In Vivo Phosphorylation Site of Hexokinase 2 in *Saccharomyces cerevisiae*[†]

Thomas M. Kriegel,[‡,§] John Rush,[∥] Anne B. Vojtek,[§,⊥] Drago Clifton,[§] and Dan G. Fraenkel[*,§]

Department of Microbiology and Molecular Genetics and Department of Genetics, Harvard Medical School, Boston Massachusetts 02115

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ABSTRACT: Yeast hexokinase 2 is known to be a phosphoprotein *in vivo*, prominently labeled from ^{32}P-inorganic phosphate after a shift of cells to medium with low glucose concentration [Vojtek, A. B., & Fraenkel D. G. (1990) *Eur. J. Biochem. 190,* 371–375]. The principal and perhaps sole site of phosphorylation is now identified as residue serine-15, by observation of a single tryptic peptide difference, its sequencing and size determination by mass spectrometry, and by mutation to alanine, which prevents phosphorylation *in vivo*. Although protein kinase A was unlikely to accomplish the phosphorylation *in vivo*, serine-15 does belong to a protein kinase A consensus phosphorylation sequence, and *in vitro* phosphorylation by protein kinase A at serine-15 could be shown by labeling and by peptide determination. The alanine-15 mutant enzyme was not phosphorylated *in vitro*.

Saccharomyces cerevisiae has three enzymes for the phosphorylation of glucose,[1] hexokinase 1 (P1, gene *HXK1*), hexokinase 2 (P2, gene *HXK2*), and glucokinase (gene *GLK1*). Mutant studies show that for growth on glucose any one of them is adequate (Lobo & Maitra, 1977; Walsh et al., 1991). In a wild-type strain, hexokinase 2 predominates in growth on glucose, hexokinase 1 appears late in growth, and glucokinase is in lower activity than the other two.

In vivo labeling with ^{32}P-inorganic phosphate and immune precipitation has shown that the two hexokinases are phosphoproteins, prominently labeled in conditions of derepression, i.e., in the 90 min after a shift from medium containing the usual 2% concentration of glucose to one containing 0.1%. Stability of label was consistent with serine or threonine phosphorylation. Mutants in the cyclic AMP effector pathway were altered in hexokinase labeling as if protein kinase A had a negative role (Vojtek & Fraenkel, 1990).

The present work shows by chemical identification and mutagenesis that for hexokinase 2 it is residue serine-15 which is phosphorylated *in vivo* in the regime employed. This residue, which is in a protein kinase A consensus sequence, Arg-Lys-Gly-Ser (Kennelly & Krebs, 1991; Denis et al., 1991; Gibbs et al., 1992), is also phosphorylated, albeit slowly, by protein kinase A *in vitro*, and the alanine-15 mutant protein (S15A) is not. A second possible protein kinase A target, serine-385 in an Arg-Arg-Leu-Ser sequence, is, as shown by mutagenesis to alanine (S385A), apparently uninvolved in phosphorylation.

MATERIALS AND METHODS

Strains and Plasmids. The *S. cerevisiae* triple kinase mutant strain DFY632 (*hxk1::LEU2 hxk2::LEU2 glk1::LEU2 lys1-1 leu2-1 ura3-52*) was described by Vojtek and Fraenkel (1990). pAV101 carries *HXK2* as a *Bam*HI–*Pvu*II fragment cloned into the corresponding sites (bp 3784 and 5475 according to New England Biolabs Catalog 1993/94, p 164) of multicopy plasmid YEp24 (Figure 1). DFY469 (α hxk1-1 hxk2::LEU2 glk1-1 leu2) and plasmid pBW111 carrying *HXK1* were described by Walsh et al. (1983). Mutant plasmid pAV101/HXK2(S15A) is decribed below, and mutant plasmid pAV101/HXK2(S385A) is described in Vojtek (1988). *Escherichia coli* strain HB101 (Bolivar & Backman, 1979) was used as bacterial host for cloning.

