Regulation of Phosphotransferase Activity of Hexokinase 2 from *Saccharomyces cerevisiae* by Modification at Serine-14†

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ABSTRACT: Isoenzyme 2 of hexokinase functions in sugar sensing and glucose repression in *Saccharomyces cerevisiae*. The degree of in vivo phosphorylation of hexokinase 2 at serine-14 is inversely related to the extracellular glucose concentration [Vojtek, A. B., and Fraenkel, D. G. (1990) *Eur. J. Biochem. 190*, 371−375]; however, a physiological role of the modification causing the dissociation of the dimeric enzyme in vitro [as effected by a serine-glutamate exchange at position 14; Behlke et al. (1998) *Biochemistry 37*, 11989−11995] is unclear. This paper describes a comparative stopped-flow kinetic and sedimentation equilibrium analysis performed with native unphosphorylated hexokinase 2 and a permanently pseudophosphorylated glutamate-14 mutant enzyme to determine the functional consequences of phosphorylation-induced enzyme dissociation. The use of a dye-linked hexokinase assay monitoring proton generation allowed the investigation of the kinetics of glucose phosphorylation over a wide range of enzyme concentrations. The kinetic data indicated that monomeric hexokinase represents the high-affinity form of isoenzyme 2 for both glycolytic substrates. Inhibition of glucose phosphorylation by ATP [Moreno et al. (1986) *Eur. J. Biochem. 161*, 565−569] was only observed at a low enzyme concentration, whereas no inhibition was detected at the high concentration of hexokinase 2 presumed to occur in the cell. Pseudophosphorylation by glutamate substitution for serine-14 increased substrate affinity at high enzyme concentration and stimulated the autophosphorylation of isoenzyme 2. The possible role of hexokinase 2 in vivo phosphorylation at serine-14 in glucose signaling is discussed.

The yeast *Saccharomyces cerevisiae* has three enzymes that phosphorylate glucose: glucokinase (encoded by *GLK1*; protein Glk1p), hexokinase 1 (*HXK1*; Hxk1p), and hexokinase 2 (*HXK2*; Hxk2p) (*5*, *6*). Isoenzyme 2 of hexokinase is the predominant hexose kinase in glucose fermentation. Glucokinase or hexokinase 1 alone is sufficient for growth on glucose; however, their expression is highest on other carbon sources (*7*−*13*). The three kinases contribute to the adaptation of metabolism to carbon source availability as reflected by glucose repression of certain genes and their derepression when glucose is exhausted (for reviews, see refs *14*−*16*). The hexokinases are specifically involved in glucose repression with isoenzyme 2 being required for the establishment of long-term repression by glucose; either hexokinase 2 or hexokinase 1 is sufficient for long-term repression by fructose and short-term repression by either sugar (*10*, *17*, *18*). Through their catalytic function in hexose phosphorylation, the three kinases are capable of triggering Ras-cAMP signaling (*19*, *20*).

Phosphorylation of isoenzyme 2 of hexokinase from *Saccharomyces cerevisiae*[1,2] at serine-14[3] occurs in vivo at low but not at high extracellular glucose concentrations (*1*, *21*−*23*) as well as in growth on other carbon sources such as galactose, raffinose or ethanol (*1*, *22*). Serine-14 is part of a sequence motif resembling the target structure of cAMP-dependent phosphorylation (*24*−*26*). Modification of this residue by phosphorylation greatly weakens subunit interaction in vitro but, like amino acid substitutions at this position, does not affect the steady-state kinetics of glucose phosphorylation (*27*). Remarkably, glucose repression is impaired

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

[2] If not specified, the term "hexokinase" refers to isoenzyme 2 of hexokinase from *Saccharomyces cerevisiae*.

[3] The in vivo phosphorylation site (*1*) and the autophosphorylation-inactivation site (*2*) of yeast hexokinase 2 are serine-14 and serine-157, respectively (cf. SwissProt P. A. No. P04807). According to *HXK2* DNA sequence (*3*, *4*), these positions correspond to codons 15 and 158, respectively.

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in an alanine-14 mutant strain (*22*) but not affected by N-terminal truncation of hexokinase removing serine-14 (*28*). The situation is different for the site of autophosphorylation-inactivation (*2*). Phosphorylation of serine-157[3] occurs in vitro in the presence of D-xylose, which is a five carbon analogue of glucose that cannot be phosphorylated, and abolishes glucose kinase activity. Substitution of serine-157 diminishes or even abolishes enzyme activity (*2*, *20*), but neither pseudophosphorylation by serine-glutamate exchange nor other substitution of this amino acid affects subunit interaction (*27*). Modification of serine-157 is not known to occur physiologically, but phosphorylation of this residue is likely to be caused in vivo by D-xylose and to result in a derepression of glucose-repressible enzymes even at high extracellular glucose concentrations (*20*, *29*−*32*).

Several enzymes are presumed to be involved in hexokinase interconversion by phosphorylation−dephosphorylation. Modification of serine-14 is accomplished in vitro by protein kinase A (*1*); however, a role for this enzyme in the in vivo phosphorylation of hexokinase is unlikely (*1*, *21*). According to genetic and phosphoproteome analyses, dephosphorylation of phosphoserine-14 hexokinase in vivo involves targeting of protein phosphatase 1 by Reg1p (*22*, *23*). Serine-157 is phosphorylated in vitro (*2*) by intramolecular catalysis (*32*), and phosphoserine-157 hexokinase is dephosphorylated and reactivated in vitro by protein phosphatase 2A (*2*).

