Articles

Autophosphorylation−Inactivation Site of Hexokinase 2 in *Saccharomyces cerevisiae*†

Katja Heidrich, Albrecht Otto,‡ Joachim Behlke,§ John Rush,|| Klaus-Wolfgang Wenzel, and Thomas Kriegel*

Technische Universität Dresden, Medizinische Fakultät Carl Gustav Carus, Institut für Physiologische Chemie, PF 800 115, D-01101 Dresden, Germany

Received September 18, 1996; Revised Manuscript Received December 13, 1996⊗

ABSTRACT: Hexokinase 2 from *Saccharomyces cerevisiae* is phosphorylated *in vivo* at serine-15 [Kriegel et al. (1994) *Biochemistry 33*, 148−152] and undergoes ATP-dependent autophosphorylation−inactivation *in vitro* when incubated in the presence of D-xylose [Fernandez et al. (1988) *J. Gen. Microbiol. 134*, 2493−2498]. This study identifies the site of inactivation by autophosphorylation as serine-158 by observation of a single tryptic peptide difference, peptide sequencing, and size determination by mass spectrometry. Mutation of serine-158 to alanine and cysteine, respectively, prevents autophosphorylation and causes a drastic decrease of the catalytic activity while mutational change to glutamate results in a complete loss of enzyme activity. The catalytically active mutant enzymes display an increased affinity for glucose and exhibit higher K_M with respect to MgATP. Phosphoserine/phosphothreonine-specific protein phosphatase-2A completely reverses the autophosphorylative inactivation of the wild-type enzyme.

Hexokinase[1] 2 (gene name *HXK2*) of *Saccharomyces cerevisiae* is the subject of the present work. It is the isoenzyme which predominates in growth on glucose (Gancedo et al., 1977; Herrero et al., 1995) and is also required for catabolite repression by glucose of expression of other genes (e.g., Entian, 1980; Ma & Botstein, 1986; Rose et al., 1991). Two other isoenzymes, hexokinase 1 (gene *HXK1*) and glucokinase (gene *GLK1*), are also singly adequate for growth on glucose, but their normal expression is highest on other carbon sources (Lobo & Maitra, 1977; Walsh et al., 1991).

In two known circumstances, hexokinase 2 and also hexokinase 1 are phosphorylated. The first situation is in derepression, when they appear to be the predominant phosphoproteins of yeast (Vojtek & Fraenkel, 1990). For hexokinase 2, the phosphorylated residue is serine-15 (*in vivo* phosphorylation site), which belongs to one of two sequence motifs (amino acids 12−15 and 382−385) resembling protein kinase A targets (Kriegel et al., 1994). *In vitro* phosphorylation by protein kinase A was observed at serine-15 for the wild-type enzyme, and a hexokinase 2 alanine-15 substitution mutant showed no observable phosphorylation either *in vivo* or by protein kinase A *in vitro* (Kriegel et al., 1994). Neither the function of the *in vivo* phosphorylation nor the enzyme responsible *in vivo* is known. *In vitro* studies have revealed no clear kinetic differences between unphosphorylated, phosphorylated, and alanine-15 mutant enzymes (Kriegel et al., 1994).

The second situation of hexokinase phosphorylation in *Saccharomyces cerevisiae* is associated with inactivation in the presence of the nonphosphorylateable glucose analogs D-xylose or D-lyxose. Observed first *in vivo* (Dela Fuente et al., 1970), *in vitro* studies then showed it to be an autophosphorylation [e.g., phosphate incorporation in hexokinase 2 (Fernandez et al., 1986); reactivation by phosphatase treatment of hexokinase 1 (Menezes & Pudles, 1976)]. Direct information on the site is uncertain. One preliminary report (Kuromizu et al., 1979) implicated serine-162 (by current notation) in hexokinase 1, while another (Moreno et al., 1992) suggested serine-79 for hexokinase 2. [The two enzymes have considerable sequence identity, including serines at positions 79, 158, and 162 (Fröhlich et al., 1985; Kopetzki et al., 1985; Stachelek et al., 1986).] Bennett and Steitz (1980) suggested on a structural basis that the residue was likely to be serine-158.

The fact that xylose-dependent autophosphorylation is an inactivation while *in vivo* phosphorylation in derepression is not makes the two phenomena different. And indeed, autophosphorylation is still observed with the hexokinase 2 alanine-15 mutant enzyme (unpublished data, this labroatory).

