Structure–function analysis of yeast hexokinase: structural requirements for triggering cAMP signalling and catabolite repression

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In baker's yeast (*Saccharomyces cerevisiae*) the hexokinases PI (Hxk1) and PII (Hxk2) are required for triggering of the activation of the Ras–cAMP pathway and catabolite repression. Specifically, Hxk2 is essential for the establishment of glucose repression, whereas either Hxk1 or Hxk2 can sustain fructose repression. Previous studies have suggested that the extent of glucose repression is inversely correlated with hexokinase catalytic activity and hence with an adequate elevation of intracellular sugar phosphate levels. However, several lines of evidence indicate that glucose 6-phosphate is not the trigger of catabolite repression in yeast. In the present study we employed site-directed mutagenesis of amino acids important for the binding of sugar and ATP, for efficient phosphoryl transfer and for the closure of the substrate-binding cleft, to obtain an insight into the structural requirements of Hxk2 for sugar-induced signalling. We show that the ATP-binding Lys-111 is not essential for catalysis *in vivo* or for signal triggering. Substitution of the

catalytic-centre Asp-211 caused loss of catalytic activity, but high-affinity sugar binding was retained. However, this was not sufficient to cause cAMP activation nor catabolite repression. Mutation of Ser-158 abrogated glucose-induced, but not fructose-induced, repression. Moreover, 2-deoxyglucose sustained repression despite an extremely low catalytic activity. We conclude that the establishment of catabolite repression is dependent on the onset of the phosphoryl transfer reaction on hexokinase and is probably related to the stable formation of a transition intermediate and concomitant conformational changes within the enzyme. In contrast, the role of Hxk2 in Ras–cAMP activation seems to be directly connected to its catalytic function. The implications of this model are discussed.

Key words: mutagenesis, phosphoryl-transfer intermediate, sugar sensing.

INTRODUCTION

Baker's yeast, *Saccharomyces cerevisiae*, like other micro-organisms, is very capable of adjusting its metabolism to the prevailing growth conditions. The control of carbon catabolism by this yeast in response to the external carbon supply has been the subject of detailed analysis in the past decades. Two well-studied regulatory mechanisms are triggered by the availability of relatively high concentrations of the fermentable sugars glucose and fructose; the 'main' or 'classical' glucose repression or catabolite repression pathway [1,2] and the Ras–cAMP pathway [3,4]. The initiation of either signalling route seems to depend on transport of the sugar into the cytoplasm and its subsequent phosphorylation. However, the triggering of catabolite repression specifically requires the action of the yeast hexokinases PI and PII (Hxk1 and Hxk2 respectively) [5–7], whereas Ras–cAMP activation can equally be triggered via glucokinase Glk1 [8].

Several components of the catabolite repression pathway have been characterized in detail [1,2]. Despite extensive research efforts, little is known at present about the actual sugar-sensing mechanism in catabolite repression. In other words, how is the activity of the signal transduction pathway regulated in response to the availability of various concentrations of glucose or fructose? It has long been known that the establishment of glucose repression primarily requires Hxk2 [9]. However, it has been shown that Hxk1 can partly substitute for Hxk2 in triggering glucose repression [10] and is as important as Hxk2 in triggering fructose repression [5]. An inverse correlation was reported between the activity of various Hxk2 mutants *in vitro* and the extent of glucose repression exerted by these mutant enzymes.

This suggested that the function of Hxk2 in glucose repression was related to an adequate elevation of intracellular sugar phosphate levels [10–12]. However, several lines of evidence indicate that glucose 6-phosphate is not the trigger for glucose repression in yeast. Glk1 cannot substitute for Hxk2, and Hxk1 can only partly substitute for it when overexpressed [10], in establishing glucose repression. Nevertheless, either enzyme can supply the yeast cell adequately with high levels of sugar phosphates. Mutations in Hxk2 have previously been reported that alleviate glucose repression but do not affect intracellular sugar phosphate levels [13]; however, this has not been substantiated. In addition, in yeast cells lacking glucose-6-phosphate isomerase, glucose 6-phosphate accumulates to high intracellular concentrations on the addition of glucose, but no, or only partial, glucose repression is observed [14,15].

We have recently characterized novel mutant alleles of *HXK2* that differentially affect catalytic activity and the ability to trigger catabolite repression [16]. Detailed analysis showed that the sugar binding affinity and the maximal phosphorylation rate *in vitro* were correlated with enzymic activity *in vivo* as monitored by the accumulation of sugar phosphates on the addition of glucose or fructose. In several of the mutants there was no correlation between sugar phosphate accumulation *in vivo* and the establishment of catabolite repression. This indicated that Hxk2 has separate regulatory properties in addition to its catalytic function [16]. In previous studies on the inverse relationship between enzymic activity and the ability to trigger glucose repression, enzyme kinetics *in vivo* and *in vitro* were not determined [11,12].

Abbreviations used: Glk1, glucokinase; Hxk1 and Hxk2, hexokinases PI and PII.

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Because the establishment of catabolite repression is primarily dependent on Hxk1 and Hxk2, and because sugar phosphates are apparently not the triggering molecules, the triggering mechanism would be on or closely connected to the hexokinases. The binding affinity for sugar and ATP are unlikely to be the sole determinants. Hxk1 and Hxk2 exhibit comparable binding constants for both glucose and fructose [10,17]. Moreover, the binding affinity of glucokinase Glk1 for glucose is even higher than that of the hexokinases [18]. ATP binding constants are comparable between the three enzymes [10,17,18]. The triggering mechanism for catabolite repression might instead be related to the large conformational changes that occur when the substrate-binding cleft closes after the synergistic binding of sugar and ATP [19–23]. This cleft closure is dependent on the presence of a 6-OH group on the sugar [24]; binding of the sugar enhances the subsequent binding of ATP [25] as well as intrinsic ATPase activity and phosphoryl transfer [20,22,25]. At present there is no clue to how changes in the three-dimensional structure of hexokinase would trigger a signalling cascade. Various attempts to detect protein–protein interactions with yeast hexokinase have been unsuccessful ([26], and L. S. Kraakman and J. H. de Winde, unpublished work). Biochemical and biophysical analyses have clearly indicated that native hexokinase exists in a monomer–dimer equilibrium [27] and that the monomer displays a higher sugar-binding affinity [28,29]. Hxk2 is phosphorylated on Ser-15 at the extreme N-terminus on depletion of glucose [30]; this phosphorylation shifts the equilibrium to the monomeric form [31,32]. Reports on the functional significance of the phosphorylation of Ser-15 for catabolite repression are, however, contradictory [31,33]. Hxk2 was recently found to reside in both the cytoplasm and the nucleus [34]; nuclear localization, but not Ser-15 phosphorylation, seems necessary for glucose repression [33]. Auto-phosphorylation of Hxk2 on inhibition of the sugar-phosphorylating activity and the enhancement of ATPase activity by unphosphorylatable D-xylose or D-lyxose [20,35] have been shown to be caused by irreversible transfer of the ATP γ -phosphate to Ser-158, which is involved in the phosphoryl transfer and cleft closure [36].

