THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 34, Issue of August 23, pp. 30477–30487, 2002
Printed in U.S.A.
© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

In Vivo and *in Vitro* Phosphorylation of Two Isoforms of Yeast Pyruvate Kinase by Protein Kinase A*

Received for publication, February 1, 2002, and in revised form, June 4, 2002
Published, JBC Papers in Press, June 12, 2002, DOI 10.1074/jbc.M201094200

Paula Portela‡, Steven Howell§, Silvia Moreno‡, and Silvia Rossi‡¶

From the §Laboratory of Protein Structure, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom and the ‡Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, Buenos Aires 1428, Argentina

***Saccharomyces cerevisiae* pyruvate kinase 1 (Pyk1) was demonstrated to be associated to an immunoprecipitate of yeast protein kinase A holoenzyme (HA-Tpk1·Bcy1) and to be phosphorylated in a cAMP-dependent process. Both glutathione *S*-transferase (GST)-Pyk1 and GST-Pyk2 were phosphorylated *in vitro* by the bovine heart protein kinase A (PKA) catalytic subunit and by immobilized yeast HA-Tpk1. The specificity constant for the phosphorylation of GST-Pyk1 and GST-Pyk2 by bovine catalytic subunit was in the range of the value for Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide). Both fusion proteins were phosphorylated *in vivo*, in intact cells overexpressing the protein, or *in vitro* using crude extracts, as source of protein kinase A, when a wild type strain was used but were not phosphorylated when using a strain with only one *TPK* gene with an attenuated mutation (*tpk1*^{w1}). The effect of phosphorylation on Pyk activity was assayed in partially purified preparations from three strains, containing different endogenous protein kinase A activity levels. Pyk1 activity was measured at different phosphoenolpyruvate concentrations in the absence or in the presence of the activator fructose 1,6-bisphosphate at 1.5 mM. Preliminary kinetic results derived from the comparison of Pyk1 obtained from extracts with the highest *versus* those from the lowest protein kinase A activity indicate that the enzyme is more active upon phosphorylation conditions; in the absence of the activator it shows a shift in the titration curve for phosphoenolpyruvate to the left and an increase in the Hill coefficient, whereas in the presence of fructose 1,6-bisphosphate it shows an n_H value of 1.4, as compared with an n_H of 2 for the Pyk1 obtained from extracts with almost null protein kinase A activity.**

Protein phosphorylation is a universal regulatory mechanism; in eukaryotic cells it is a phenomenon with implications in almost every aspect of cell physiology and biochemistry. In *Saccharomyces cerevisiae* protein kinase A (PKA)[1] lies at the heart of an important signal transduction pathway with key elements identified principally in the 1980s. The PKA regulatory subunit is encoded by the *BCY1* gene, and the catalytic subunits are encoded by the redundant *TPK1*, *TPK2*, and *TPK3* genes (1, 2). In yeast, the PKA pathway controls many different targets at the post-translational and transcriptional levels and has been implicated in several different but interdependent cellular reactions in response to changes in environmental conditions (3). Glucose is the preferred carbon source of *S. cerevisiae* and as such is a crucial regulator of cellular physiology. Transport and phosphorylation of the sugar are required for most of its regulatory effects. PKA is involved in one of the main regulatory systems transducing glucose signals. Glucose triggers a rapid and transient increase in the levels of the second messenger cAMP, synthesized by adenylate cyclase, which is stimulated either by Ras proteins (possibly by reacting to intracellular acidification) or by glucose via the Gpr1-Gpa2 G-protein system (3, 4). Yeast cells, like other organisms or cells able to carry out glycolysis and gluconeogenesis, have developed multiple regulatory mechanisms to avoid the simultaneous operation of these antagonistic metabolic pathways (5, 6). PKA has an input on this process by regulation of some key enzymes either transcriptionally or post-translationally. Although it may be clear that the activity of the enzyme targets is regulated in response to PKA, it is frequently difficult to be sure that the activation or inhibition of their activity is a direct effect. Well known (direct or indirect) targets of PKA phosphorylation are 1) enzymes involved in trehalose synthesis and degradation, including trehalase and trehalose-6-phosphate synthase; 2) those involved in glycogen synthesis and degradation, such as glycogen synthase and glycogen phosphorylase; and 3) those enzymes involved in the glycolytic flux, such as phosphofructokinase and fructose-bisphosphatase (3, 7). A less known target is pyruvate kinase, whose *in vitro* phosphorylation by PKA has recently been reported (8, 9). The level of expression of *PFK26* and *PFK27* genes, coding for 6-phosphofructo-2-kinase, depend on the carbon source, via a cAMP pathway (10). In the presence of fermentable sugars yeast glycolysis is fully activated; the initiation of the glycolytic flux seems to rely on the coordinated triggering of multiple events, including allosteric regulation of enzymatic activities, protein modification, and modulation of gene expression.