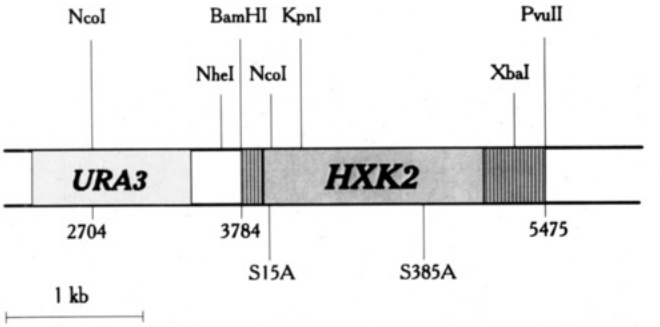

FIGURE 1: Plasmid pAV101. The restriction sites indicated (except *Nco*I) are unique in pAV101. Coordinates refer to the vector YEp24. Positions of mutations (serine–alanine exchanges) are also indicated.

Media and Growth. For enzyme preparation, most experiments employed the regime of growth in low-phosphate yeast extract peptone medium (LP medium; Rubin, 1973) with 2% glucose, harvest at A_{600} of 2 or less, resuspension in fresh medium with 2% or 0.1% glucose, respectively, and 90 min of further incubation before proceeding (Vojtek & Fraenkel, 1990). For labeling *in vivo*, ^{32}P-inorganic phosphate was included in the latter incubation. Growth otherwise generally employed YNB-A medium, which is yeast nitrogen base (Sherman, 1991) supplemented with 0.2% casamino acids and the indicated carbon sources at 2% concentration. For growth with glucose and galactose, harvest was also at A_{600} of 2 or less.

Enzyme Purification. Cells were collected by centrifugation, washed with and resuspended in 50 mM potassium phosphate buffer containing 2 mM ethylene diamine tetraacetate (EDTA), pH 7.4 (yeast extract buffer, 3 mL of buffer per gram of wet cell pellet) and disrupted in the presence

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* To whom correspondence should be addressed.

‡ Present address: Universität Leipzig, Universitätsbereich Medizin, Institut für Biochemie, Liebigstrasse 16, D-(O) 7010 Leipzig, Germany.

§ Department of Microbiology and Molecular Genetics.

∥ Department of Genetics.

⊥ Present address: Fred Hutchinson Cancer Research Center, Seattle WA 98104.

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

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of 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 5%(v/v) glycerol at 20 000 psi using an Aminco French pressure cell press. After centrifugation and treatment with protamine sulfate (2 mg/mL), the supernatant was fractionated by HPLC on a Waters no. 088044 DEAE cellulose column equilibrated with 20 mM Tris buffer, pH 7.4, containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 5% (v/v) glycerol, using a linear gradient of NaCl from 0 to 350 mM. After dialysis against 10 mM potassium phosphate, pH 7.0, containing 1 mM DTT, 1 mM PMSF, and 5% (v/v) glycerol, the enzyme was further purified by HPLC using a Bio-Rad no. 125-0177 hydroxlyapatite column. Hexokinase was eluted with a linear gradient of potassium phosphate, 10–200 mM, at pH 7.0. Hexokinase 2(S15A) was purified from strain DFY632/pAV101(S15A). Hexokinase 2(S385A) was purified from strain DFY632/pAV101(S385A). Hexokinase 1 was purified by the same procedure as hexokinase 2 from strain DFY469/pBW111-(*HXK1*).

Tryptic Peptide Mapping and Peptide Sequencing. Hexokinase 2 was recovered from suspensions saturated in $(NH_4)_2SO_4$ by centrifugation and was redissolved in water. Before digestion the protein concentration of each sample was measured by amino acid analysis using a Beckman 6300 amino acid analyzer. Volumes containing 0.6 nmol of protein were taken to dryness in a Speedvac centrifuge and were reduced, carboxamidomethylated, and digested with trypsin as described (Stone et al., 1990). Tryptic peptide maps were obtained by reverse-phase HPLC on a Vydac C_{18} column (0.46 × 25 cm; Stone et al., 1990), using either a modified Applied Biosystems/Kratos HPLC system or a Hewlett-Packard model 1090M HPLC unit. Peaks of interest were manually collected and sequenced on polybrene-treated filters with an Applied Biosystems model 477A protein sequencer, modified as described by Tempst and Riviere (1989).