To obtain more insight into the structural requirements of Hxk2 for establishing catabolite repression and triggering cAMP signalling, we have mutated amino acid residues important for the binding of sugar and ATP, for efficient phosphoryl transfer and for cleft closure and conformational change. The resulting mutant proteins were analysed biochemically and their abilities to support fermentative metabolism and to initiate catabolite repression and cAMP accumulation were studied. Results show that the ATP-binding Lys-111 is not essential for catalysis or for sugar-induced signalling. Mutation of the highly conserved Asp-211 causes a loss of catalytic activity but high sugar-binding affinity is retained. This is, however, not sufficient to maintain the capacity to initiate and sustain catabolite repression. Mutation of Ser-158 indicates that the initiation of catabolite repression is closely connected to sugar conversion, presumably involving the stable formation of a phosphoryl transfer intermediate.

MATERIALS AND METHODS

Strains and growth conditions

Yeast strains used in this study were W303-1A (*MATa leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100 GAL SUC mal*) [37] and isogenic YSH7.4-3C (*hxx1Δ::HIS3 hxx2Δ::LEU2 glk1Δ::LEU2*) [5]. Wild-type and mutated alleles of *HXX2* were introduced into YSH7.4-3C and cloned as single-copy YCplac33 [38] constructs YCpHXX2, YCpHXX2^{K111R}, YCpHXX2^{K111V},

YCpHXX2^{D211I}, YCpHXX2^{D211S} and YCpHXX2^{S158A}. Yeast cells were cultured in standard yeast nitrogen base/(NH₄)₂SO₄ media with a synthetic 'drop-out' amino acid/nucleotide mixture as required and supplemented with 4% (w/v) glucose (SCD), 4% (w/v) fructose (SCF), 2% (w/v) galactose (SCGal), 2% (w/v) raffinose (SCRaf) or 2% (w/v) ethanol with 2% (w/v) glycerol and 0.05% glucose (SCEG medium) as indicated. YP medium contained 1% (w/v) yeast extract and 2% (w/v) peptone. Solid media contained 2% (w/v) Bacto-agar in addition. 2-Deoxyglucose and antimycin-A were added to liquid and solid media as indicated in the figure legends. The effectiveness and toxicity of 2-deoxyglucose were carefully tested on solid and liquid media: catabolite repression was apparent from 0.004% and was complete at 0.02%; toxicity and general growth arrest occurred at concentrations above 0.05%.

Cloning and site-directed mutagenesis

The K111R and K111V mutations were introduced into the *HXX2* gene by using the Altered Sites[®] *in vitro* Mutagenesis system from Promega Corp., with a few modifications. A 2.6 kb *Bam*HI–*Eco*RI fragment containing the complete *HXX2* gene was excised from pMR17 [10] and subcloned into phagemid mutagenesis vector pSelect[®], yielding pSelHXX2. Transformants of *Escherichia coli* JM109 were cultured in Luria–Bertani medium containing tetracyclin, with helper phage R408 for 7 h at 37 °C. Cells were pelleted and the supernatant was mixed with 0.25 vol. of 3.75M ammonium acetate (pH 7.5)/20% (w/v) poly(ethylene glycol) (molecular mass 8000 Da). The precipitate was dissolved in 10 mM Tris/HCl, pH 7.5/1 mM EDTA and extracted once with chloroform and three times with phenol/chloroform (1:1, v/v). Single-stranded DNA was precipitated with ethanol and dissolved in TE buffer. The AmpR-repair and desired mutagenic oligonucleotides (Table 1) were phosphorylated with T4 polynucleotide kinase and annealed to single-stranded pSelHXX2. Second-strand synthesis was performed with T4 DNA polymerase and T4 DNA ligase for 90 min at 37 °C. The reaction product was transformed to *E. coli* BMH71-18 to suppress mismatch repair *in vivo*. The presence of the desired mutations in transformants was verified by screening for the newly introduced restriction sites (Table 1) on mini-DNA isolates. DNA isolates were retransformed to *E. coli* TOP10F' to segregate wild-type and mutant plasmids fully. The presence of the desired mutations was confirmed by restriction analysis and DNA sequence analysis.

The D211I, D211S and S158A mutations were introduced into the *HXX2* gene with the use of PCR mutagenesis. The 2.6 kb *Bam*HI–*Eco*RI fragment from pMR17 [10] (see above) was subcloned into single-copy vector YCplac33 and multi-copy vector YEplac195 [38], yielding YCpHXX2 and YEphXX2 respectively. Mutations were introduced in two sequential PCR reactions. The first reaction used YEphXX2 as a template, with the 5'-HK2 oligonucleotide and the mutagenic oligonucleotide of interest (Table 1), yielding a 400 bp PCR fragment for the D211I and D211S mutations and a 240 bp PCR fragment for the S158A mutation. These fragments were gel-purified and used as a mega-primer in the second PCR reaction, with YEphXX2 as a template and the 3'-HK2 oligonucleotide (Table 1). The resulting 900 bp PCR fragments were tested for the presence of the desired mutation by restriction analysis [except for the S158A mutation (Table 1)].

All mutations were introduced into YCpHXX2 and YEphHXX2 by subcloning an *Asp*718–*Bcl*I fragment from either pSelHXX2 or a 900 bp PCR fragment bearing the mutation of interest, followed by restriction analysis of the mutated site and

Table 1 Oligonucleotides and mutations used in this study

Position is indicated relative to the ATG start codon and in the 5' → 3' direction of the oligomer. Nucleotides that are mutated compared with the wild-type sequence are underlined.

Name	Mutation	Restriction site introduced	Position	Sequence
K111R	Lys-111/Arg	<i>Xba</i> I	344/320	5'-GGTAATCTGTATCTAGATTGAGTGG-3'
K111V	Lys-111/Val	<i>Acl</i> I	344/320	5'-GGTAATCTGTATACAGATTGAGTGG-3'
D211I	Asp-211/Ile	<i>Ssp</i> I	644/619	5'-GTACCGGTAGT <u>AAT</u> ATTATCAAGC-3'
D211S	Asp-211/Ser	<i>Sca</i> I	644/620	5'-GTACCGGTAGTACTGTTTATCAAG-3'
S158A	Ser-158/Ala	—	481/460	5'-CTGGGAAAGCAAAGGTGAAACC-3'
5'-HK2	—	—	240/261	5'-TGATTCTTGGCCATTGATTG-3'
3'-HK2	—	—	1168/1147	5'-CACCAATCAATTCAGATAAACG-3'

DNA sequencing of the entire *Asp718–Bcl*I fragment with a T7 Sequencing[®] kit (Pharmacia Biotech).