The present work identifies the site of autophosphorylative inactivation of yeast hexokinase 2 as serine-158 and describes basic kinetic properties of mutant enzymes constructed by substituting alanine, cysteine, and glutamate, respectively, for that serine residue. The reversibility of the inactivation process by enzymatic dephosphorylation of phosphoserine-158 hexokinase 2 accomplished by protein phosphatase-2A is demonstrated.

† Supported by DFG Grant Kr 1162/3-1 (to T.K.).

* To whom correspondence should be addressed. Phone: (49) (351) 8832-871. Fax: (49) (351) 8832-875.

‡ Present address: Max-Delbrück-Centrum für Molekulare Medizin, Proteinchemie, D-13122 Berlin, Germany.

§ Present address: Max-Delbrück-Centrum für Molekulare Medizin, Proteinkristallisation, D-13122 Berlin, Germany.

|| Present address: Department of Genetics, Harvard Medical School, Boston, MA 02115.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1997.

[1] Abbreviation: hexokinase, ATP:D-hexose 6-phosphotransferase (EC 2.7.1.1).

S0006-2960(96)02364-1 CCC: $14.00

EXPERIMENTAL PROCEDURES

Strains and Plasmids. DFY632 (*hxk1::LEU2 hxk2::LEU2 glk1::LEU2 lys1-1 leu2-1 ura3-52*), a triple kinase mutant strain of *Saccharomyces cerevisiae* (Vojtek & Fraenkel, 1990), was used for wild-type and mutant hexokinase 2 expression. Plasmid pAV101 carries the hexokinase 2 gene of *Saccharomyces cerevisiae* as a *Bam*HI−*Pvu*II fragment cloned into YEp24. The construction of mutant plasmids pAV101(S158A), pAV101(S158C), and pAV101(S158E) is described below. *Escherichia coli* strain HB101 (Bolivar & Backman, 1979) was used as bacterial host for cloning.

Media. Yeast cells were grown as before (Kriegel et al., 1994) in either LP medium (low-phosphate yeast extract peptone medium; Rubin, 1973) or YNB-A medium [yeast nitrogen base (Sherman, 1991) with 0.2% casamino acids] with harvest at an OD_{600} of ~2. The concentration of glucose or galactose was 2%. *E. coli* growth was in tryptone yeast extract medium consisting of 10 g of Bacto tryptone, 5.0 g of yeast extract, and 8.0 g of NaCl per liter. Ampicillin concentration was 200 μg/mL when required.

Enzyme Assay (Standard Conditions). Hexokinase activity was determined spectrophotometrically in 50 mM triethanolamine hydrochloride buffer containing 5.0 mM glucose, 10 mM $MgCl_2$, 1.0 mM ATP, and 0.3 mM $NADP^+$ at pH 7.4 and 25 °C with glucose-6-phosphate dehydrogenase as auxiliary enzyme.

Enzyme Purification. Hexokinase 2 was prepared according to Kriegel et al. (1994). The procedure was modified by substituting rechromatography on a Waters 088044 type DEAE column for the hydroxylapatite step. Wild-type enzyme and mutant hexokinases were isolated from strains DFY632/pAV101, DFY632/pAV101(S158A), and DFY632/pAV101(S158C), respectively, after growth in LP medium supplemented with 2% glucose. Mutant enzyme from DFY632/pAV101(S158E) lacking hexokinase activity was isolated from cells grown in medium A containing 2% galactose and was identified by SDS−PAGE and peptide sequencing.

In Vitro Autophosphorylation−Inactivation. Purified hexokinase (5 μg) was incubated according to Fernandez et al. (1988) in 50 mM Hepes buffer containing 12 mM $MgCl_2$, 4.0 mM ATP, 100 mM D-xylose, and 1.0 mM DTT at pH 7.5 and 30 °C in a total volume of 30 μL. Inactivation was monitored by measuring enzyme activity during incubation. For radioactive labeling, 1 μCi of [γ-^{32}P]ATP (5.000 Ci/mmol, 10 mCi/mL) was added. The assay was proportionally upscaled when necessary.