We have previously hypothesized that the phosphorylation of hexokinase at serine-14 occurring in vivo when the extracellular glucose is limiting, and the subsequent dissociation of the homodimeric enzyme is part of a molecular mechanism that allows the cell to improve glucose utilization and enables hexokinase to participate in the signal transduction mediating glucose derepression (*27*). To experimentally address this question, we have now investigated the kinetic properties of native hexokinase at two different enzyme concentrations shown by sedimentation analysis to favor the predominance of either monomeric or dimeric enzyme. Glutamate-14 mutant hexokinase was employed as a chemically stable substitute of the phosphoserine-14 enzyme (*27*). N-terminally well-characterized preparations of both forms of hexokinase were used to exclude interference by N-terminal proteolysis (*33*) or covalent modification of serine-14 (*27*). The requirement to measure extremely high substrate turnover rates was met by the adaptation of a pH-dependent, dye-linked assay monitoring stoichiometric proton generation due to phosphoryl transfer by hexokinase employing the stopped-flow technique. The results prompted us to investigate the influence of serine-14 modification on hexokinase in vitro autophosphorylation and to observe an unexpected stimulation of the process. The novel finding of hexokinase modification at serine-14 resulting in regulation of substrate affinity and phosphoryl transfer is discussed in a hypothetical model of involvement of isoenzyme 2 in sugar sensing and glucose derepression in *S. cerevisiae*.

EXPERIMENTAL PROCEDURES

Purification of Hexokinase. The triple kinase mutant strain DFY632 (*hxk1*::*LEU2 hxk2*::*LEU2 glk1*::*LEU2 lys1-1 leu2-1 ura3-52*) of *S. cerevisiae* (*21*) was used for the expression and isolation of plasmid encoded serine-14 and glutamate-14 hexokinase. The construction of the mutation at amino acid position 14 is described elsewhere (*27*). Serine-14 hexokinase [Hxk2p (S14/S157), i.e., the nonphosphorylated wild-type enzyme] was isolated after growth in low phosphate yeast extract peptone medium (*34*) with 2% glucose in the first and second cultivation step (*1*). The strain expressing glutamate-14 hexokinase Hxk2p (S14E/S157) was grown in the usual yeast peptone medium (*35*) with 2% glucose without recultivation. For large scale purification of the Hxk2p (S14/S157) enzyme, the chromatographic procedure outlined in ref *1* was modified by substituting repeated separation on HiLoad 26/10 Q Sepharose HP (Amersham Pharmacia Biotech) for (diethylamino)ethyl (DEAE)[4] -cellulose and hydroxyapatite chromatography using buffer A (20 mM Tris/HCl containing 1.0 mM EDTA, pH 7.4) and buffer B (buffer A containing 1.0 M NaCl, linear gradient 0−350 mM) as the solvents. Hxk2p (S14E/S157) was purified employing Fractogel EMD DEAE-650 (S) 150−10 chromatography (Merck; buffers A and B as above), followed by HiLoad 26/10 Q Sepharose HP (Amersham Pharmacia Biotech; buffers A and B as above) and Bio-Silect SEC 125−5 (Bio-Rad Laboratories; buffer A containing 150 mM NaCl) or Superdex 75 HR 10/30 (Amersham Pharmacia Biotech; buffer A containing 150 mM NaCl) columns. DTT (1.0 mM) and PMSF (0.50 or 1.0 mM) were present throughout all purification steps. The purified enzymes were stored as ammonium sulfate suspensions at 90% saturation of the salt in the presence of 5.0 mM glucose at 2−4 °C.

Molecular Characterization of Hexokinase. The N-terminal integrity as well as the state of phosphorylation and the amino acid exchange at position 14 were checked by Edman degradation and mass spectrometric MS/MS sequencing, respectively, after tryptic digestion of the hexokinase protein as described (*2*, *27*).

Analytical Ultracentrifugation. Molecular mass studies were performed in a XL-A type analytical ultracentrifuge (Beckman Instruments) equipped with UV absorbance scanner optics. Sedimentation equilibrium distribution of hexokinase was analyzed using externally loaded six-channel centerpieces with 12 mm optical path length with the capacity to handle three solvent−solution pairs of 75 μL of liquid; 50 mM potassium phosphate buffer, pH 7.4, containing 1.0 mM EDTA, 1.0 mM PMSF, and 1.0 mM DTT was used as the solvent. To minimize error in the determination of association constants, equilibrium distributions corresponding to a broad range of loading enzyme concentrations (2−400 and 10−1000 μg/mL of serine-14 and glutamate-14 hexokinases, respectively) were analyzed. Sedimentation equilibrium was reached after 2 h of overspeed at 24 000 rpm, followed by an equilibrium speed of 20 000 rpm for 24−30 h at 10 °C. Depending on the loading enzyme concentration, the radial absorbance in each compartment was scanned at three different wavelengths between 230 and 300 nm, usually at 280, 285, and 290 nm, using the molar absorption coefficients indicated in ref *27*. Molecular mass calculations employed the simultaneous fitting of three radial absorbance

[4] Abbreviations: DEAE, (diethylamino)ethyl; DTT, D,L-dithiothreitol; EDTA, ethylenediaminetetraacetic acid (disodium salt); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TEA, triethanolamine.

distribution curves described by

$$A_r = A_{rm} \exp[MK(r^2 - r_m^2)] \quad (1)$$

with

$$K = [(1 - \rho\bar{\nu})\omega^2]/2RT \quad (2)$$

using our program Polymole (*36*). In this equation, r means solvent density, $\bar{\nu}$ is the partial specific volume, ω is the angular velocity, R is the gas constant, and T is the absolute temperature. A_r is the radial absorbance while A_{rm} represents the corresponding value at meniscus position. The program Polymole (*36*) allows the analysis of self-associating proteins including the determination of partial concentrations and association constants. The experimental data obtained with both hexokinase species were examined assuming monomer−dimer equilibria.