Hexokinase assay and kinetic analysis

For the determination of specific hexokinase activity, the maximum phosphorylation rate $V_{\max}$ and the Michaelis constant K_m , cells were grown in SCGal medium to mid-exponential growth phase and harvested by centrifugation at 4 °C. The preparation of crude enzyme extracts and measurement of activity were essentially as described previously [16,39]. Specifically, extracts were prepared in either 100 mM potassium phosphate buffer, pH 6.0, or in imidazole buffer, pH 7.0, containing 50 mM imidazole, 100 mM KCl, 10 mM MgCl₂ and 0.1 mM EDTA. PMSF (1 mM) was added to each buffer. Protein concentrations in the extracts were between 1.0 and 2.0 mg/ml, except for the Asp-211 mutants, which were between 10 and 20 mg/ml. Hexokinase activities determined with either extraction method were fully comparable. For kinetic analysis, protein extracts were diluted 1:25 in imidazole buffer containing 8 mM NADP and glucose-6-phosphate dehydrogenase (and phosphoglucosomerase for the determination of fructose-phosphorylating activity [18]). For the Asp-211 mutants, protein extracts were diluted only 1:5. Glucose concentrations in the hexokinase assays were between 0.05 and 100 mM, fructose concentrations between 1.0 and 200 mM, and ATP concentrations between 0.1 and 10 mM. Kinetic constants were calculated by non-linear least-squares analysis, with the Excel template ANEMONA [40].

Determination of concentrations of cAMP and sugar phosphates

For determination of cAMP and glycolytic intermediates, cells were grown in SCEG medium to mid-exponential growth phase, harvested by centrifugation at 4 °C and washed twice in ice-cold YP medium. Subsequently the cells were resuspended in YP medium and preincubated at 30 °C before the addition of 100 mM glucose or fructose. Sampling in cold methanol, the preparation of extracts and the measurement of cAMP and metabolites were performed essentially as described previously [16].

Medium shift experiments, RNA isolation and Northern blot analysis

Medium shifts, sampling at various time points and the subsequent isolation and Northern blot analysis of total RNA were performed essentially as described previously [5]. The evaluation and quantification of Northern blots were performed with Fuji BAS-1000 PhosphorImager technology and software. Gene-specific probe fragments for actin, *SSA3*, *SUC2* and *HXK2* were obtained by PCR [5].

Reproducibility of data

All experiments were performed at least in triplicate from independent cultures, and gave highly consistent trends, i.e. differences between strains and mutants were highly reproducible. Absolute values for enzyme activities, metabolite concentrations and relative mRNA levels varied between independent experiments by not more than 25%. In all cases, results from representative experiments are shown.

RESULTS

Mutagenesis of amino acids that are important for phosphoryl transfer and cleft closure

We mutated several amino acid residues of Hxk2 that had previously been implicated in the binding of sugar and ATP, in phosphoryl transfer and in closure of the sugar-binding cleft. Lys-111 is fully conserved among hexokinases and other ATP-binding proteins and is specifically involved in glucose-enhanced ATP binding on hexokinase [41]. This residue is not part of the PHOSPHATE 1 and PHOSPHATE 2 elements of the ATP-binding fold common to actin, hsp70 proteins and sugar kinases [42] (Figure 1). Nevertheless, molecular modelling predicts this residue to move into the active site and to be near the γ -phosphate when sugar and ATP bind to hexokinase and the phosphate side chain is 'bracketed' between the two PHOSPHATE elements [22,43]. Accordingly, the ATP analogue pyridoxal 5'-diphospho-5'-adenosine can be cross-linked to this acceptor lysine residue [41]. Lys-111 was mutated to arginine (K111R) and to valine (K111V) (Table 1), thereby respectively maintaining and abolishing a positive charge at this position.

From tertiary structure determination, Asp-211 of yeast Hxk2 was predicted to participate in glucose binding by hydrogen-bonding to OH groups of the sugar [19,44]. Moreover, this residue was implicated in promoting the nucleophilic attack of the glucose 6-OH group on the γ -phosphate of ATP [19]. A central role in catalysis has been confirmed for the equivalent residue Asp-205 of human pancreatic β -cell glucokinase, which bears considerable similarity to yeast Hxk2 in both amino acid sequence and tertiary structure [45,46] (Figure 1). Mutagenesis of this highly conserved aspartic residue in the mammalian enzyme caused a drastic decrease in k_{cat} but the glucose binding affinity was not affected [47]. Accordingly, this aspartic residue is in the middle of the CONNECT 1 helix between the PHOSPHATE 1 and PHOSPHATE 2 elements of the ATP-binding fold [42] (Figure 1) and therefore in the vicinity of both the 6-OH group and the γ -phosphate on the binding of sugar and ATP. Asp-211 of yeast Hxk2 was mutated to isoleucine (D211I) and to serine (D211S) (Table 1).

Figure 1 Protein sequence alignment of eukaryotic hexokinases

Protein sequence alignments for a large number of eukaryotic hexokinases were obtained with BLAST 2.0, PSI-BLAST [53] and DIALIGN [54] algorithms. For clarity, only a representative subalignment is shown containing: ScHxk2, *S. cerevisiae* hexokinase PII; ScHxk1, *S. cerevisiae* hexokinase PI; RnGlbK, *Rattus norvegicus* pancreatic β -cell glucokinase (hexokinase IV). Identical amino acids are indicated with dark grey shading and white lettering and conservative substitutions are shown in light shading. Regions of the hexokinases constituting the ATPase domain conserved in several protein families [42] are indicated underneath the sequences with grey shading. The positions of α -helices and β -sheets in the three-dimensional structure are indicated above the aligned sequences. The mutated residues Lys-111, Asp-211 and Ser-158 are highlighted in white-on-black lettering and indicated with asterisks.

A comparison of the crystal structures of yeast hexokinase in the native and glucose-bound conformations [44] and molecular modelling of yeast hexokinase and rat liver glucokinase [48] (Figure 1) have predicted Ser-158 to be a conserved glucose-binding residue, positioned in the vicinity of the glucose 6-OH group. This residue is a critical determinant of enzyme conformation, sugar binding affinity and catalytic activity and is probably involved directly in the hydrolysis of ATP [48]. Recently, Ser-158 was identified as the site of autophosphorylative inactivation when Hxk2 is inhibited by non-phosphorylatable D-xyllose [36]. This underscores the central role of this amino acid in the phosphoryl transfer reaction. Ser-158 was mutated to alanine (S158A) (Table 1).

HXK2 alleles carrying each of the above-mentioned mutations were introduced on single-copy plasmids into a yeast strain lacking the endogenous *HXK1*, *HXK2* and *GLK1* genes. The stable and unperturbed expression of all mutant enzymes was verified by Northern and Western blot analyses (results not shown).