Dephosphorylation−Reactivation of Autophosphorylated Hexokinase. Inactivated enzyme was equilibrated using a Bio-Spin type 6 column (BioRad) with 40 mM Hepes buffer, pH 7.4, containing 40 mM NaCl, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.1% Triton X-100. Treatment with phosphoserine/phosphothreonine-specific protein phosphatase-2A (Biomol; 5.0 units/mL of dephosphorylation mixture, 36 °C) was essentially according to Levin et al. (1995) leaving out $MnCl_2$.

Tryptic Digestion, Peptide Mapping, and Sequencing. Prior to proteolysis, hexokinase was bound to 10 mg of Poros 20 R1 reverse-phase material (PerSeptive Biosystems), washed with 1 mL of 0.1% trifluoroacetic acid, and eluted with 0.3 mL of 60% acetonitrile containing 0.1% trifluoroacetic acid. Volumes containing 200 pmol of protein were taken to dryness in a Speedvac centrifuge and were digested at 36 °C for 15 h with 0.5 μg of sequencing-grade modified trypsin (Promega) dissolved in 100 μL of 0.1 M Tris/HCl buffer, pH 8.1. Tryptic peptide maps were obtained by reverse-phase HPLC on a μRPC C2/C18 SC 2.1/10 column (Pharmacia Biotech) using the Smart system (Pharmacia Biotech). The flow rate was 100 μL/min at 26 °C employing a gradient of acetonitrile in 0.1% trifluoroacetic acid. Peptides of interest were loaded onto a Biobrene-coated glass filter fiber of a Procise sequencer (Applied Biosystems). For sequencing of ^{32}P-labeled autophosphorylated hexokinase, the radioactive peptide was bound to a 4 mm^2 piece of Sequelon membrane using the Sequelon AA Reagent Kit (Millipore). Sequencing was carried out on an Applied Biosystems type 477A sequencer employing a modified program using 50% aqueous methanol as solvent S3 instead of *n*-butyl chloride. Measurement of ^{32}P-initiated Cerenkov irradiation of the amino acid anilinothiazolinone derivatives was in a Beckman type LS 6000 LL liquid scintillation system.

Mass Spectrometry. Peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a Fisons/VG TofSpec instrument. A saturated solution of α-cyano-4-hydroxycinnamic acid in aqueous 40% acetonitrile/0.1% trifluoroacetic acid was used as matrix. Samples dissolved in 25% acetonitrile/0.1% trifluoroacetic acid (0.8 μL) and matrix (1.2 μL) were mixed directly on target, air-dried, and analyzed in the linear mode. Data of 20−50 laser shots were collected and signal-averaged before analysis.

Mutations. Point mutations causing changes in the hexokinase 2 gene at codon 158 from serine to alanine (S158A), cysteine (S158C), and glutamate (S158E), respectively, were constructed by oligonucleotide-directed mutagenesis employing the two-primer polymerase chain reaction technique (Mullis & Faloona, 1987; Saiki et al., 1988) as applied by Hemsley et al. (1989), resulting in complete plasmid amplification (inverse PCR). Antisense oligonucleotides were designed according to codon usage (Fröhlich et al., 1985). The back-to-back sense primer employed covers bp 485−513 of hexokinase 2. pAV101 was used as template. Mutations and ligation were confirmed by manual sequencing (Sanger, 1977) prior to transformation. Mutated plasmids were transformed into triple kinase mutant strain DFY632 according to Gietz et al. (1995), recovered from transformants (Hoffmann & Winston, 1987), and subjected to sequencing of the hexokinase 2 ORF using the ALFexpress DNA Sequencer (Pharmacia Biotech).

Chemicals, Molecular Biology Reagents. Media constituents were from Difco. [γ-^{32}P]ATP (5.000 Ci/mmol) was from Amersham-Buchler. Restriction enzymes and buffers were obtained from Boehringer. Mutagenizing PCR was done on a Gene ATAQ Controller (Pharmacia Biotech) employing the Expand High Fidelity System (Boehringer). 5′-Phosphorylated oligonucleotides were custom-made by Perkin-Elmer. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The Blunting End Ligation Set was an MBI Fermentas product. DNA was isolated from agarose gels by use of the QIAEX II Gel Extraction Kit (Qiagen). The Sequenase 2.0 DNA Sequencing Kit used was from U.S. Biochemicals. For automated sequencing, the Cy5 Auto Read Sequencing Kit (Pharmacia Biotech) was used.