Hexokinase Assay (*Standard Method*). Glucose kinase activity of hexokinase was determined spectrophotometrically at 340 nm in 50 mM TEA/HCl buffer containing 10 mM $MgCl_2$, 5.0 mM glucose, 1.0 mM ATP, and 0.5 mM $NADP^+$ at pH 7.4 using glucose 6-phosphate dehydrogenase as an auxiliary enzyme at 25 °C.

Stopped-Flow Kinetic Analysis of Glucose Kinase Activity of Hexokinase. The acid−base dye-indicator linked assay (*37*) employed responds to the stoichiometric proton liberation catalyzed by hexokinase according to the following reaction scheme:

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose 6-phosphate} + \text{ADP} + \text{H}^+$$

$$p\text{-nitrophenolate}^- + \text{H}^+ \rightarrow p\text{-nitrophenol}$$

Kinetic experiments were carried out with an Applied Photophysics BioSequential DX.17 MV stopped-flow spectrometer. The decrease of absorbance due to *p*-nitrophenolate protonation was recorded at 400 nm. The optical path length was 10 mm. The slit widths of both monochromators were set to 5 mm. Photomultiplier voltage was adjusted using the water baseline. Prior to analysis, hexokinase was desalted and equilibrated on a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) with incomplete assay buffer consisting of 4.0 mM TEA/HCl, 10 mM $MgCl_2$, and 1.0 mM DTT at pH 7.4. The kinetic studies were carried out at 25 °C in a complete assay buffer obtained by supplementing the incomplete buffer with glucose, ATP, and *p*-nitrophenol(ate) and its subsequent mixing with hexokinase solution to give the final substrate and enzyme concentrations indicated. Determination of glucose kinase activity considered the initial part of the linear decrease in the kinetic traces (Figure 1A). Primary data treatment employed a linear regression of at least four traces each corresponding to a single stopped-flow experiment performed at a given substrate and enzyme concentration (Figure 1B). Nonlinear regression according to Michaelis−Menten assumed multiple substrate binding to best describe the dependence of enzyme activity on glucose; data indicating substrate inhibition by ATP were excluded from consideration in the determination of substrate affinity and maximum reaction rate (Figure 4; Table 2). Because the assay system contained buffering substances (TEA, ATP, bicarbonate used for ATP neutralization), only a fraction of the enzymatically generated protons

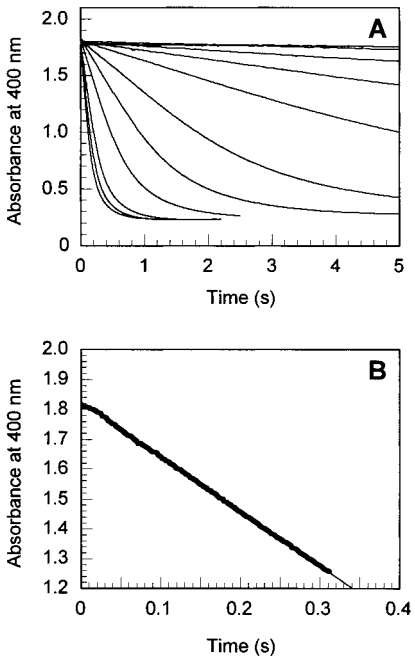

FIGURE 1: Determination of glucose kinase activity of yeast hexokinase 2 by stopped-flow *p*-nitrophenolate titration. (A) Measurements were carried out in 4.0 mM TEA/HCl buffer containing 10 mM $MgCl_2$, 1.0 mM DTT, 200 μM *p*-nitrophenol(ate), 5.0 mM glucose, and 2.0 mM ATP at pH 7.4. Reaction was started by mixing equal volumes of assay buffer and enzyme solution (100 μL each) in a stopped-flow machine and was monitored at 25 °C following the decrease of absorbance at 400 nm. Progress curves of product formation were obtained at final concentrations of glutamate-14 hexokinase ranging between 0.5 μg/mL and 1.0 μg/mL. (B) Demonstration of primary data (●) treatment employing linear regression (—). Initial reaction rate corresponds to a final concentration of serine-14 hexokinase of 750 μg/mL.

contributed to *p*-nitrophenolate protonation. This complication was taken into account by determining the relative absorption coefficient of *p*-nitrophenol(ate) by stopped-flow titration of the complete assay buffer with HCl at different concentrations of ATP (Figure 2). The primary data for this figure indicated a linear correlation between absorbance change and amount of protons formed/added when initial slopes corresponding to the formation of up to 0.5 mM glucose 6-phosphate were considered. Importantly, these data also demonstrated that proton distribution occurred much faster than proton formation.

In Vitro Autophosphorylation of Hexokinase. Serine-14 and glutamate-14 hexokinase were incubated with ATP according to ref *38* in 40 mM HEPES/NaOH buffer containing 10 mM $MgCl_2$ and 1.0 mM DTT at pH 7.4. Total ATP was a mixture of unlabeled nucleotide and [γ-^{32}P]ATP giving final assay concentrations of 20 μM and 100 mCi/L, respectively. The final concentration of enzyme was 100 μg/mL. Incubation time was 60 min at 30 °C. Reaction was terminated by sample treatment with SDS/DTT loading dye buffer at 95 °C for 5 min. Denatured samples were subjected to SDS−PAGE followed by PhosphorImager (Molecular Dynamics) scanning of the stained and dried gels.