Lysine-111 is essential neither for catalysis nor for sugar-induced signalling

Although Lys-111 of yeast Hxk2 is specifically involved in ATP binding [41] and is fully conserved in all eukaryotic hexokinases [49], no structure-function studies have addressed the role of this residue in enzyme activity and regulation. We mutated Lys-111, applying the 'conservative' mutation K111R and the more

drastic mutation K111V. Both mutations conferred growth on glucose and fructose even in the presence of the respiration inhibitor antimycin (Figure 2), and therefore supported high fermentation activity *in vivo*.

Both mutant enzymes exhibited a K_m for binding of glucose that was comparable to that of the wild type; the glucose affinity for Hxk2^{Val111} seemed to be slightly enhanced (Table 2). In contrast, fructose affinity was partly compromised. In agreement with a specific role for Lys-111 in ATP binding, both mutations caused a significant decrease in ATP binding affinity with either glucose or fructose as co-substrate. The K111V mutation caused an approx. 2.5-fold increase in the K_m^{ATP} (Table 2). Accordingly, the V_{max} of Hxk2^{Val111} for both glucose and fructose was significantly decreased. However, the V_{max} of Hxk2^{Arg111} was unaffected or only slightly affected (Table 2). These kinetic properties are apparently sufficient to support normal fermentative growth (Figure 2). With 2-deoxyglucose as a substrate, the activities of both Hxk2^{Arg111} and Hxk2^{Val111} were decreased to a comparable extent to that with glucose and fructose, although the overall activities were considerably lower than with the latter sugars (Table 2). The K_m of wild-type hexokinase for 2-deoxyglucose was approx. 3-fold that for glucose; both Lys-111 mutations increased this to a comparable extent (Table 2).

To estimate the catalytic activity of Hxk2^{Arg111} and Hxk2^{Val111} *in vivo*, we measured the accumulation of sugar phosphates on the addition of either glucose or fructose to the respective mutant strains, after growth on a non-fermentable carbon source. In comparison with the otherwise isogenic wild type, both mutants

Figure 2 Growth pattern of HXK2 mutants

Single-copy constructs YCpHXK2 (wt), YCpHXK2^{K111R}, YCpHXK2^{K111V}, YCpHXK2^{D211I}, YCpHXK2^{D211S} and YCpHXK2^{S158A} and 'empty' vector YCplac33 ($\Delta 3$), were transformed to strain YSH7.4-3C. Transformants were pregrown on SCGal plates to stationary phase. Subsequently, cells were resuspended and diluted in sterile water to equal attenuances and spotted on SCGal, SCD, SCF and SCEG plates. Antimycin A (antM) was added to SCD and SCF plates, with a final concentration of 2 μ g/ml. 2-Deoxyglucose (2-dog) was added to SCEG plates at a final concentration of 0.02%. Growth was checked at the indicated number of days.

Table 2 Kinetic constants of yeast Hxk2 wild-type and mutant proteins

Cells were grown in SCGal medium and harvested; enzyme kinetics were determined in cleared extracts. Glucose concentrations in the hexokinase assay were between 0.05 and 100 mM; fructose concentrations were between 1.0 and 200 mM; 2-deoxyglucose concentrations were between 0.01 and 10 mM; ATP concentrations were between 0.1 and 10 mM. Kinetic constants were calculated by non-linear least-squares analysis, with the Excel template ANEMONA [26]. Abbreviation: n.d., not determined. Protein concentrations used for the kinetic analysis of the D211I and D211S mutants with glucose and fructose were approx. 50-fold the standard values. Kinetics for glucose and fructose were determined in the presence of 10 mM ATP; kinetics for ATP were determined in the presence of 10 mM glucose or 50 mM fructose.

Substrate	Protein	$V_{\max}$ (nmol/min per mg)	Relative $V_{\max}$ (%)	K_m (mM)	$V_{\max}/K_m$	K_m^{ATP} (mM)
Glucose	HXK2	590 $\pm$ 50	100	0.22 $\pm$ 0.02	2680	0.16 $\pm$ 0.13
	K111R	526 $\pm$ 30	90	0.21 $\pm$ 0.01	2505	0.31 $\pm$ 0.10
	K111V	305 $\pm$ 20	52	0.15 $\pm$ 0.01	2033	0.38 $\pm$ 0.08
	D211I	0.97 $\pm$ 0.09	0.16	0.16 $\pm$ 0.10	6.0	0.32 $\pm$ 0.09
	D211S	0.72 $\pm$ 0.07	0.12	0.16 $\pm$ 0.10	4.5	0.31 $\pm$ 0.08
	S158A	67 $\pm$ 4	10	0.025 $\pm$ 0.010	2680	1.31 $\pm$ 0.05
Fructose	HXK2	600 $\pm$ 20	100	1.19 $\pm$ 0.02	505	0.088 $\pm$ 0.03
	K111R	600 $\pm$ 20	100	1.70 $\pm$ 0.03	350	0.17 $\pm$ 0.06
	K111V	365 $\pm$ 30	60	1.48 $\pm$ 0.06	245	0.23 $\pm$ 0.06
	D211I	2.0 $\pm$ 0.2	0.33	2.20 $\pm$ 0.09	1.0	0.37 $\pm$ 0.10
	D211S	1.2 $\pm$ 0.1	0.12	2.03 $\pm$ 0.10	0.6	0.35 $\pm$ 0.11
	S158A	292 $\pm$ 20	50	1.21 $\pm$ 0.03	240	1.05 $\pm$ 0.04
2-Deoxyglucose	HXK2	93 $\pm$ 5	100	0.55 $\pm$ 0.06	170	n.d.
	K111R	77 $\pm$ 3	85	0.79 $\pm$ 0.04	95	n.d.
	K111V	42 $\pm$ 2	45	0.83 $\pm$ 0.04	50	n.d.
	D211I	n.d.	—	n.d.	—	n.d.
	D211S	n.d.	—	n.d.	—	n.d.
	S158A	7.0 $\pm$ 0.4	7.5	0.021 $\pm$ 0.06	335	n.d.

accumulated sugar phosphates equally to a slightly lower level on the addition of glucose (Figure 3A) and to a distinctly lower level after the addition of fructose (Figure 3B). These results suggest that the K111R and K111V mutations affected the kinetic properties of Hxk2 *in vivo* to a comparable extent, which was not fully in line with the results *in vitro* (Table 2). Nevertheless, although Lys-111 is important for normal ATP binding, this residue appeared not to be essential for enzymic activity.

Transient activation of cAMP synthesis in yeast is dependent on sugar phosphorylation [8]. The accumulation of cAMP was comparable to that in the wild type or was slightly less efficient in both Lys-111 mutant strains after the addition of either glucose (Figure 4A) or fructose (Figure 4B). This is consistent with the catalytic activity of Hxk2^{Arg111} and Hxk2^{Val111} being only marginally affected *in vivo* (Figure 3). The addition of 2-deoxyglucose is known not to trigger cAMP accumulation, even in wild-type strains (F. Rolland, personal communication).