Mass Spectrometry. Peptides were analyzed by matrix-assisted laser-desorption time-of-flight mass spectrometry using a Fisons/VG TofSpec instrument. Samples or standards were mixed with equal volumes of saturated solutions of α-cyano-4-hydroxycinnamic acid in 0.05% trifluoroacetic acid and 50% acetonitrile directly on targets, dried, and analyzed in positive ion mode. The instrument was calibrated with mixtures of gramicidin S (1142.0 Da) and bovine insulin (5734.5 Da) immediately before collecting data for peptide samples. Thirty spectra of each sample were collected and signal-averaged before analysis.

HXK2(S15A) Mutation. The hexokinase 2 point mutation causing a change at codon 15 from serine to alanine (S15A) was constructed by oligonucleotide-directed mutagenesis employing the two-primer polymerase chain reaction technique (Mullis & Faloona, 1987; Saiki et al., 1988). Oligonucleotides covering the *Nco*I sites in *HXK2* (oligo no. 1, 25 bases) and *URA3* (oligo no. 2, 24 bases) of pAV101 were made with an Applied Biosystems model 394 DNA synthesizer:

oligo #1 5' GGC ACA TCG GC↓C ATG GCA CCC TTT C 3'

oligo #2 5' GAC TGA TTT TTC↓**CAT GG**A GGG CAC 3'.

The *Nco*I recognition sequence (arrows indicate the position of cut) is printed in boldface, the A-to-C mutation in the antisense strand is underlined. Plasmid pAV101 was used as template. The mutated ~1.3 kb *HXK2* fragment obtained by *Nco*I digestion of the PCR product was substituted for the 1.3 kb *Nco*I fragment in plasmid pAV101, giving pAV101-(S15A), which was transformed into the triple kinase mutant strain DFY632. The mutant plasmid was recovered from the transformed strain DFY632/pAV101(S15A), and the mutation was confirmed by sequencing (Sanger et al., 1977).

In Vivo Labeling. The procedure was described by Vojtek and Fraenkel (1990). SDS–polyacrylamide gels (5% stacking gel, 10% separating gel; Laemmli, 1970) of ribonuclease-treated supernatants were analyzed by PhosphorImager (Molecular Dynamics) scanning.

In Vitro Labeling. The conditions were modified from Glass et al. (1978): Hexokinase (1–2 μg) was incubated at 30 °C for 30–180 min in a phosphorylation mixture (30 μL) containing 60 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.25 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA), 12 mM $MgCl_2$, 4 mM DTT, 250 μM ATP (containing 1 μCi of [γ-^{32}P]ATP), 0.1 μg of bovine serum albumin, and 6.5–100 ng of cAMP-protein kinase catalytic subunit at pH 6.9. Samples treated with SDS/DTT loading dye buffer at 95 °C/5 min were applied to SDS–PAGE. Gels were analyzed by PhosphorImager scanning employing internal ^{32}P standards or by radioautography.

Chemicals. Media constituents were from Difco (Detroit, MI). ^{32}P-inorganic phosphate and [γ-^{32}P]ATP were from Amersham (Arlington Heights, IL). TPCK-treated trypsin was a product of Worthington Biochemicals (Freehold, NJ), and DTT and iodoacetamide were from Calbiochem (San Diego, CA). cAMP-protein kinase catalytic subunit (bovine heart) was from E. G. Krebs or purchased from Sigma (St. Louis, MO). The Tpk-1 yeast cAMP-protein kinase catalytic subunit was from M. J. Zoller (Zoller et al., 1988). Restriction enzymes and buffers were obtained from Boehringer (Mannheim, Germany) or Sigma. GeneAmp polymerase chain reaction reagent kit with AmpliTaq DNA polymerase was from Perkin Elmer Cetus (Norwalk, CT). The Sequenase version 2.0 DNA sequencing kit was obtained from U.S. Biochemicals (Cleveland, OH). The DNA ligation kit used was a Takara Biochemical (Berkeley, CA) product.