Chemicals and Enzymes. Media constituents were from Difco. The dye-indicator *p*-nitrophenol was a Merck product. ATP (special quality disodium salt), glucose 6-phosphate (disodium salt), and auxiliary enzymes were from Boeh-

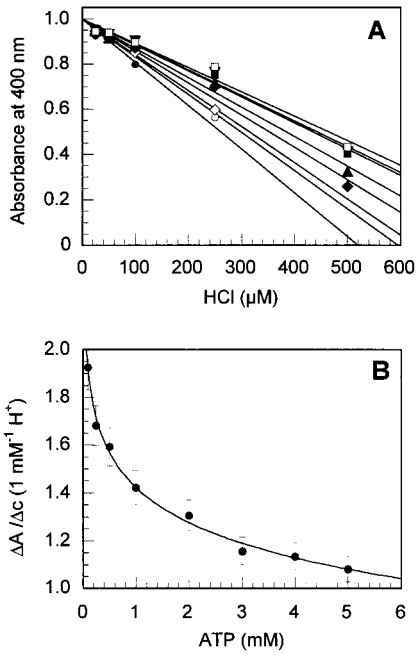

FIGURE 2: Determination of the relative absorption coefficient of *p*-nitrophenol(ate). (A) Titration of the complete assay buffer [4.0 mM TEA/HCl, 10 mM $MgCl_2$, 5.0 mM glucose, 200 μM *p*-nitrophenol(ate), ATP as indicated] with HCl (diluted with 4.0 mM TEA/HCl containing 10 mM $MgCl_2$) at pH 7.4 and 25 °C in a stopped-flow machine. The change of absorbance was monitored at 400 nm. Absorbance values reached after mixing equal volumes (100 μL each) of buffer and acid were plotted against the concentration of HCl using a normalized absorbance scale. The different symbols correspond to different concentrations of ATP (●, 0.10; ○, 0.25; ◇, 0.50; ◆, 1.0; ▲, 2.0; ■, 3.0; ▼, 4.0; and □, 5.0 mM). (B) Dependence of the relative absorption coefficient of *p*-nitrophenol(ate) on the concentration of ATP. The values of $\Delta A/\Delta c$(1 mM^{-1} H$^+$) indicating the absorbance change at 400 nm caused by the addition/generation of 1.0 mM H$^+$ were calculated from the slopes of the regression lines in panel A. Data were fitted by a logarithmic function providing the relative absorption coefficients that were used in the calculation of enzyme activity from the slope of the initial decrease of absorbance (see Figure 1B).

ringer/Roche. [γ-^{32}P]ATP (Isoblue stabilized nucleotide, >4.000 Ci/mmol) was purchased from ICN Biomedicals. All other substances used were analytical grade.

RESULTS

Molecular and Functional Properties of Wild-Type and Mutant Hexokinases. Serine-14 and glutamate-14 hexokinases were purified from French press extracts of yeast strains DFY632/pAV101 (S14/S157) and DFY632/pAV101 (S14E/S157), respectively. According to protein staining (Figure 6), these preparations were approximately 95% pure; their average specific catalytic activity was 350 U/mg (standard conditions).

N-terminal intactness, identity of the amino acid at position 14 and lack of serine-14 phosphorylation, respectively, were verified by Edman degradation and/or by mass spectrometric MS/MS sequencing to match type of strain and glucose availability in cell growth for all hexokinase preparations employed in this study.

Monomer−Dimer Equilibrium of Hexokinase. The effect of enzyme concentration and modification at amino acid position 14 on the oligomeric stability of hexokinase was analyzed by equilibrium sedimentation as a basis for a

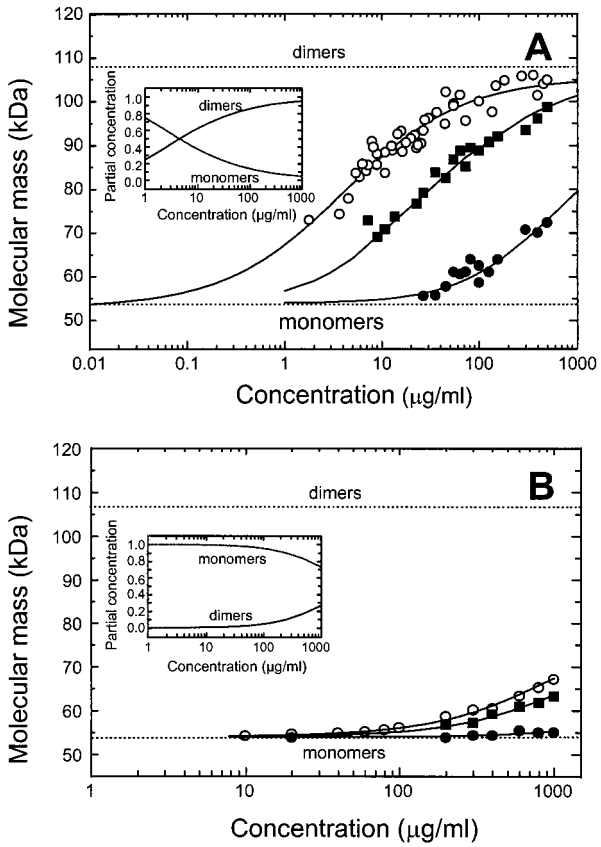

FIGURE 3: Dependence of the weight average molecular mass of serine-14 (A) and glutamate-14 (B) yeast hexokinase 2 on the enzyme concentration. Equilibrium sedimentation was performed at 10 °C in the absence of substrate (○), in the presence of 5.0 mM glucose (●), and in the presence of 2.0 mM glucose 6-phosphate (■) employing 50 mM potassium phosphate buffer, pH 7.4, supplemented with 1.0 mM EDTA, 1.0 mM PMSF, and 1.0 mM DTT as the solvent. Insets: Effect of enzyme concentration on the monomer−dimer proportions of serine-14 and glutamate-14 hexokinase (effector-free condition). Calculation of the relative proportions of monomers and dimers employed the association constants for dimerization given in Table 1. Relative protein concentrations illustrating the equilibrium distribution of monomers and dimers correspond to the respective total concentration of hexokinase.