The Lys-111 mutants did not support growth on a non-fermentable carbon source in the presence of low concentrations of 2-deoxyglucose (see the Materials and methods section), a potent elicitor of catabolite repression in yeast [13] (Figure 2). This indicated that both the HXK2^{K111R} and HXK2^{K111V} alleles support the glucose repression of respiratory metabolism. The ability of the mutant hexokinases to initiate catabolite repression was investigated in more detail through a Northern blot analysis of the expression of the *SUC2* gene, which encodes invertase. Repression of *SUC2* by fermentable sugars occurs via a biphasic mechanism [5,50]. Both short-term and long-term repression, induced by either glucose or fructose, were absent in a yeast strain lacking all three hexose kinase enzymes (Figure 5). As shown previously [5], derepression with glucose is much more marked than with fructose. Expression of HXK2^{K111R} and HXK2^{K111V} restored the repression induced by glucose (Figure 5A) and by fructose (Figure 5B), which is in line with the observation that with both alleles, growth was inhibited in the presence of 2-deoxyglucose (Figure 2). Only short-term glucose repression triggered via Hxk2^{Val111} seemed to be somewhat retarded (Figure 5A). Thus Lys-111 is not essential in the establishment of catabolite repression.

High-affinity sugar binding does not trigger catabolite repression

The mutagenesis of Asp-211 into isoleucine (D211I) or serine (D211S) created HXK2 alleles that did not confer growth on fermentable sugar and that enabled growth on a non-fermentable carbon source in the presence of 2-deoxyglucose, thus resembling the absence of hexokinase activity (Figure 2). As described for rat liver glucokinase [47], mutation of this essential amino acid virtually, but not fully, abolished phosphorylating activity (Table 2). This confirmed the central role of this conserved aspartic residue in catalysis by hexokinase. Determination of the K_m for glucose and fructose was less straightforward for these mutants than for enzymes with appreciable hexokinase activity and required much higher protein concentrations in the enzyme assays. The glucose binding affinity for both Hxk2^{Ile211} and Hxk2^{Ser211} was slightly enhanced compared with the wild type, whereas the fructose binding affinity was decreased (Table 2). In contrast with the Asp-205 mutation in rat liver glucokinase [47], the D211I and D211S mutations in yeast Hxk2 clearly decreased the ATP binding affinity with either glucose or fructose as co-substrate. Owing to an extremely low residual activity the Michaelis–Menten constants could not be determined with 2-deoxyglucose as a substrate. Consistent with the no-growth phenotype on fermentable sugars and with abrogated catalytic activity was the observation that neither mutant enzyme sup-

Figure 3 Accumulation of sugar phosphates *in vivo* in HXK2 mutants

The accumulation of glucose 6-phosphate (left panels), fructose 6-phosphate (middle panels) and fructose 1,6-bisphosphate (right panels) is shown in mM, after the addition of 100 mM glucose (A) or 100 mM fructose (B) to wild-type and hexokinase mutant strains grown on SCEG medium. Symbols: ●, wild-type HXK2; ○, $\Delta hvk1 \Delta hvk2 \Delta glk1$; ■, HXK2^{K111R}; □, HXK2^{K111V}; ▲, HXK2^{S158A}.

Figure 4 Activation of cAMP signalling in HXK2 mutants

Shown is cAMP accumulation after the addition of 100 mM glucose (A) or 100 mM fructose (B) to wild-type and hexokinase mutant strains grown on SCEG medium. Symbols: ●, wild-type HXK2; ○, $\Delta hvk1 \Delta hvk2 \Delta glk1$; ■, HXK2^{K111R}; □, HXK2^{K111V}; ▲, HXK2^{S158A}.

ported the accumulation of sugar phosphates or of cAMP after the addition of glucose or fructose (results not shown).

In line with the fact that the Asp-211 mutants grew in the presence of 2-deoxyglucose, the HXK2^{D211I} and HXK2^{D211S} alleles did not restore the loss of glucose- or fructose-induced *SUC2*

repression in an *hvk1Δhvk2Δglk1Δ* mutant (Figure 5C). These results indicated that mere high-affinity sugar binding on yeast hexokinase is not sufficient to trigger glucose repression. Apparently, the phosphoryl transfer reaction constitutes an additional prerequisite.

Figure 5 Northern blot analysis of *HXK2* mutants during transition to fermentative growth

Expression of repressible *SUC2* was monitored at the indicated time points after the addition of 200 mM glucose (**A**) or 200 mM fructose (**B**) to cells pregrown on SC_{Raf}Gal. *SUC2* mRNA levels were quantified relative to the corresponding actin mRNA levels for wild-type *HXK2*, $\Delta hxx1 \Delta hxx2 \Delta glk1$ (**A, B, C**), *HXK2*^{K111R}, *HXK2*^{K111V}, *HXK2*^{S158A} (**A, B**), and *HXK2*^{D211I} and *HXK2*^{D211S} (**C**). (C) Abbreviations: D, 200 mM glucose, F, 200 mM fructose.

Figure 6 S158A mutation supports 2-deoxyglucose- but not glucose-induced repression of *SUC2*

Expression of repressible *SUC2* was monitored at the indicated time points and quantified relative to the corresponding actin mRNA levels after the addition of 4% (w/v) glucose or 0.04% 2-deoxyglucose (2-dog) to wild-type (HXK2) or S158A mutant cells pregrown on SCEG medium.

Differential roles for Ser-158 in catalysis and signalling

Ser-158 has recently been identified as a critical determinant of the catalytic activity and conformation of hexokinase [48]. Because this residue is directly involved in the phosphoryl transfer reaction, we decided to investigate the effects of its mutation on enzyme activity and signalling capacity of yeast Hxk2 *in vitro* and *in vivo*. The *HXK2*^{S158A} allele conferred growth with glucose and fructose, although slightly more slowly than that of the wild type and the *HXK2*^{K111R} and *HXK2*^{K111V} alleles (Figure 2). In the presence of the respiration inhibitor antimycin, growth was severely inhibited on glucose and, less severely, on fructose. This suggested that hexokinase activity and therefore the influx of glucose and (to a lesser extent) of fructose into glycolysis via Hxk2^{Ala158} was severely restricted. Indeed, the accumulation of intracellular free glucose was observed in the *HXK2*^{S158A} mutant after the addition of glucose to glucose-depleted cells (results not shown).