RESULTS

Enzyme Purification. The initial intent was to compare peptide maps of (unlabeled) hexokinase 2 obtained from the same conditions employed previously (Vojtek & Fraenkel, 1990), i.e., a 90-min incubation in enriched medium depleted for inorganic phosphate (LP medium) and containing 2% or 0.1% glucose (known low and high labeling, respectively, by ^{32}P-inorganic phosphate). We used a triple kinase mutant strain (*hxk1 hxk2 glk1*) carrying *HXK2* in multicopy and hence hexokinase 2 in large amount. With this strain (DFY632/pAV101), chromatography on DEAE of a protamine sulfate treated crude extract gives an enzyme fraction of at least 80% purity, and a second chromatographic step on hydroxylapatite improves the SDS–PAGE pattern and removes contaminating protein kinase A activity. Unexpectedly, while previous use of this protocol with enzyme obtained from exponential growth in the usual enriched medium with 2% glucose gave a single peak of protein and activity in hydroxylapatite chromatography, as did the enzyme from the incubation in LP medium with 2% glucose (Figure 2, lower panel, peak I, corresponding to fraction HA-I), the enzyme obtained from the incubation with 0.1% glucose gave two protein and activity peaks, the first in the familiar position and a second (Figure 2, upper panel, peak II, fraction HA-II) eluting several fractions later.

Peptide Separation. Fractions HA-I and HA-II from hydroxylapatite chromatography (Figure 2, upper panel) were subjected to tryptic digestion and reverse-phase chromatog-

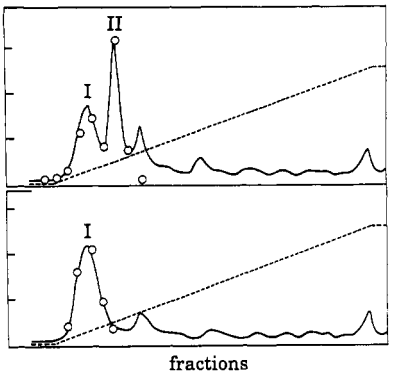

FIGURE 2: HPLC hydroxylapatite chromatography of hexokinase 2. Hexokinase-containing fractions from DEAE cellulose were chromatographed on hydroxylapatite. Elution at pH 7.0 used a linear gradient of potassium phosphate, 10–200 mM (dashed line). Solid line: absorption at 280 nm (linear scale). Open circles: hexokinase activity (linear scale). (Lower panel) Hexokinase 2 from cells incubated 90 min in LP medium with 2% glucose. (Upper panel) Hexokinase 2 from cells incubated 90 min inLP medium with 0.1% glucose.