The main objective of our group is to study the mechanism of *in vivo* activation of PKA by cAMP and the participation of the substrate in this process using lower eukaryotic models (11–

* This research was supported in part by grants from the University of Buenos Aires and Ministerio de Salud through the Carrillo-Oñativia Fellowship. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel./Fax: 54-11-4576-3342; E-mail: srossi@qb.fcen.uba.ar.

[1] The abbreviations used are: PKA, protein kinase A; C_b, protein kinase A catalytic subunit from bovine heart; PEP, phosphoenolpyruvate; FBP, fructose 1,6-bisphosphate; HA, hemagglutinin; GST, glutathione *S*-transferase; ORF, open reading frame; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Pyk1, -2, pyruvate kinases 1 and 2; WT, wild type.

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015

TABLE I
List of strains and nomenclature

Strain	Genotype	Genetic nomenclature
EJ758-YOR347C[a]	*Mata his3–200 leu2–3 112 ura3–52 pep4::HIS3* + pYEX4T1-*GSTYOR347C*	*EJ758 + GST-PYK2*
EJ758-YAL038W[a]	*Mata his3–200 leu2–3 112 ura3–52 pep4::HIS3* + pYEX4T1-*GSTYAL038W*	*EJ758 + GST-PYK1*
KT 1115[b]	*Mata leu2 ura3 his3 pep4Δ*	*1115*
KT 1115[b]	*Mata leu2 ura3 his3 pep4Δ* + Ycp50-*HATPK1*	*1115 + HA-TPK1*
KT 1115[b]	*Mata leu2 ura3 his3 pep4Δ* + Ycp50-*HATPK1* + YEp51-*BCY1*	*1115+HA-TPK1* + YEp51-*BCY1*
KT 1115[b]	*Mata leu2 ura3 his3 pep4Δ* + pEG(KG)	*1115 + GST*
KT 1451[b]	*Matα leu2 ura3 his3 pep4Δ bcy1–14*	*1451*
RS13–58[a] - 1[c]	*Mata his3 leu2 ura3 trp1 ade8 tpk1[w1] tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*	*tpk1[w1]*
RS13–58[a] - 1	*Mata his3 leu2 ura3 trp1 ade8 tpk1[w1] tpk2::HIS3 tpk3::TRP1 bcy1::LEU2* + pYEX4T1-*GSTYOR347C*	*tpk1[w1] + GST-PYK2[d]*
RS13–58[a] - 1	*Mata his3 leu2 ura3 trp1 ade8 tpk1[w1] tpk2::HIS3 tpk3::TRP1 bcy1::LEU2* + pYEX4T1-*GSTYAL038W*	*tpk1[w1] + GST-PYK1[d]*

These strains have been previously described in the literature: [a](18), [b](13), [c](19).
[d] Strains developed in this report.