functional characterization of the enzyme by stopped-flow kinetics. Serine-14 hexokinase and the glutamate-14 mutant enzyme were subjected to sedimentation analysis alone and in the presence of hexose substrate and product (Figure 3). Weight-average molecular mass data were determined from the radial concentration distributions fitted by eq 1. Association constants were calculated from partial concentrations assuming monomer−dimer equilibria (Table 1; see Experimental Procedures). The dependence of the monomer−dimer proportions of hexokinase on the enzyme concentration calculated according to the constants in Table 1 is illustrated for the effector-free condition in the insets of Figure 3. The molecular mass data indicated that at a high enzyme concentration (1 mg/mL) the unphosphorylated wild type enzyme was mainly dimer in the absence of effector and in the presence of glucose 6-phosphate (Figure 3A), whereas glutamate-14 hexokinase was largely dissociated at these conditions (Figure 3B). The latter enzyme is fully dissociated at low protein concentrations (Figure 3B) where wild-type hexokinase is monomeric only in the presence of glucose and glucose 6-phosphate (Figure 3A). The data in Figure

Table 1: Effector Influence on Monomer−Dimer Equilibrium of Serine-14 and Glutamate-14 Yeast Hexokinase 2 As Analyzed by Equilibrium Sedimentation

effector	association constant of dimerization, K_a (M^{-1}) Hxk2p (S14/S157)[a]	Hxk2p (S14E/S157)[b]
none	1.2×10^7	1.3×10^4
glucose (5.0 mM)	4.5×10^4	7.0×10^2
glucose 6-phosphate (2.0 mM)	1.5×10^6	7.1×10^3

[a] Wild-type yeast hexokinase 2 carrying serine-14 and serine-157 unsubstituted/unmodified. [b] Glutamate-14 mutant enzyme. Experimental conditions as described in legend to Figure 3. Association constants were determined by numerical integration of the areas below the radial distribution curves of monomeric and dimeric hexokinases calculated from the concentration distributions of the enzyme at sedimentation equilibrium by assuming monomer−dimer equilibria using our program Polymole (*36*).

3A demonstrate that the oligomeric state of unmodified hexokinase is essentially controlled by the enzyme concentration at a protein concentration range between 1 μg/mL and 1 mg/mL. In contrast, dimerization of pseudophosphorylated glutamate-14 hexokinase requires significantly higher enzyme concentrations (Figure 3B). Sugar substrate or product impaired dimer stability of both forms of hexokinase with glucose representing the more powerful effector.

Steady-State Kinetic Properties of Hexokinase. The effect of substrate concentration on the initial kinetics of glucose phosphorylation by serine-14 and glutamate-14 hexokinase was analyzed at two enzyme concentrations (1 μg/mL and 1 mg/mL) differing by 3 orders of magnitude (Figure 4). According to the results of sedimentation analysis (Figure 3A), wild-type hexokinase was expected to predominantly exist in either the monomeric or dimeric form at these conditions. Permanently pseudophosphorylated glutamate-14 hexokinase was studied to determine the functional consequences of serine-14 phosphorylation occurring in vivo and greatly weakening dimer stability in vitro (*27*; Figure 3B).

The dependence of enzyme activity on the concentration of glucose (Figure 4) is presented in panels A and B for serine-14 and in panels E and F for glutamate-14 hexokinase. The corresponding kinetic parameters, $S_{0.5}$ and V_{max}, are summarized in Table 2. Compared with the $S_{0.5}$ value of serine-14 hexokinase determined at the high enzyme concentration, both wild-type and mutant enzyme exhibited a 3-fold higher affinity for the sugar substrate when acting at a low enzyme level. Remarkably, glutamate-14 hexokinase displayed an intermediate substrate affinity at the high enzyme concentration. Determination of V_{max} indicated smaller differences and identified glutamate-14 hexokinase as the more active enzyme species (Table 2; see also Figure 5). The numerical value of V_{max} obtained with serine-14 hexokinase at the low enzyme concentration usually employed in steady-state kinetics (Table 2) was identical within a limit of error of ±10% with the value measured in the coupled optical test (see Experimental Procedures). Due to the inhibition of hexokinase by ATP at the low enzyme level (Figure 4C,G), the final concentration of ATP was 0.75 mM in the experiments corresponding to panels A and E (see Discussion).