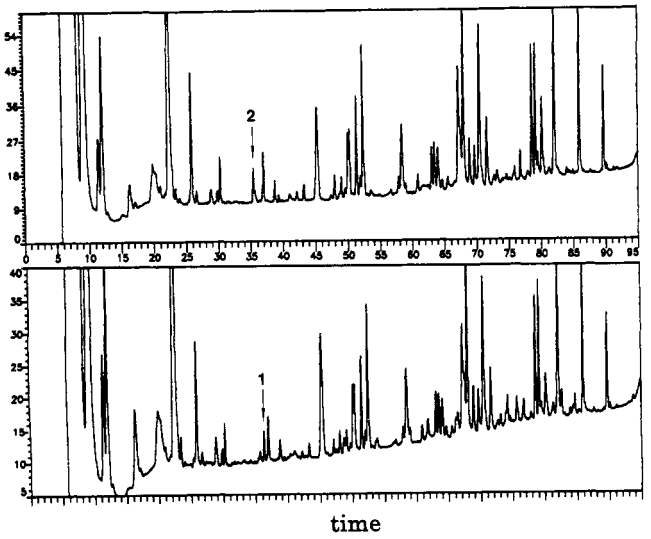

FIGURE 3: HPLC C_{18} reverse-phase separation of hexokinase 2 tryptic peptides. Solid line: absorption at 214 nm. Starting solvent (A): 0.060% trifluoroacetic acid in water. Developing solvent (B): 0.054% trifluoroacetic acid in 80% acetonitrile. Flow rate at 36 °C: 0.5 mL/min. Gradient: 2% solvent B for 3 min, 2–37% solvent B in 60 min, 37–75% solvent B in 35 min, 75–98% solvent B in 10 min, 98% solvent B for 10 min. The time scale (min) refers to both panels. (Lower panel): Hydroxylapatite fraction HA-I (300 pmol; Figure 2, upper panel, peak I). (Upper panel): Hydroxylapatite fraction HA-II (300 pmol; Figure 2, upper panel, peak II). The arrows indicate the tryptic peptides, 1 and 2, subjected to further analysis.

raphy (Figure 3, lower and upper panels, respectively). Despite the complexity of the separation pattern (a complete tryptic digestion would be expected to give 47 different peptides), the two chromatograms were remarkably similar but for a difference at ca. 35-min elution, with one peak (labeled "1" in Figure 3) from fraction HA-I almost absent in the digest from fraction HA-II, which had a new or considerably increased peak eluting a little earlier (labeled "2" in Figure 3). This change in profiles of the tryptic digests was observed with enzyme obtained from two different experiments, whereas enzyme prepared from cells in LP medium with 2% glucose (Figure 2, lower panel) or from the usual high phosphate medium with 2% glucose, which also gave the single HA-I peak on hydroxylapatite, had tryptic digests profiles essentially identical with that shown in the lower panel of Figure 3. Their 35-min region is shown in Figure 4 (panels A–C).

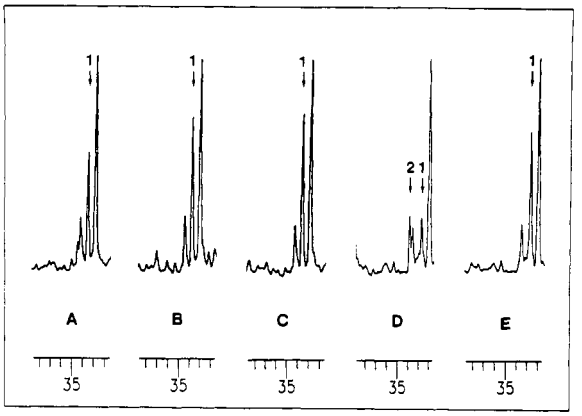

FIGURE 4: 35-min region of HPLC C_{18} reverse-phase separation patterns. The tryptic digests of 200 pmol of HA-I-type enzyme were from cells grown in YNB-A medium with 2% galactose (A), in YNB-A medium with 2% glucose (B), and in LP medium with 2% glucose (C). Pretreatment *in vitro* with ATP and yeast Tpk-1 cAMP protein kinase of a sample analogous to B is shown in lanes D (complete incubation) and E (the protein kinase omitted).

Edman degradation showed peak 1 from Figure 3 to have the sequence:

H_2N-Lys-Gly-Ser-Met-Ala-Asp-Val-Pro-Lys-COOH

This sequence corresponds to residues 13–21 predicted from the nucleotide sequence of the *HXK2* gene (Fröhlich et al., 1985; Stachelek et al., 1986). Peak 2 from Figure 3 gave the same order of residues but no signal at the third cycle of degradation. This is the expected result of the conversion of serine to serine phosphate, producing a derivative that cannot be efficiently extracted from the sequencing support by the apolar organic solvents employed (Meyer et al., 1991). Mass spectrometry of the two peptides, 1 and 2, respectively, showed the nonapeptide masses expected, 936.6 Da with serine unsubstituted and 1015.3 Da with phosphoserine (Figure 5).