In agreement with the growth properties on fermentable sugar, and with results on the equivalent S151A mutation in pancreatic β -cell glucokinase ([48]; see also [36]), the S158A mutation strongly decreased the $V_{\max}$ for glucose, whereas the $V_{\max}$ for fructose decreased to approx. 50% of that of the wild type (Table 2). The loss of enzyme activity was correlated with a severe decrease in ATP binding affinity with either glucose or fructose as co-substrate. In contrast, glucose binding affinity was increased approx. 10-fold but fructose binding affinity was not affected. The latter results contrast with those on β -cell glucokinase, where the S151A mutation also caused a decrease in the K_m for fructose [48]. As with glucose, the S158A mutation severely diminished the $V_{\max}$ for 2-deoxyglucose, whereas binding affinity was drastically increased (Table 2).

The accumulation of sugar phosphates after glucose addition was markedly lowered in the S158A mutant (Figure 3A), whereas fructose-induced accumulation was comparable to the K111R and K111V mutant profiles (Figure 3B). These results *in vivo* corroborated the growth characteristics and the kinetics *in vitro*, in that the S158A mutation has a more marked effect on glucose-phosphorylating activity than on fructose phosphorylation. In particular, the accumulation of fructose 1,6-bisphosphate was

strongly decreased, confirming that the flux through glycolysis was severely compromised (see the Discussion section). In line with this, the stimulation of cAMP synthesis by glucose through Hxk2^{Ala158} was clearly decreased (Figure 4A), whereas fructose-dependent cAMP accumulation was comparable to that of the wild type and the K111R and K111V mutant profiles (Figure 4B).

Interestingly, the S158A mutation inhibited growth on non-fermentable carbon sources in the presence of 2-deoxyglucose (Figure 2; see the Materials and methods section). This suggested that Hxk2^{Ala158} would trigger normal glucose repression, despite a much lower (10%) $V_{\max}$ for glucose phosphorylation. Surprisingly, however, the *HXK2*^{S158A} allele did not restore the glucose-induced repression of *SUC2* expression in an *hxx1* Δ *hxx2* Δ *gk1* Δ mutant, whereas fructose repression was normal (Figures 5A and 5B). In line with the observed growth inhibition, 2-deoxyglucose did indeed repress *SUC2* expression through both the wild-type *HXK2* and the *HXK2*^{S158A} allele (Figure 6). This was remarkable with respect to the very low catalytic activity of Hxk^{Ala158} with 2-deoxyglucose as a substrate, being only approx. 1% of that with glucose (Table 2).

Because Ser-158 has a central role in the transfer of the γ -phosphate from ATP to the sugar and in the associated closure of the substrate-binding cleft, our results indicate that an efficient phosphoryl transfer and the concomitant conformational change of hexokinase are connected with the triggering of the glucose repression signal. However, the role of Ser-158 in hexokinase catalysis, conformational change and hence the establishment of catabolite repression seems to vary with different substrates. Accordingly, the structural requirements for hexokinase-mediated signalling might vary in a substrate-dependent manner.

DISCUSSION

Site-directed structure-function analysis of yeast Hxk2

Until recently, the central role of yeast Hxk2 in glucose repression seemed to be tightly correlated with its catalytic activity [10–12]. However, we have shown that the catalytic and regulatory properties of Hxk2 can be separated, by a detailed analysis of a series of Hxk2 mutants isolated as spontaneous suppressors of the growth deficiency of *tps1* Δ strains [16]. The various mutations characterized affected sugar binding, sugar phosphorylation and sugar-induced signalling to different extents, without any apparent correlation between those properties. The different amino acids mutated were scattered throughout the enzyme, and their function in sugar binding or protein folding could be inferred in part, according to the known three-dimensional structure [19,44,46]. To obtain further insight into the structural requirements for Hxk2-mediated catabolite repression and cAMP signalling, we chose to mutate amino acid residues that had previously been implicated in the binding of sugar and ATP, in the phosphoryl transfer reaction and in closure of the substrate-binding cleft. A detailed explanation of our choice for the amino acid residues studied in this paper is given at the beginning of the results section.

Substrate binding affinity and catalysis

Mutation of Lys-111 into either Arg (K111R) or Val (K111V) did not seriously affect the glucose binding affinity; however, the affinities for fructose and 2-deoxyglucose were somewhat decreased. In line with a specific role for Lys-111 in ATP binding,

both mutations caused a clear decrease in ATP binding affinity with either glucose or fructose as a co-substrate, the hydrophobic substitution causing the most pronounced effect. Surprisingly, the decreased ATP affinity caused by the K111R substitution did not affect catalytic activity, whereas the hydrophobic substitution K111V resulted in a halving of $V_{\max}$ with either glucose or fructose. However, the apparent activity *in vivo*, as measured by the accumulation of sugar phosphates, was not severely affected with glucose and was halved with fructose. Probably, although this lysine residue is highly conserved in eukaryotic hexokinases, merely the positive charge at this position is important for normal ATP binding and efficient phosphoryl transfer. The presence of a lysine residue might be important for the efficient phosphorylation of sugars other than glucose but it is not essential for ATP binding and enzymic activity.

Asp-211 is of central importance to the enzymic activity of yeast Hxk2 and can be regarded as the genuine catalytic centre. Mutation of this residue virtually abolished sugar phosphorylating activity, decreasing the k_{cat} to approx. 1/500. However, the glucose binding affinity was comparable to that of the wild type, whereas in the equivalent rat liver glucokinase mutant [47] glucose binding was enhanced 2–3-fold. This difference from the rat liver mutants might be correlated with the fact that the sugar binding affinity is already high in the yeast enzyme. A naturally occurring variation was recently described in the otherwise fully conserved amino acid position immediately flanking the catalytic centre in *Schizosaccharomyces pombe* hxl1⁺ [51]. This yeast enzyme shows an unusually high K_m for glucose, which is decreased by replacement of the serine residue at stake by the ‘normal’ asparagine. In contrast, a normal K_m for fructose is increased by this Ser → Asn substitution. Hence the highly conserved region including and surrounding the catalytic centre of hexokinase is involved in determining both the sugar binding affinity and specificity.

Our results confirm the importance of Ser-158 in determining the binding affinity for sugars and ATP, and in phosphoryl transfer on hexokinase. The S158A mutation greatly enhanced the affinity for both glucose and 2-deoxyglucose; however, the affinity for fructose was not affected. Interestingly, mutation of Pro-160 within the same highly conserved P¹⁵²LGFTFSFP¹⁶⁰ motif does not affect glucose affinity but severely decreases the affinity for fructose [16]. Apparently, the binding sites for the various sugar substrates on hexokinase are different, at least in part. The binding affinity for ATP decreased to less than 10% for both the S158A and P160A mutations, indicating the additional importance of this region for ATP binding and therefore catalysis. Indeed, the Ser → Ala mutation completely abolishes the ATPase activity of hexokinase *in vitro* [48]. The S158A mutation is predicted to abolish hydrogen-bonding of the serine-OH group with neighbouring residues within the substrate-binding cleft [43,44,48,52] and to favour the interaction of the carbonyl oxygen of residue 158 with glucose. This would explain the strongly enhanced glucose affinity and the decrease in $V_{\max}$ and apparent activity *in vivo* with glucose (and 2-deoxyglucose). The marked decrease in fructose 1,6-bisphosphate accumulation is probably caused by the concomitant decrease in the transient cAMP accumulation and hence a reduced activation of phosphofructokinase (see below) [8]. $V_{\max}$ and activity with fructose *in vivo* are halved, which is comparable with effects of the Lys-111 mutations. A likely explanation is that fructose binding, phosphorylation and fructose-induced conformational change are primarily dependent not on Ser-158 but on Pro-160 (see above). This presents a strong case for different structural requirements of hexokinase for glucose and fructose binding and for phosphorylation.