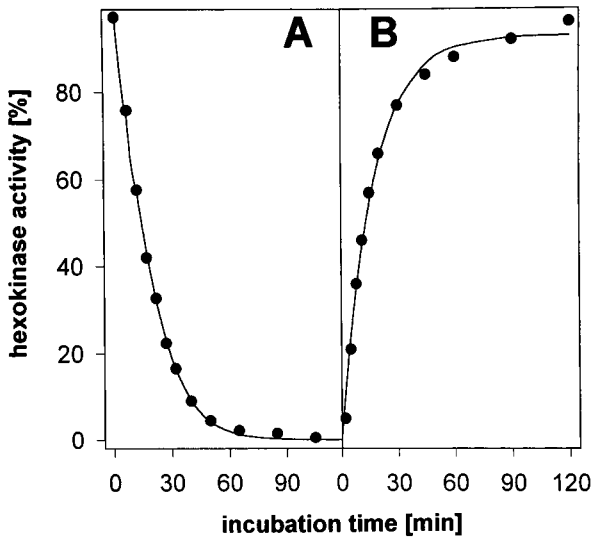

FIGURE 1: Time course of *in vitro* autophosphorylation−inactivation and dephosphorylation−reactivation of yeast hexokinase 2. (A) Wild-type enzyme Hxk2(S158) was incubated in the presence of 100 mM D-xylose with 4.0 mM MgATP at 30 °C as described (in a control, D-xylose was omitted). (B) Wild-type hexokinase completely inactivated by autophosphorylation−incubation for 120 min (cf. Figure 1A) was subjected to protein phosphatase-2A treatment at 36 °C as described (in a control, protein phosphatase-2A was omitted). Aliquots were assayed for hexokinase activity under standard assay conditions. Hexokinase activity is referred to the respective control the activity of which remained essentially unchanged during incubation.

RESULTS

Enzyme Purification, Autophosphorylation−Inactivation, and Dephosphorylation−Reactivation. The isolation of hexokinase 2 from yeast strain DFY632/pAV101 as described above gives enzyme preparations of more than 95% purity with a minimum specific catalytic activity of 350 units/mg (standard assay). The purified enzyme was completely inactivated (Figure 1A) and autophosphorylated from [γ-^{32}P]-ATP (Figure 4, lane 2) upon *in vitro* incubation with D-xylose; neither inactivation (control to Figure 1A, data not shown) nor phosphate incorporation (Figure 4, lane 1) was observed when the D-xylose was omitted. Incubation of the autophosphorylated enzyme with the phosphoserine/phosphothreonine-specific mammalian protein phosphatase-2A resulted in almost complete recovery of catalytic activity (Figure 1B).

Tryptic Peptide Analysis. The intent was to identify the autophosphorylation site of hexokinase 2 by analysis of tryptic peptides obtained by digestion of both nonphosphorylated and *in vitro* autophosphorylated enzyme. Figure 2 shows the complex reverse-phase HPLC separation pattern (a complete tryptic digestion would be expected to give 45 different peptides). The upper elution profile (I) is for nonphosphorylated hexokinase and the lower one (II) for the autophosphorylated enzyme. These profiles are extremely similar though not identical. At elution times of ~70 min, chromatogram I reproducibly exhibits a major peak (labeled "1") being almost absent in chromatogram II which has a new or considerably increased peak (labeled "2") eluting slightly earlier. Edman degradation of peak 1 gave the following sequence: H_2N-Ala-Phe-Ile-Asp-Glu-Gln-Phe-Pro-Gln-Gly-Ile-Ser-Glu-Pro-Ile-Pro-Leu-Gly-Phe-Thr-Phe-Ser-Phe-Pro-Ala-Ser-Gln-Asn-Lys-COOH. This sequencing coincides with amino acids 137−165 of hexokinase 2 predicted