Alanine-15 Mutant. A mutant gene, *HXK2(S15A)*, was made and enzyme prepared after the usual regime of culture, i.e., incubation in the LP medium with 2% or 0.1% glucose. Both hydroxylapatite profiles gave only peak-I enzyme. The alanine substitution was confirmed by sequencing of the tryptic peptide (data not shown).

In vivo labeling (Figure 6), i.e., the same regime but including ^{32}P-inorganic phosphate, showed essentially no labeling of hexokinase 2(S15A) (size ~50 kDa) from either 2% or 0.1% glucose (lanes 3 and 4, respectively) as compared with the prominent labeling of the wild-type enzyme (lanes 1 and 2) in the same regime from 0.1% glucose. Hexokinase activities of the two strains were similar.

In Vitro Phosphorylation with Protein Kinase A. Inspection of the hexokinase 2 gene sequence (Fröhlich et al., 1985; Stachelek et al., 1986) shows at least two sites in hexokinase 2 with similarity to a protein kinase A consensus sequence (Kennelly & Krebs, 1991; Denis et al., 1991; Gibbs et al., 1992): Arg-Lys-Gly-Ser (serine-15) and Arg-Arg-Leu-Ser (serine-385). Mutational change of serine-385 to alanine-385 did not alter *in vivo* phosphorylation (Vojtek, 1988), and phosphorylation *in vitro* with protein kinase A was found for hexokinase 2, hexokinase 2 mutant S385A, as well as hexokinase 1 (Figure 7, upper panel). [Hexokinase 1 has considerable sequence similarity with hexokinase 2, including the same 15 N-terminal residues, but a cysteine at the position equivalent to serine-385 (Kopetzki et al., 1985; Fröhlich et al., 1985; Stachelek et al., 1986).] The S-form of hexokinase 2, which arises by proteolysis of the N-terminal 12 amino