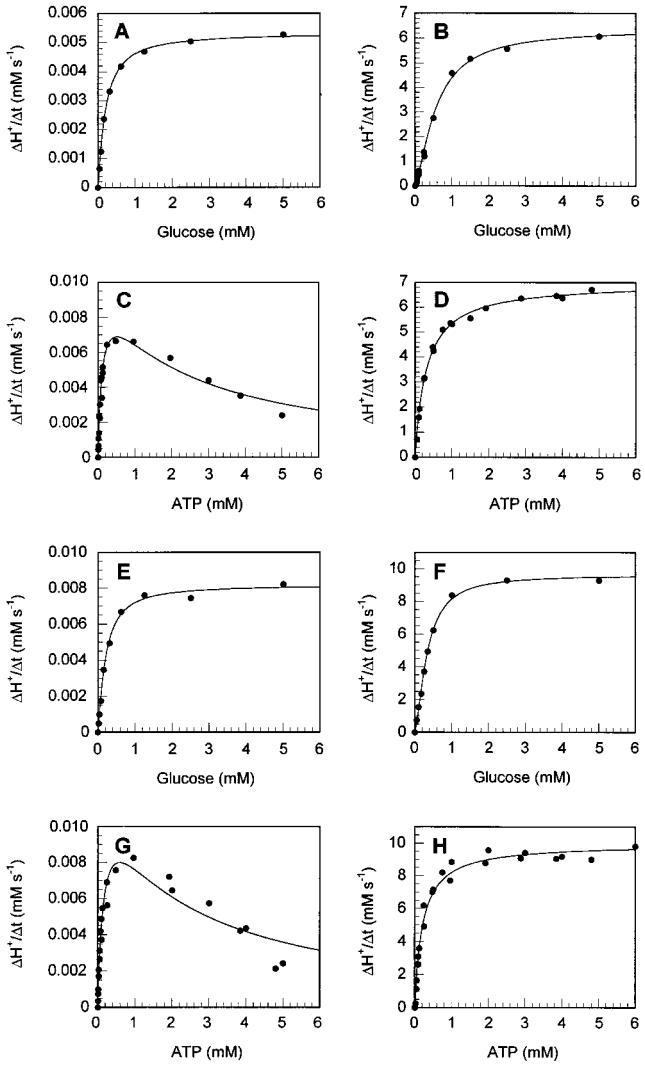

FIGURE 4: Dependence of glucose kinase activity of yeast hexokinase 2 on the concentration of substrate and enzyme. Measurements were carried out in 4.0 mM TEA/HCl buffer containing 10 mM $MgCl_2$, 1.0 mM DTT, and 200 μM *p*-nitrophenol(ate) at pH 7.4. Protonation of *p*-nitrophenolate by serine-14 (A−D) and glutamate-14 hexokinase (E−H) was analyzed at a low (1.0 μg/mL; A, C, E, and G) and high (1.0 mg/mL; B, D, F, and H) enzyme concentration. The concentration of glucose was 5.0 mM (C, D, G, and H); ATP was employed at an optimal concentration of 0.75 mM (A and E) and 2.0 mM (B and F). Reaction was started by mixing equal volumes of assay buffer and enzyme solution (100 μL each) in a stopped-flow machine and monitored at 25 °C following the decrease of absorbance at 400 nm. For primary data treatment and nonlinear regression analysis see Experimental Procedures.

The results of analogous investigations employing ATP as the variable substrate are summarized in Figure 4C,D,G,H. The prominent finding of this study was the dependence of the kinetic response on the enzyme concentration. Comparison of data in panels C and G vs panels D and H indicated that hexokinase was inhibited by ATP at low enzyme level (1 μg/mL) while no inhibition was observed at 1000-fold higher hexokinase concentration. Since data treatment assuming substrate inhibition alone was inappropriate to adequately describe the inhibitory effect of ATP (Figure 4C,G), V_{max} and $S_{0.5}$ were calculated by omitting the data reflecting a loss of enzyme activity at increasing ATP concentrations (Table 2). As similarly observed for the sugar substrate, both serine-14 and glutamate-14 hexokinases

Table 2: Effect of Enzyme Concentration and Substitution/Modification at Amino Acid Position 14 on the Initial Kinetics of Glucose Phosphorylation by Yeast Hexokinase 2

hexokinase 2 species (concentration)	dependence of hexokinase activity on glucose			dependence of hexokinase activity on ATP		
	V_{max} [$\Delta H^+/\Delta t$ (mM s^{-1})] (μmol min^{-1} mg^{-1})	$S_{0.5}$ (mM)	Hill coeff	V_{max} [$\Delta H^+/\Delta t$ (mM s^{-1})] (μmol min^{-1} mg^{-1})	$S_{0.5}$ (mM)	K_i (mM)
Hxk2p (S14/S157) (1 mg mL^{-1})[a]	[6.36 ± 0.16][c] 381 ± 10[c]	0.57 ± 0.053[c]	1.44 ± 0.07	[7.00 ± 0.07] 420 ± 4	0.31 ± 0.013	
Hxk2p (S14/S157) (1 μg mL^{-1})[a]	[0.0053 ± 0.6 e^{-4}][d] 318 ± 4[d]	0.21 ± 0.013[d]	1.17 ± 0.04	[0.0075 ± 4.3 e^{-4}][e] 450 ± 26[e]	0.071 ± 0.011[e]	2.21 ± 0.52
Hxk2p (S14E/S157) (1 mg mL^{-1})[b]	[9.63 ± 0.18][c] 578 ± 11[c]	0.35 ± 0.027[c]	1.55 ± 0.09	[9.97 ± 0.19] 598 ± 11	0.22 ± 0.018	
Hxk2p (S14E/S157) (1 μg mL^{-1})[b]	[0.0082 ± 1.9 e^{-4}][d] 492 ± 11[d]	0.20 ± 0.029[d]	1.25 ± 0.09	[0.0092 ± 3.6 e^{-4}][e] 552 ± 22[e]	0.098 ± 0.010[e]	1.99 ± 0.56

[a] Wild-type hexokinase carrying serine-14 and serine-157 unsubstituted/unmodified. [b] Glutamate-14 mutant enzyme. [c] The concentration of ATP was 2.0 mM. [d] The concentration of ATP was 0.75 mM. [e] Primary data indicating substrate inhibition by ATP were excluded from consideration. Experimental conditions as outlined in legend to Figure 4. Protein determination at 280 nm used the absorption coefficient of 0.92 cm^2/mg (*27*). For determination of hexokinase activity, the relative absorption coefficients of *p*-nitrophenol(ate) were taken from Figure 2B. Nonlinear regression analysis according to Michaelis−Menten assumed cooperative kinetics to best describe the dependence of enzyme activity on glucose. K_i was obtained by fitting the data according to substrate excess inhibition.