Hexokinase and cAMP signalling

In line with previous findings [16], our present results show a good correlation between, on the one hand, the apparent $V_{\max}$ and the activity of wild-type and mutant hexokinases *in vivo* and, on the other, the glucose- and fructose-induced transient cAMP accumulation in the strains concerned. A decrease of approx. 90% in $V_{\max}$ and a strong decrease in the apparent activity with glucose *in vivo* caused by the S158A mutation results in a strong decrease in the cAMP signal. In contrast, with fructose as a substrate, $V_{\max}$ and activity *in vivo* are affected only partly and, as an apparent consequence, cAMP accumulation is not affected. We therefore conclude that the role of yeast hexokinase in the sugar-induced activation of cAMP signalling is closely connected to the catalytic function of the enzyme. The mechanism through which the yeast hexokinases fulfil the intracellular prerequisite for the activation of cAMP signalling is currently under investigation (F. Rolland, J. H. de Winde and J. Winderickx, unpublished results).

Structural determinants for hexokinase-mediated catabolite repression

The present study clearly indicates that the role of yeast hexokinase in establishing catabolite repression lies beyond its catalytic function. Indeed, the S158A mutation, which decreases $V_{\max}$ and lowers the apparent activity with glucose *in vivo*, abolishes the glucose-induced repression of *SUC2* expression. However, 2-deoxyglucose, with a $V_{\max}$ that is only 1% of the wild-type activity with glucose, still represses *SUC2* as well as respiratory growth. Moreover, glucose phosphorylation on Hxk2^{Ala158} triggers sugar monophosphate accumulation to an extent comparable to fructose phosphorylation on the same mutant enzyme and on Hxk2^{Arg111} and Hxk2^{Val111}, whereas repression is established only with fructose. Mere high-affinity binding of sugar is clearly not sufficient to establish catabolite repression, as exemplified by the absence of glucose-induced repression in the Asp-211 mutants and in the Ser-158 mutant with even a 10-fold enhanced affinity. What, then, determines the initiation of catabolite repression on hexokinase?

Closure of the substrate-binding cleft as well as phosphoryl transfer is essentially dependent on the 6-OH group on the sugar [24]. Catalysis and the formation of a phosphoryl transfer intermediate are initiated by combined nucleophilic attack by Asp-211 and the OH group of Ser-158, at least for glucose phosphorylation. Formation of the transition state with other sugars is apparently dependent on additional or other interactions, because determinants for binding affinity and specificity vary with different sugars. Moreover, mutation of Ser-158 has no or only a minor effect on affinity and catalysis with fructose; accordingly, fructose-induced repression is normal. With 2-deoxyglucose the S158A mutant shows a 25-fold increase in affinity compared with wild-type hexokinase; however, k_{cat} is elevated despite a lowered $V_{\max}$. This suggests that amino acid residues that normally stabilize the glucose ring in the substrate-binding cleft through hydrogen-bonding of the 2-OH group on binding of 2-deoxyglucose participate in catalysis and favour phosphoryl transfer. Sugar binding and cleft closure have indeed been shown to depend on the nature of the C-2 substituent on the sugar [19]. Subsequently, 2-deoxyglucose phosphorylation triggers repression. In this respect it is important to note that the S158A mutation does not affect the monomer–dimer equilibrium or other hydrodynamic properties [32] (see below). We propose that the critical determinant for the establishment of catabolite repression through yeast hexokinase is closely connected with the

onset of catalysis and is most probably related to the ability of the enzyme to form a stable transition intermediate efficiently.

Hexokinase function in the initiation of catabolite repression

The present evidence indicates that the establishment of catabolite repression through yeast hexokinase depends on the efficient progress of phosphoryl transfer and would therefore be correlated with the large conformational changes occurring in the enzyme on binding of the substrates, on catalysis and/or on product release. It is attractive to hypothesize that such a conformational change would trigger catabolite repression via protein–protein interaction and protein modification. However, we and others have been unsuccessful in detecting proteins that would directly interact with yeast hexokinase PII, other than the known homodimer. The monomer–dimer equilibrium shifts to the monomeric form on the phosphorylation of Ser-15 [31,32]. The monomer is the predominant form of the enzyme under glucose-depleted conditions and has a higher sugar binding affinity than the dimer [28,29]. Recently, Hxk2 was found to reside partly in the nucleus of glucose-grown cells [34] and to constitute part of a protein–DNA complex recognizing the upstream activating sequence (UAS) of *SUC2* [33]. It has been reported that the glucose repression of *SUC2* [31] but not the nuclear localization of Hxk2 [33] depend on Ser-15 phosphorylation. However, the latter report refuted the involvement of Ser-15 phosphorylation in repression signalling. Therefore the involvement of either the monomeric or the dimeric form of the enzyme, as well as the importance of nuclear translocation, in the establishment of catabolite repression remains unresolved. Our present results clearly indicate that repression signalling depends on the onset of catalysis on hexokinase. Whether this is correlated with nuclear translocation and interaction with other regulators or transcription factors remains to be established.