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015

14). While looking for PKA substrates in *S. cerevisiae*, we found that both isoforms of pyruvate kinase, Pyk1 and Pyk2, are substrates of PKA. Pyk 1 is the last enzyme in the glycolytic pathway of sugar catabolism; it catalyzes the irreversible conversion of phosphoenolpyruvate (PEP) to pyruvate by the addition of a proton and the loss of a phosphate group, which is transferred to ADP. The enzyme is allosterically activated by fructose 1,6-bisphosphate (FBP), and its gene expression is dependent on the presence of glucose in the culture medium (15). Mck1, a member of the glycogen synthase kinase 3 family, has been reported to be a negative regulator of Pyk1 (16) through the negative regulation of its phosphorylation by PKA (9). With the completion of the yeast genome sequencing project, an open reading frame coding for a protein with high homology to Pyk1 has been reported. The characterization of this gene indicated the existence of a Pyk2 protein, which is insensitive to FBP and whose expression is repressed by glucose (17). In this work we identify both Pyk1 and Pyk2 as *in vitro* and *in vivo* substrates of PKA, characterize their behavior as PKA substrates, and present a preliminary study of the effect of phosphorylation on the kinetics of pyruvate kinase.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade. Yeast growth medium supplies were from Difco Laboratories and Merck. HA-probe antibody (F-7), protein A/G Plus-agarose, anti-GST antibody GST(Z-5), and anti-goat IgG-peroxidase-conjugated, Chemiluminescence Luminol reagent were from Santa Cruz Biotechnology, Inc. [γ-^{32}P]ATP and [^{32}P]orthophosphate were from PerkinElmer Life Sciences. Phosphocellulose paper (P81) was from Whatman. Colloidal Coomassie Blue stain was from Novex. Protein kinase catalytic subunit from bovine heart, protein kinase inhibitor amide fragment 6-22, IgG-peroxidase conjugated, PEP, NADH, ATP, ADP, FBP, lactate dehydrogenase, and phosphatase alkaline-biotinamidocaproyl (P1318) and streptavidin immobilized on iron oxide (S2415) were from Sigma Chemical Co. Polyclonal anti-pyruvate kinase (rabbit muscle) was from Rockland. Nitrocellulose membrane was from MSI. Trypsin, of modified sequencing grade, was from Promega, Madison, WI.

Yeast Strains and Plasmids—The strains, genotype, and genetic nomenclature used in this study are listed in Table I. The plasmid Yep51-*BCY1* expresses the *BCY1* gene under the control of the *GAL10* promoter (13). The plasmid Ycp50-*HATPK1* codes for a HA-Tpk1 fusion protein under the control of the *TPK1* promoter (generously provided by Stephen Garrett, Department of Microbiology and Molecular Genetics, New Jersey Medical School). Strains EJ758-YOR347C and EJ758-YAL038W expressing the respective open reading frames fused to glutathione *S*-transferase (GST) from pYEX4T1 plasmid under control of the *CUP1* promoter were purchased from Research Genetics, Inc. The plasmids contained in the EJ758-YOR347C and EJ758-YAL038W strains were recovered for further experiments using a plasmid preparation protocol described for yeast cells (20). The sequence of each ORF was confirmed using the primer F, 5′-GAATTCCAGCTGACCAC-3′, and primer R, 5′-GATCCCCGGGAATTGCCAT-3′.

The vector pEG(KG) contains the GST gene cloned downstream of the galactose-inducible *CYC1* promoter (21). Yeast strains were transformed by the lithium acetate method (22).

Growth Media—Yeast media were prepared as described previously (23). Strains were grown on rich medium containing 2% Bactopeptone, 1% yeast extract, and 2% galactose (YPgal) or 2% glucose (YPG). Synthetic media containing 0.67% yeast nitrogen base without amino acids and 2% glucose plus the necessary additions to fulfill auxotrophic requirements were used to maintain the selectable plasmids (SD). Solid media contained 2% agar.

Standard PKA Assay—C subunit activity was determined by assay of phosphotransferase activity with Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) as substrate. The assay was started by mixing the different amounts of PKA coming from different sources (protein kinase catalytic subunit from bovine heart (C_b), semi-purified HA-Tpk1, HA-Tpk1·Bcy1 complex, or crude extract) with assay mixture to give 50 mM potassium phosphate, pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 15 mM $MgCl_2$, 10 mM 2-mercaptoethanol (buffer PA), 0.1 mM [γ-^{32}P] ATP (700 dpm/pmol) plus 200 μM Kemptide and 10 μM cAMP, when added. After 15 min at 30 °C, aliquots were processed according to the phosphocellulose paper method (24). PKA assays were linear with time and protein concentration. PKA activity is expressed in units: one unit is defined as the amount of enzyme catalyzing the incorporation of 1 pmol of phosphate to protein substrate per minute at pH 7.4 at 30 °C. C_b was reconstituted in 50 mM dithiothreitol and the units used in the assays (expressed as picomoles of phosphate incorporated to a synthetic substrate per minute) were estimated according to the instructions of the manufacturer, except when indicated, where the activity was assayed under our standard assay conditions.