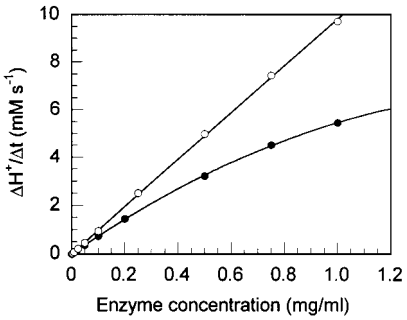

FIGURE 5: Dependence of glucose kinase activity of yeast hexokinase 2 on enzyme concentration. Measurements were carried out in 4.0 mM TEA/HCl buffer containing 10 mM $MgCl_2$, 1.0 mM DTT, 200 μM *p*-nitrophenol(ate), 5.0 mM glucose, and 2.0 mM ATP at pH 7.4. Reaction was started by mixing equal volumes of assay buffer and serine-14 (●) or glutamate-14 (○) hexokinase (100 μL each) in a stopped-flow machine and was monitored at 25 °C following the decrease of absorbance at 400 nm. For primary data treatment see Experimental Procedures.

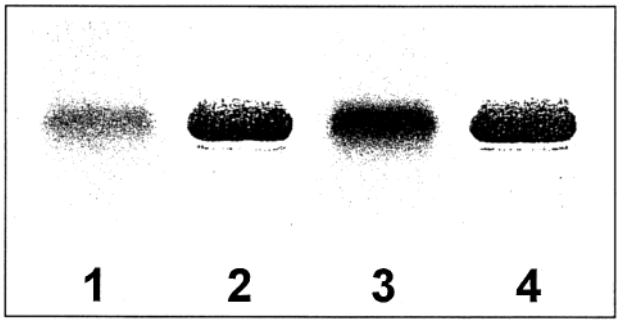

FIGURE 6: In vitro autophosphorylation of serine-14 and glutamate-14 yeast hexokinase 2. The purified enzymes were incubated with ATP in 40 mM HEPES/NaOH buffer containing 10 mM $MgCl_2$ and 1.0 mM DTT at pH 7.4 and 30 °C for 60 min. Total ATP was a mixture of unlabeled nucleotide and [γ-^{32}P]ATP giving final assay concentrations of 20 μM and 100 mCi/L, respectively. The final concentration of hexokinase was 100 μg/mL. Reaction was terminated by sample treatment with SDS/DTT loading dye buffer at 95 °C for 5 min. Samples of 5 μg of denatured protein were subjected to SDS−PAGE followed by PhosphorImager scanning. Lanes 1 and 2, unphosphorylated serine-14 hexokinase; lanes 3 and 4, glutamate-14 mutant enzyme; lanes 1 and 3, phosphor image; lanes 2 and 4, protein stain (Coomassie Blue).

exhibited significantly higher affinity for ATP at the low enzyme concentration where $S_{0.5}$ was reduced 3−4-fold as compared with the value determined for the wild-type enzyme at the high hexokinase concentration (Table 2). The intermediate $S_{0.5}$ value displayed by glutamate-14 hexokinase at the high enzyme level paralleled the finding obtained for the sugar substrate. Determination of V_{max} at the high enzyme concentration gave data that were very similar to the values calculated from the glucose dependence of enzyme activity (Table 2). The numerical values of V_{max} determined for both enzyme species are consistent with the results of an independent investigation of the effect of enzyme concentration on the initial kinetics of hexokinase (Figure 5).

In Vitro Autophosphorylation of Glutamate-14 Hexokinase. To further assess the influence of the chemical nature of amino acid residue 14 on hexokinase function, the phosphorylation of pseudophosphorylated glutamate-14 hexokinase was investigated in vitro in the absence of any hexose substrate or substrate analogue. Serine-14 hexokinase was used as a control. Both enzymes were incubated with [γ-^{32}P]-ATP under conditions previously applied to study protein kinase activity of hexokinase (*38*). According to the results of equilibrium sedimentation (Figure 3), the concentration of hexokinase employed was appropriate to ensure a maximum difference in the oligomeric state of both enzymes. Figure 6 shows that there was significantly higher phosphate incorporation into glutamate-14 hexokinase (lane 3) as compared with the unphosphorylated wild-type enzyme (lane 1). This difference was not due to different amounts of the hexokinase protein as revealed by Coomassie staining (lanes 2 and 4) and immunodetection (not shown).

DISCUSSION

This paper presents molecular and functional studies performed to explore the effect of hexokinase phosphorylation at serine-14 occurring in the yeast cell under physiological glucose limitation (*1*, *21*). The experimental strategy considered that glucose phosphorylation alone is not sufficient to account for the indispensable involvement of isoenzyme 2 in glucose repression (*18*, *20*) and that hexokinase interconversion by phosphorylation-dephosphorylation is likely to be part of the underlying signal transduction (*22*, *23*). In view of data suggesting different kinetic properties for monomeric and dimeric yeast hexokinases (*39*−*41*), the present study was designed to evaluate our

hypothesis that serine-14 phosphorylation and subsequent dissociation of the homodimeric enzyme may improve glucose utilization and enable hexokinase to transmit the glucose derepression signal when glucose becomes the limiting fermentable substrate (*27*). In contrast to previous work employing partially purified enzyme and/or enzyme of unknown N-terminal identity (*39*−*43*), N-terminally well-characterized preparations of purified hexokinase were used to allow a clear assignment of functional and structural data.

There are three new findings that require special consideration: First, native monomeric hexokinase represents the high affinity form of the alternatively homodimeric enzyme for the two glycolytic substrates (Figure 4; Table 2). Second, ATP inhibition of glucose phosphorylation is restricted to the monomeric form of hexokinase (Figure 4). Third, autophosphorylation of hexokinase is stimulated by the serine-glutamate exchange performed to permanently mimic serine-14 phosphorylation (Figure 6).