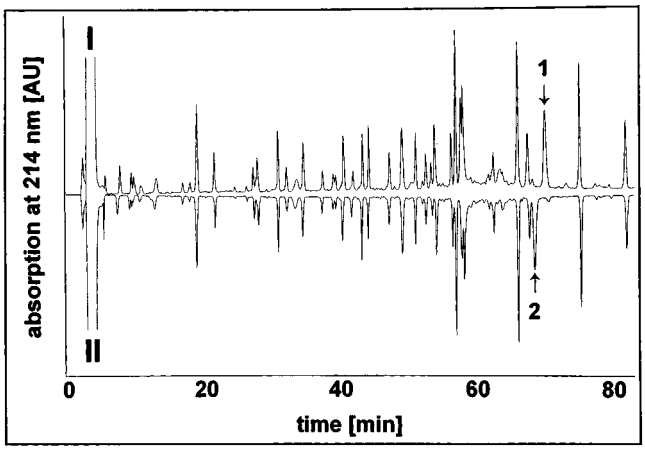

FIGURE 2: HPLC C_{18} reverse-phase separation of yeast hexokinase 2 tryptic peptides. Solid line: Absorption at 214 nm. Starting solvent (A): 0.1% trifluoroacetic acid in water. Developing solvent (B): 0.085% trifluoroacetic acid in acetonitrile. Flow rate at 26 °C: 100 μL/min. Gradient: 5% solvent B for 5 min, 5−45% solvent B in 80 min, 45−85% solvent B in 20 min, 85% solvent B for 10 min. Chromatogram I (upper panel): Nonphosphorylated hexokinase 2 (200 pmol). Chromatogram II (lower panel): Autophosphorylated enzyme (200 pmol). The arrows indicate the tryptic peptides, 1 and 2, subjected to further analysis.

from the DNA primary structure (Fröhlich et al., 1985; Stachelek et al., 1986). Sequencing of peptide 2 showed the same order of residues but with one exception: no signal was detected in the 22nd cycle of degradation corresponding to serine-158. Assuming serine phosphorylation to occur at that position, this finding is the expected result of the formation of an amino acid derivative that cannot be efficiently extracted from the sequencing support by the apolar organic solvents employed (Meyer et al., 1991). In addition, when peptide 2 from ^{32}P-labeled autophosphorylated enzyme was stepwise degraded (cf. Experimental Procedures), a radioactivity profile was obtained showing a peak at the very same degradation step (data not shown), thus confirming the result of conventional sequencing of the unlabeled phosphopeptide.

When peptides 1 and 2 were subjected to mass spectrometry (Figure 3), molecular masses of 3214.5 Da (upper panel) and 3294.4 Da (lower panel) were determined which are identical within the limits of error with the values calculated for this peptide carrying serine unsubstituted and phosphoserine, respectively.

Serine-158 Mutation and Mutant Enzyme. Site-directed mutagenesis was employed to verify the result of tryptic peptide analysis and to evaluate the role of serine-158 in hexokinase catalysis and regulation. Point mutations were constructed causing amino acid exchanges from serine to alanine, cysteine, and glutamate, respectively. Wild-type enzyme and the mutant hexokinases Hxk2(S158A), Hxk2(S158C), and Hxk2(S158E) were prepared (comparative DEAE rechromatography of these enzymes revealed almost identical elution behavior). Amino acid substitutions were confirmed by DNA sequencing as well as by mass spectrometry and sequencing of the tryptic peptides (data not shown). Assay of autophosphorylation (Figure 4) showed, by contrast to a prominent labeling of Hxk2(S158) observed in the presence of 100 mM D-xylose (lane 2), no labeling with the mutant enzymes (lanes 4, 6, and 8).

Table 1 summarizes the basic kinetic parameters, V_{MAX} and K_M, of wild-type hexokinase and mutant enzymes as