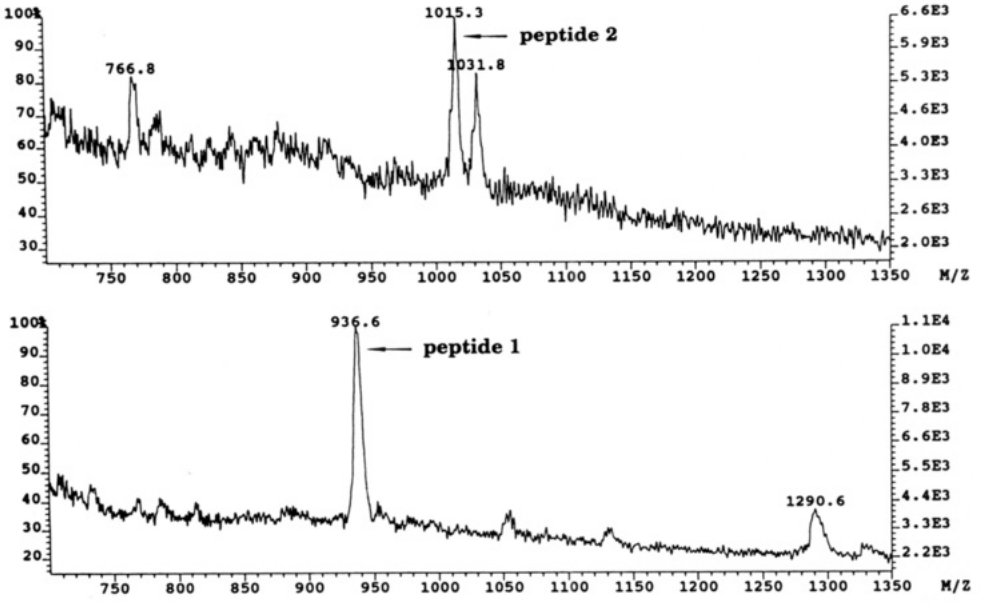

FIGURE 5: Mass analysis of yeast hexokinase 2 tryptic peptides. Solid line: Signal intensity. m/z: Mass/charge ratio. Peptides 1 and 2 (see Figure 3) from tryptic digests of hydroxylapatite fractions HA-I and HA-II (see Figure 2) were analyzed by laser-desorption mass spectrometry using α-cyano-4-hydroxycinnamic acid as a matrix. Raw, unsmoothed spectra are shown. Spectral peaks are labeled with centroided masses. Expected masses: 933.1 Da for the nonphosphorylated, 1013.1-Da for the phosphorylated peptide. The 1031.7-Da component may result from Met-16 oxidation during sample preparation.

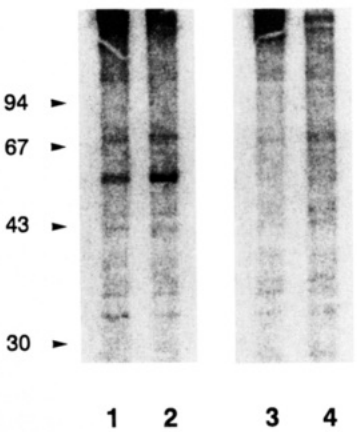

FIGURE 6: *In vivo* labeling of yeast hexokinase 2. DFY632/pAV101, lanes 1 and 2; DFY632/pAV101(S15A), lanes 3 and 4. Cells initially grown in LP medium with 2% glucose were transferred to and incubated for 90 min at 30 °C in LP medium containing ^{32}P-labeled inorganic phosphate and 2% glucose (lanes 1 and 3) or 0.1% glucose (lanes 2 and 4). Cell extracts prepared as described (see Materials and Methods) were subjected to SDS–PAGE. Dried gels were scanned using a PhosphorImager. Migration of standard is indicated in kilodaltons (left column).

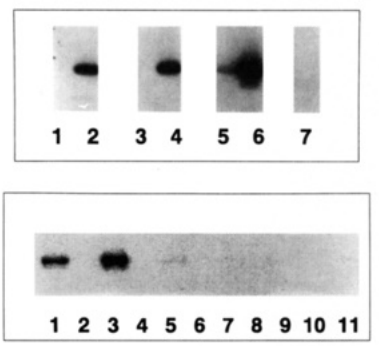

FIGURE 7: *In vitro* phosphorylation. (Upper panel) Phosphorylation with [γ-^{32}P]ATP employed beef heart cAMP-protein kinase catalytic subunit (6.5 ng, from E. G. Krebs), 180-min incubation at 30 °C (see Materials and Methods). The hexokinases were purified from cultures grown in YNB-A medium with glycerol plus lactate, and hydroxylapatite fractions were employed except for mutant S385A, which was material from DEAE. Lanes 1 and 2, hexokinase 1, 1 μg (strain DFY469/pBW111); lanes 3 and 4, hexokinase 2, 1 μg (strain DFY632/pAV101); lanes 5 and 6, hexokinase 2 mutant S385A, 5 μg. In lanes 1, 3, and 5, the protein kinase was omitted, and in lane 7 hexokinase was omitted. (Lower panel) Phosphorylation employed beef heart cAMP-protein kinase catalytic subunit (20 ng, Sigma product), 30-min incubation at 30 °C. The hexokinases were prepared from cultures grown in LP medium with glucose as indicated, and 2 μg of hydroxylapatite (HA) fractions was employed. Lanes 1 and 2, hexokinase 2 from 2% glucose (strain DFY632/pAV101), fraction HA-I; lanes 3 and 4, (same strain) after shift to 0.1% glucose, fraction HA-I; and lanes 5 and 6, (same strain) after shift to 0.1% glucose, fraction HA-II. Lanes 7 and 8, hexokinase 2 mutant S15A [strain DFY632/pAV101(S15A)] from 2% glucose, fraction HA-I; lanes 9 and 10, (same strain) after shift to 0.1% glucose, fraction HA-I. In lanes 2, 4, 6, 8, and 10 the protein kinase was omitted, and in lane 11 hexokinase was omitted.

acids (Schmidt & Colowick, 1973), was not phosphorylated by protein kinase A *in vitro* (Vojtek, 1988).