Assessment of the kinetic data (Table 2) has to take into account that the concentrations of glucose and ATP employed correspond to a physiological condition (*42*, *44*); however, neither the in situ concentrations of the two substrates nor their dependence on extracellular glucose availability are known. Therefore, the differences in substrate affinity and catalytic activity of monomeric and dimeric hexokinase summarized in Table 2 reflect a tendency but do not necessarily describe the in situ situation in quantitative terms. Interestingly, the 10-fold increase of glucose affinity observed after proteolytic modification and subsequent dissociation of a dimeric yeast hexokinase using GTP as the nucleotide substrate (*39*) is consistent with the above results. The finding that substrate affinity of glutamate-14 hexokinase depends on the enzyme concentration with higher $S_{0.5}$ values determined at the high enzyme level (Table 2) suggests a stabilization of the dimeric structure of the mutant enzyme under reaction conditions.

The inhibition of hexokinase by ATP (Figure 4) confirms findings obtained with partially purified isoenzyme 2 of unreported N-terminal identity in an experimental condition presumably corresponding to a low rather than to a high enzyme concentration (*42*, *43*). The former authors demonstrated that inhibition was related to the concentration of the Mg^{2+}-free nucleotide, and it was concluded that free Mg^{2+} has no effect on the inhibition of hexokinase (*42*). According to these data, the calculated concentration of Mg^{2+}-free ATP present in our assay assuming the association constant $K_a = 10^4\ M^{-1}$ of the Mg−ATP complex (*45*) was high enough to account for the inhibition of hexokinase seen in Figure 4 (this concentration was 0.10 mM at 5 mM total ATP). The important and new finding, however, is the lack of inhibition by the nucleotide substrate at a high hexokinase concentration (Figure 4), because a similarly high enzyme level is likely to exist in vivo (*5*, *39*, *46*, *47*). On the basis of the observation that serine-14 phosphorylation promotes dissociation of the hexokinase dimer at the same high enzyme concentration (*27*), we suggest that one consequence of this modification is the conversion of the ATP-insensitive homodimeric enzyme into a monomeric one, which is sensitive to ATP inhibition in the physiological range of ATP concentrations (*42*, *44*) even at the micromolar level of hexokinase expected to occur in the cell (*5*, *39*, *46*−*48*). Remarkably, glucose kinase activity of isoenzyme 1 exhibiting similar molecular and functional properties was not inhibited by free ATP (*42*).

Hexokinase is phosphorylated at serine-14 in vivo (*1*, *21*) and is reversibly inactivated in vitro by autophosphorylation at serine-157 (*32*), which is located in the immediate vicinity of the active site (*49*). The enzyme is also capable of acting as a protein kinase (*38*). These features as well as speculations on a role of conformational changes of hexokinase in initiating glucose repression (*20*, *31*, *32*), led us to evaluate the influence of the serine-14 modification on the autophosphorylation of the enzyme. The novel finding of glutamate substitution for serine-14 resulting in stimulation of ATP-dependent phosphate labeling at a hitherto unknown site (Figure 6) is consistent with a multifunctional role of isoenzyme 2 (*30*, *32*, *38*, *50*). Identification of the modified residue of glutamate-14 hexokinase is the subject of current investigations that are complicated by a low labeling stoichiometry. This work takes into account that the identity of serine-157, which is modified in the presence of D-xylose (*2*), and the site of autophosphorylation, which is affected in the absence of any substrate or substrate analogue (*32*), remains to be shown. According to X-ray crystallography studies, however, modification of the same residue is likely to occur in the two conditions (*49*), suggesting serine-157 to be the site in question.

The results of the present study support the following hypothetical model of hexokinase involvement in glucose sensing and in signaling in glucose derepression: Glucose exhaustion causes hexokinase phosphorylation at serine-14 (cf. refs *1* and *21*−*23*) by a still unknown mechanism and thereby promotes the dissociation of the homodimeric enzyme even at a physiologically high enzyme concentration (*27*; this work). The formation of monomeric phosphohexokinase, representing the high affinity form of the enzyme for the two glycolytic substrates (this work), contributes to the improvement of glucose utilization from low extracellular concentration. The inhibition of monomeric (but not dimeric) hexokinase by the free ATP (*42*; this work) originating from Mg−ATP due to the loss of intracellular magnesium ions in a situation of glucose exhaustion (*42*) allows the cell to integrate the intracellular ATP concentration into the control of glucose phosphorylation. Simultaneously, phosphorylation at serine-14 stimulates hexokinase autophosphorylation (this work). The new conformation of the diphosphoenzyme may affect enzyme targeting via exposure of its N-terminal bipartite motif of basic residues resembling a nuclear localization sequence (*27*, *50*, *51*) and/or modifiy its interaction with components involved in the transduction of the glucose derepression signal (*22*, *23*, *38*).

Essential questions remain to be answered. First, what is the role of hexokinase 1 in glucose signaling ? In vivo labeling studies have shown that under glucose limitation both isoenzymes are major phosphoproteins (*21*), and both enzymes exhibit an identical N-terminal motif of 23 amino acids (*3*, *4*) suggesting a functional role for this sequence (*5*). Second, which protein kinase accomplishes serine-14 phosphorylation and is there a protein kinase other than hexokinase itself which phosphorylates the enzyme in vivo at another site ? Finally, what is the substrate specificity of phosphoserine-14 hexokinase when acting as a protein kinase, and what is the catalytic and/or regulatory function of a diphosphohexokinase ? Experiments addressing these questions have to consider that regardless of a possible low

extent of phosphate incorporation into phosphoserine-14 hexokinase even a few appropriately modified enzyme molecules could transmit the glucose depletion signal to upstream located components of the glucose derepression signaling cascade.

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