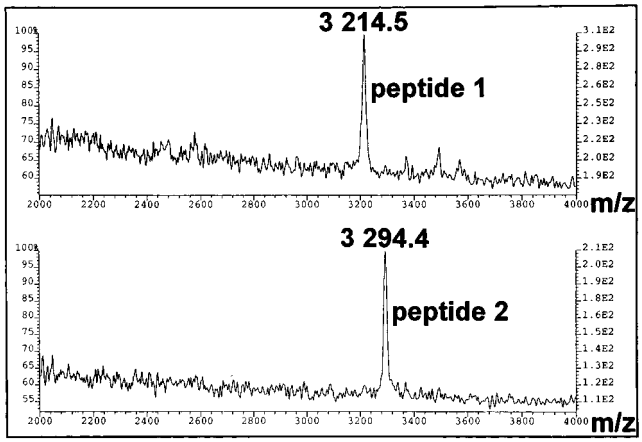

FIGURE 3: Mass analysis of tryptic peptides of yeast hexokinase 2. Solid line: Signal intensity. *m*/*z*: Mass/charge ratio. Peptides 1 and 2 from tryptic digests of autophosphorylated hexokinase and control enzyme (cf. Figure 2) were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using α-cyano-4-hydroxycinnamic acid as matrix. Mass peaks are labeled with centroided masses. Expected masses: 3213.6 Da for the nonphosphorylated peptide (cf. upper panel), 3293.6 Da for the autophosphorylated peptide (cf. lower panel).

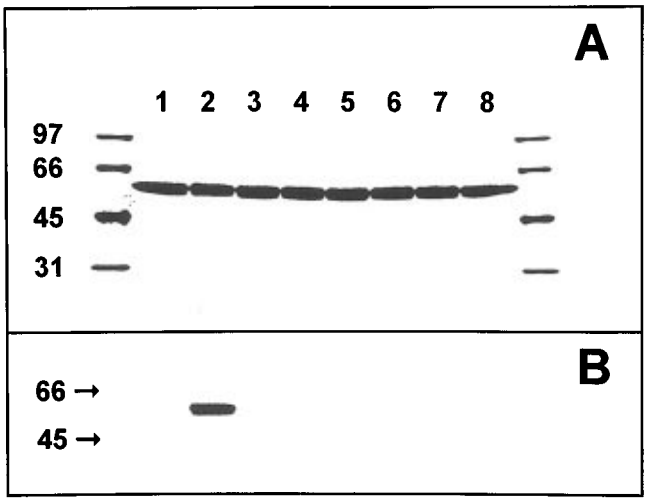

FIGURE 4: *In vitro* autophosphorylation of yeast hexokinase 2. Wild-type enzyme and mutant hexokinases were incubated for 120 min in the absence (odd-numbered lanes) and presence (even-numbered lanes) of 100 mM D-xylose with 4.0 mM MgATP/1 μCi of [γ-^{32}P]ATP as described. Samples treated immediately after radioactive labeling with SDS/DTT loading buffer at 95 °C for 5 min were subjected to SDS−PAGE (acrylamide gradient 4−20%; Laemmli, 1970) and protein staining (panel A). The dried gels were also analyzed by PhosphorImager (Molecular Dynamics) scanning (panel B). Wild-type hexokinase (lanes 1, 2); Hxk2-(S158A) (lanes 3, 4); Hxk2(S158C) (lanes 5, 6); Hxk2(S158E) (lanes 7, 8). MW standards: Migration indicated in kilodaltons.

determined for glucose and MgATP. The data calculated by nonlinear least-squares fitting reveal that substitution of serine-158 by alanine and cysteine, respectively, caused a decrease of catalytic activity by a factor of 20−70, as well as increased affinity for glucose (3−6-fold) and decreased affinity for ATP (3-fold). Like autophosphorylation itself (Figure 1), the introduction of a negatively charged group [glutamate-158 in Hxk2(S158E)] resulted in complete hexokinase inactivation.

DISCUSSION

Yeast and animal hexokinases are similarly susceptible to specific inactivation induced by D-xylose in the presence of MgATP (Dela Fuente, 1970; Lazo & Sols, 1979). For the hexokinases from *Saccharomyces cerevisiae*, it has been shown that this inactivation is mediated by protein phosphorylation (Cheng et al., 1973; Menezes & Pudles, 1976; Kuromizu et al., 1979; Fernandez et al., 1986). Bennett and Steitz (1980) already speculate that the binding of 5-carbon analogs of glucose results [according to Schirch and Wilson (1987)] in positioning of the serine-158 hydroxyl group close to the site normally occupied by the 6-hydroxyl of glucose. Indeed, this paper identifies serine-158 as the autophosphorylation−inactivation site of yeast hexokinase 2. It is known from X-ray diffraction (Bennett & Steitz, 1980), peptide mapping (Schirch & Wilson, 1987), and cDNA sequencing (Andreone et al., 1989) as well as from mutagenesis and functional studies (Arora et al., 1991; Xu et al., 1994, 1995) that this residue is highly conserved and critically involved in determining the conformational state, hexose affinity, and catalytic activity of yeast and mammalian hexose kinases. Thus, the drastic decrease of V_{MAX} (Table 1) as a consequence of serine-158 substitution by alanine and cysteine, respectively, might reflect an impaired interaction of hexokinase with MgATP at the substrate site but could also result from an increase of glucose affinity (Table 1) raising the energetic barrier of the transitional state in hexokinase catalysis.