Since the present results showed that the residue of hexokinase 2 phosphorylated *in vivo* was in fact serine-15, *in vitro* phosphorylation of the S15A mutant enzyme was tested. Figure 7 (lower panel) shows phosphorylation of wild-type hexokinase 2 fraction HA-I (lanes 1 and 3), but not of fraction HA-II (lane 5), and no phosphorylation of hexokinase 2 mutant S15A (lanes 7 and 9). Similar results (not shown) were obtained with 20 ng of the yeast Tpk-1 cAMP protein kinase, except that this kinase also was autophosphorylated, as reported (Zoller et al., 1988).

Tryptic digestion of the wild-type enzyme fraction HA-I after incubation with Tpk-1 type protein kinase A and (unlabeled) ATP showed a shift similar to that resulting from *in vivo* phosphorylation, i.e., reduction of peak 1 (the serine-15 peptide) and appearance of peak 2 (the phosphoserine-15 peptide; Figure 4, panels D and E).

DISCUSSION

This paper identifies serine-15 as a primary residue phosphorylated *in vivo* in yeast hexokinase 2. The loss of labeling *in vivo* in the alanine-15 mutant suggests it may even be the only site or be required for phosphorylation of other

sites. The extent of phosphorylation can be quite high, since the phosphorylated form of the enzyme observed on hydroxylapatite chromatography (fraction HA-II) comprised ca. 50% of the protein (Figure 2, upper panel). In cells grown with 2% glucose, neither the hydroxylapatite form II of the enzyme nor the phosphopeptide in question were clearly observed (Figure 4, panels B and C), but considering the methods it might be present in smaller amount, consistent with low but observable labeling *in vivo* of wild-type but not of S15A mutant enzyme from that condition (Figure 6).

Several issues have not been addressed in the present work. First, we have used enzyme from cells containing it in unusually high amount (or, for *in vivo* labeling, the cells themselves), and the situation in normal cells is not known, other than the fact that *in vivo* labeling with ^{32}P-inorganic phosphate did identify hexokinase 2 as a prominent phosphoprotein (Vojtek & Fraenkel, 1990). Second, major phosphorylation has only been shown for cells from the low phosphate/low glucose regime, and it is not yet known for cells treated in other ways. Third, there is no knowledge of function of the modification. Limited assessment of V_{max} and K_M has not revealed large differences between wild-type enzyme from several conditions, or for the S15A or S385A mutant enzymes. Indeed, extensive earlier work on the S-form, which has been N-terminally truncated *in vitro*, did not show differences either (Easterby & Rosemeyer, 1972), nor were they seen with a 15 amino acid deletion obtained genetically (Ma & Botstein, 1989). The latter work also pointed to this region (and by implication serine-15 itself) not being required for glucose repression. To suggest other functions would be even more speculative. Fourth, the mechanism of *in vivo* phosphorylation is unknown. A role for cAMP-dependent protein kinase(s) in hexokinase 2 phosphorylation is problematical. As mentioned, *in vivo* experiments with mutants *bcy1* or *tpk1^{w1}* (Vojtek & Fraenkel, 1990) suggested a negative role for yeast protein kinase A, and yet, serine-15 seems to be the major, and perhaps the only, site of protein kinase A phosphorylation *in vitro*. It should be remarked, however, that the rate of protein kinase A phosphorylation *in vitro* is low and need not reflect a physiological activity.

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