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REFERENCES

- de Winde, J. H., Thevelein, J. M. and Winderickx, J. (1997) in *Yeast Stress Responses* (Hohmann, S. and Mager, W. H., eds.), pp. 7–52. Springer–Verlag, Berlin.
- Gancedo, J. M. (1998) *Microbiol. Mol. Biol. Rev.* **62**, 334–361.
- Wigler, M., Field, J., Powers, S., Broek, D., Toda, T., Cameron, S., Nikawa, J., Michaeli, T., Colicelli, J. and Ferguson, K. (1988) *Cold Spring Harb. Symp. Quant. Biol.* **53**, 649–655.
- Thevelein, J. M. (1992) *Antoni van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **62**, 109–130.
- De Winde, J. H., Crauwels, M., Hohmann, S., Thevelein, J. M. and Winderickx, J. (1996) *Eur. J. Biochem.* **241**, 633–643.
- Entian, K. D. and Mecke, D. (1982) *J. Biol. Chem.* **257**, 870–874.
- Ma, H. and Botstein, D. (1986) *Mol. Cell. Biol.* **6**, 4046–4052.
- Beullens, M., Mbonyi, K., Geerts, L., Gladines, D., Detremere, K., Jans, A. W. H. and Thevelein, J. M. (1988) *Eur. J. Biochem.* **172**, 227–231.
- Entian, K. D. (1980) *Mol. Gen. Genet.* **178**, 633–637.
- Rose, M., Albig, W. and Entian, K. D. (1991) *Eur. J. Biochem.* **199**, 511–518.
- Ma, H., Bloom, L. M., Walsh, C. T. and Botstein, D. (1989) *Mol. Cell. Biol.* **9**, 5643–5649.
- Ma, H., Bloom, L. M., Zhu, Z., Walsh, C. T. and Botstein, D. (1989) *Mol. Cell. Biol.* **9**, 5630–5642.
- Entian, K. D. and Froehlich, K. U. (1984) *J. Bacteriol.* **158**, 29–35.
- Cereghino, G. P. and Scheffler, I. E. (1996) *EMBO J.* **15**, 363–374.
- Sierkstra, L. N., Sillje, H. H. W., Verbakel, J. M. A. and Verrips, C. T. (1993) *Eur. J. Biochem.* **214**, 121–127.
- Hohmann, S., Winderickx, J., de Winde, J. H., Valckx, D., Cobbaert, P., Luyten, K., De Meersman, C., Ramos, J. and Thevelein, J. M. (1999) *Microbiology* **145**, 703–714.
- Kopetzki, E. and Entian, K. D. (1985) *Eur. J. Biochem.* **146**, 657–662.
- Maitra, P. K. (1975) *Methods Enzymol.* **42C**, 25–30.
- Anderson, C. M., Stenkamp, R. E., McDonald, R. C. and Steitz, T. A. (1978) *J. Mol. Biol.* **123**, 207–219.
- DeLaFuente, G., Lagunas, R. and Sols, A. (1970) *Eur. J. Biochem.* **16**, 226–233.
- McDonald, R. C., Steitz, T. A. and Engelman, D. M. (1979) *Biochemistry* **18**, 338–342.
- Wilson, J. E. and Schwab, D. A. (1996) *FASEB J.* **10**, 799–801.
- Woolfitt, A. R., Kellett, G. L. and Hoggett, J. G. (1988) *Biochim. Biophys. Acta* **952**, 238–243.
- Shoham, M. and Steitz, T. A. (1982) *Biochim. Biophys. Acta* **705**, 380–384.
- Viola, R. E., Raushel, F. M., Rendina, A. R. and Cleland, W. W. (1982) *Biochemistry* **21**, 1295–1302.
- Muller, S., Boles, E. and Zimmermann, F. K. (1996) *Eur. J. Biochem.* **236**, 626–631.
- Easterby, J. S. and Rosemeyer, M. A. (1972) *Eur. J. Biochem.* **28**, 241–252.
- Hoggett, J. G. and Kellett, G. L. (1976) *Eur. J. Biochem.* **66**, 65–77.
- Hoggett, J. G. and Kellett, G. L. (1992) *Biochem. J.* **287**, 567–572.
- Kriegel, T. M., Rush, J., Vojtek, A. B., Clifton, D. and Fraenkel, D. G. (1994) *Biochemistry* **33**, 148–152.
- Randez-Gil, F., Sanz, P., Entian, K.-D. and Prieto, J. A. (1998) *Mol. Cell. Biol.* **18**, 2940–2948.
- Behlke, J., Heidrich, K., Naumann, M., Müller, E.-C., Otto, A., Reuter, R. and Kriegel, T. (1998) *Biochemistry* **37**, 11989–11995.
- Herrero, P., Martinez-Campa, C. and Moreno, F. (1998) *FEBS Lett.* **434**, 71–76.
- Randez-Gil, F., Herrero, P., Sanz, P., Prieto, J. A. and Moreno, F. (1998) *FEBS Lett.* **425**, 475–478.
- Fernandez, R., Herrero, P., Fernandez, E., Fernandez, T., Lopez-Boado, Y. S. and Moreno, F. (1988) *J. Gen. Microbiol.* **134**, 2493–2498.
- Heidrich, K., Otto, A., Behlke, J., Rush, J., Wenzel, K.-W. and Kriegel, T. (1997) *Biochemistry* **36**, 1960–1964.
- Thomas, B. J. and Rothstein, R. J. (1989) *Cell* **56**, 619–630.
- Gietz, R. D. and Sugino, A. (1988) *Gene* **74**, 527–534.
- Ernandes, J. R., De Meersman, C., Rolland, F., Winderickx, J., de Winde, J. H., Brandao, R. L. and Thevelein, J. M. (1998) *Yeast* **14**, 255–269.
- Hernandez, A. and Ruiz, M. T. (1998) *Bioinformatics* **14**, 227–228.
- Tamura, J. K., LaDine, J. R. and Cross, R. L. (1988) *J. Biol. Chem.* **263**, 7907–7912.
- Bork, P., Sander, C. and Valencia, A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7290–7294.
- Shoham, M. and Steitz, T. A. (1980) *J. Mol. Biol.* **140**, 1–14.
- Bennet, Jr., W. S. and Steitz, T. A. (1980) *J. Mol. Biol.* **140**, 183–230.
- Charles, R. S., Harrison, R. W., Bell, G. I., Pilakis, S. J. and Weber, I. T. (1994) *Diabetes* **43**, 784–91.
- Gidh-Jain, M., Takeda, J., Xu, L. Z., Lange, A. J., Vionnet, N., Stoffel, M., Froguel, P., Velho, G., Sun, F., Cohen, D. et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1932–1936.
- Lange, A. J., Xu, L. Z., van Poelwijk, F., Lin, K., Granner, D. K. and Pilakis, S. J. (1991) *Biochem. J.* **277**, 159–163.
- Xu, L. Z., Harrison, R. W., Weber, I. T. and Pilakis, S. J. (1995) *J. Biol. Chem.* **270**, 9939–9946.
- Griffin, L. D., Gelb, B. D., Wheeler, D. A., Davison, D., Adams, V. and McCabe, E. R. B. (1991) *Genomics* **11**, 1014–1024.
- Sanz, P., Nieto, A. and Prieto, J. A. (1996) *J. Bacteriol.* **178**, 4721–4723.
- Petit, T., Herrero, P. and Gancedo, C. (1998) *Biochem. Biophys. Res. Commun.* **251**, 714–719.
- Xu, L. Z., Weber, I. T., Harrison, R. W., Gidh-Jain, M. and Pilakis, S. J. (1995) *Biochemistry* **34**, 6083–6092.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) *Nucleic Acids Res.* **25**, 3389–3402.
- Morgenstern, B., Dress, A. and Werner, T. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12098–12103.

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