Table 1: Effect of Mutational Substitution of Serine-158 on Yeast Hexokinase 2 Catalysis

Hxk-2	rel V_{max}[a] (%)	K_M(GLC)[b] (μmol/L)	K_M(ATP)[b] (μmol/L)	V_{max}/ K_M(GLC)[b]
wild-type	100	170	140	0.588
S158A	5.3	28	460	0.189
S158C	1.4	57	470	0.024
S158E	—[c]	—[c]	—[c]	—[c]

[a] Average specific catalytic activity of wild-type hexokinase 2 (Hxk-2) corresponding to a relative V_{max} of 100% was 380 units/mg. [b] Enzyme activity was measured with glucose as sugar substrate under standard assay conditions otherwise. Protein determination was according to Bradford (1976). Nonlinear regression analysis assumed Michaelis−Menten kinetics. Deviations from the mean did not exceed 12%. [c] No activity detectable.

Based on the different positions and interactions of serine-158 in the open and closed conformation of native yeast hexokinase, the low K_M for glucose of the mutant enzymes may be attributed to the elimination of side-chain interactions occurring in the open conformation of wild-type hexokinase which involves serine-158, asparagine-86, and lysine-176 (Bennett & Steitz, 1980; Xu et al., 1995). Accordingly, the "pseudo-closed" conformation readily formed by the catalytically active mutant hexokinases lacking the serine-158 hydroxyl residue would have the 3-hydroxyl group of glucose positioned closer to the carbonyl group of amino acid 158 and, hence, exhibit enhanced glucose affinity. Similar changes of V_{MAX} and K_M have been reported for tumor hexokinase (Arora et al., 1991) and for human glucokinase (Xu et al., 1995) when serine residues equivalent to yeast hexokinase serine-158 were mutationally substituted.

The finding that serine-158 substitution by alanine or cysteine, respectively, is compatible with residual catalytic activity (Table 1) supports the view that phosphoenzyme intermediates are not involved in hexokinase catalysis (Kuromizu et al., 1979). The serine−glutamate exchange apparently imitates serine-158 autophosphorylation by introducing a similar type of negatively charged residue which

might prevent glucose binding and/or transphosphorylation, both causing the complete loss of enzyme activity detected. Phosphoserine-158 as well as glutamate-158 might also form ionic interactions with lysine-176 which would stabilize the open conformation and thus complicate or prevent catalysis.

Finally, we note that reactivation of the autophosphorylated enzyme was achieved by the action of mammalian phosphoserine/phosphothreonine-specific protein phosphatase-2A (Figure 1B). The catalytic subunit of this enzyme is 80% identical to the protein phosphatase-2A-like proteins encodedby yeast genes *PPH21* and *PPH22* which are known to be essential in *Saccharomyces cerevisiae* (Ronne et al., 1991).

ACKNOWLEDGMENT

This work was initiated with Dan G. Fraenkel in the Department of Microbiology and Molecular Genetics at Harvard Medical School with the support of NIH Grant GM 21098. We thank Drago P. Clifton (Harvard) for technical assistance, Eva-Christina Müller (Max-Delbrück-Centrum für Molekulare Medizin) for support in mass spectrometry, and Wolfgang Schellenberger (Leipzig) for helpful discussions.

REFERENCES

Andreone, T. L., Printz, R. L., Pilkis, S. J., Magnuson, M. A., & Granner, D. K. (1989) *J. Biol. Chem. 264*, 363−369.

Arora, K. K., Filburn, C. R., & Pedersen, P. L. (1991) *J. Biol. Chem. 266*, 5359−5362.

Bennett, W. S., Jr., & Steitz, T. A. (1980) *J. Mol. Biol. 140*, 183−230.

Bolivar, F., & Backman, K. (1979) *Methods Enzymol. 68*, 245−267.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Cheng, L.-Y., Inagami, T., & Colowick, S. P. (1973) *Fed. Proc. 32*, 667 Abs.

