, clearly has lower initial fructose 6-phosphate levels and a similar ethanol-production rate when compared to *hxk1Δ hxk2-129 glk1Δ*, but does show normal fructose repression.

Since it has previously been difficult to separate the regulatory and catalytic functions of the sugar kinases, both appear to be closely related. One possible scenario could be related to substrate binding and recognition. What the mutant proteins Hxk2-39 and Hxk2-129 have in common (also partly with Hxk2-36), apart from their very similar defects in catabolite repression, is a dramatically altered proportion of the affinities for sugar and ATP, albeit in opposite directions. If binding of both substrates does not occur in the proper coordinated fashion, this might affect the nature or the timing of a conformational change in the protein. If such a conformational change were the trigger for (a) signalling pathway(s) some protein(s) should recognize this switch and this protein(s) would then remain to be identified. An alternative explanation is that the mutations identified in this study affect to different degrees the domain of the Hxk2 protein that would interact with a downstream component of the glucose-repression pathway.

Role of sugar kinases in cAMP signalling

We have shown previously that sugar phosphorylation, but not further metabolism of the sugar, is essential for glucose- and fructose-induced activation of cAMP synthesis (Beullens *et al.*, 1988). The sugar phosphate produced, however, did not seem to act as the trigger since no correlation was observed between the increase in glucose 6-phosphate and the increase in cAMP when cells were exposed to different glucose concentrations. In the present experiments there is a fair correlation between the increase in glucose 6-phosphate for the different hexokinase alleles, although this correlation is not perfect. For instance, the strains *HXK1 GLK1 hxk2Δ* and *hxk1Δ hxk2-37 glk1Δ* have the same increase in glucose 6-phosphate but the former has a higher cAMP increase. Nevertheless, the data tend to indicate that the role of hexokinase in sugar-induced activation of cAMP synthesis is closely connected to its catalytic function. In that case, the conformational change in the protein during catalysis might trigger activation of a component of the cAMP pathway. As observed for the role of hexokinase in glucose repression, such a mechanism would tend to result in a general correlation between catalytic activity and regulatory activity for induction of cAMP synthesis. Only with mutations in very specific residues would this correlation disappear. Hence, as we have accomplished in this paper for hexokinase catalytic activity and glucose repression function, very specific selection schemes might be needed to find hexokinase mutants in which catalytic activity and cAMP-synthesis activation are separated. It has been suggested that the rapid glucose-induced mRNA disappearance involves cAMP-dependent protein kinase (Cereghino & Scheffler, 1996). The data presented here appear to contradict this idea, since in our mutants there is no correlation between the cAMP spike and the ability to mediate short-term (or long-term) catabolite repression. The present results clearly demonstrate that glucose-repression activity and the capacity to activate the cAMP pathway are distinct properties of hexokinase.

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