DelaFuente, G. (1970). *Eur. J. Biochem. 16*, 240−243.

Entian, K.-D. (1980) *Mol. Gen. Genet. 178*, 633−637.

Fernandez, R., Herrero, P., Fernandez, M. T., & Moreno, F. (1986) *J. Gen. Microbiol. 132*, 3467−3472.

Fernandez, R., Herrero, P., Fernandez, E., Fernandez, T., Lopez-Boado, Y. S., & Moreno F. (1988) *J. Gen. Microbiol. 134*, 2493−2498.

Fröhlich, K.-U., Entian, K.-D., & Mecke, D. (1985) *Gene 36*, 105−111.

Gancedo, J. M., Clifton, D., & Fraenkel, D. G. (1977) *J. Biol. Chem. 252*, 4443−4444.

Gietz, R. D., Schiestl, R. H., Willems, A. R., & Woods, R. A. (1995) *Yeast 11*, 355−360.

Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G., & Galas, D. J. (1989) *Nucleic Acids Res. 17*, 6545−6551.

Herrero, P., Galindez, J., Ruiz, N., Martinez-Campa, C., & Moreno, F. (1995) *Yeast 11*, 137−144.

Hoffman, C. S., & Winston, F. (1987) *Gene 57*, 267−272.

Kopetzki, E., Entian, K.-D., & Mecke, D. (1985) *Gene 39*, 95−102.

Kriegel, T. M., Rush, J., Vojtek, A. B., Clifton, D., & Fraenkel, D. G. (1994) *Biochemistry 33*, 148−152.

Kuromizu, K., Cheng, L.-Y., Takahashi, N., Fletcher, P., Inagami, T., & Colowick, S. P. (1979) *Fed. Proc. 38*, 324 Abs.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Lazo, P. A., & Sols, A. (1979) *FEBS Lett. 98*, 88−90.

Levin, G., Keren, T., Peretz, T., Chikvashvili, D., Thornhill, W. B., & Lotan, I. (1995) *J. Biol. Chem. 270*, 14611−14618.

Lobo, Z., & Maitra, P. K. (1977) *Arch. Biochem. Biophys. 182*, 637−643.

Ma, H., & Botstein, D. (1986) *Mol. Cell. Biol. 6*, 4046−4652.

Menezes, L. C., & Pudles, J. (1976) *Eur. J. Biochem. 65*, 41−47.

Meyer, H. E., Hoffman-Posorske, E., & Heilmeyer, L. M. G., Jr. (1991) *Methods Enzymol. 201*, 169−185.

Moreno, F., Martinez-Campa, C., & Herrero, P. (1992) in *Control of gene expression in yeast* (Gancedo, C., & J. M., Eds.) pp 31−32, Ediciones Peninsular, Madrid.

Mullis, K. B., & Faloona, F. A. (1987) *Methods Enzymol. 155*, 335−350.

Ronne, H., Carlberg, M., Hu, G. Z., & Nehlin, J. O. (1991) *Mol. Cell. Biol. 11*, 4876−4884.

Rose, M., Albig, W., & Entian, K.-D. (1991) *Eur. J. Biochem. 199*, 511−518.

Rubin, G. (1973) *J. Biol. Chem. 248*, 3860−3875.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Ehrlich, H. A. (1988) *Science 239*, 487−491.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Schirch, D. M., & Wilson, J. E. (1987) *Arch. Biochem. Biophys. 257*, 1−12.

Sherman, F. (1991) *Methods Enzymol. 194*, 3−20.

Stachelek, C., Stachelek, J., Swan, J., Botstein, D., & Konigsberg, W. (1986) *Nucleic Acids Res. 14*, 945−963.

Vojtek, A. B., & Fraenkel, D. G. (1990) *Eur. J. Biochem. 190*, 371−375.

Walsh, R. B., Clifton, D., Horak, J., & Fraenkel, D. G. (1991) *Genetics 128*, 521−527.

Xu, L. Z., Zhang, W., Weber, I. T., Harrison, R. W., & Pilkis, S. J. (1994) *J. Biol. Chem. 69*, 27458−27465.

Xu, L. Z., Weber, I. T., Harrison, R. W., Gidh-Jain, M., & Pilkis, S. J. (1995) *Biochemistry 34*, 6083−6092.

BI9623643