Immunoprecipitation—Strain *1115+HA-TPK1*+YEp51-*BCY1* was grown in YPgal medium during 16 h at 30 °C. Cells were lysed by disruption with glass beads at 4 °C in 0.1 M potassium phosphate, pH 7; 1 mM EDTA, 0.1 mM EGTA, 5% glycerol, 0.1 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin, 3 μg/ml antipain, and 0.5% Triton X-100 (buffer A). Cell debris was pelleted by centrifugation at 5000 × g for 5 min, and the crude extract was used to immunoprecipitate HA-Tpk1·Bcy1 complex. A 0.5-ml aliquot of crude extract (2.5 mg/ml) was incubated with anti-HA (4 mg/ml) antibody or nonspecific antibody during 1 h at 4 °C, followed by addition of A/G agarose (30 μl), and incubation for 16 h at 4 °C. The immunoprecipitates were washed with 5 ml of buffer A plus 0.1 M NaCl. Phosphorylation of immunoprecipitates was performed by incubation with 0.1 mM [γ-^{32}P]ATP (700 dpm/pmol) in buffer PA, plus 10 μM cAMP, when added. The immunoprecipitates were subjected to SDS-PAGE. The gel was stained with colloidal Coomassie Blue, destained, and autoradiographed. Protein kinase activity of aliquots of each immunoprecipitate was assayed under the standard assay conditions.

Peptide Mass Mapping by MALDI—The selected protein bands were excised, washed, reduced, and *S*-alkylated essentially as described (25). A sufficient volume of 2 ng/μl trypsin in 5 mM NH_4HCO_3 was added to cover the gel pieces, and digestion was performed overnight at 32 °C in an incubator. The digests were then acidified by

the addition of a 1/10 vol. of 2% trifluoroacetic acid prior to MALDI analysis. A Reflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with a 337-nm nitrogen laser and a Scout-384 probe, was used to obtain positive ion mass spectra of digested protein with pulsed ion extraction in reflectron mode. An accelerating voltage of 26 kV was used with detector bias gating set to 2 kV and a mass cut-off of m/z 650. Thin-layer matrix surfaces of α-cyano-4-hydroxycinnamic acid mixed with nitrocellulose were prepared as described previously (26). An aliquot (0.4 μl) of acidified digestion supernatant was deposited onto the thin layer and allowed to dry prior to desalting with water. Mass spectra were clean and interpretable. Peptide mass fingerprints thus obtained were searched against the non-redundant protein data base placed in the public domain by NCBI, using the program MASCOT (27).

Induction of GST-ORF and Purification of Fusion Proteins—Cell patches of EJ758-YOR347C and EJ758-YAL038W were inoculated in SD-Ura liquid medium, grown overnight, and re-inoculated into SD-Ura-Leu, grown to A_{600} = 0.8, and induced with 0.5 mM Cu_2SO_4 for 2 h before harvest. Cells were homogenized with glass beads in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 5 mM dithiothreitol, 10% glycerol, and 0.5 M NaCl plus 2 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 3 μg/ml antipain (buffer B). The GST protein was purified from crude extract of *1115+GST* cultures induced by adding galactose to a final concentration of 2% (21). GST protein and GST-Pyk1 and GST-Pyk2 fusion proteins were purified by glutathione agarose chromatography in buffer B plus 1 M NaCl. Proteins were eluted by 1 h incubation at 37 °C with buffer B, 1 M NaCl, 40 mM glutathione, 20 mM dithiothreitol. After dialysis in buffer, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM dithiothreitol, 55 mM NaCl, 50% glycerol, the preparations were stored in aliquots at −20 °C. The efficiency of elution of either GST-Pyk1 and GST-Pyk2 was low; therefore, for the experiments of Figs. 4 and 5, where a high concentration of the proteins was needed, the GST fusion proteins were used bound to the glutathione-agarose. The protein concentration of the immobilized enzyme was estimated by Coomassie Blue staining of the proteins eluted with loading buffer and run on SDS-PAGE.

In Vitro Phosphorylation of Pyk1-GST and Pyk2-GST—Semi-purification of HA-Tpk1 was performed by immunoprecipitation from crude extract of *1115+HA-TPK1* strain grown for 16 h at 30 °C in SD-Ura. The immunoprecipitation was performed using anti-HA (4 mg/ml) or nonspecific antibody (rabbit preimmune serum) as described above. The immunoprecipitates were washed first with buffer A plus 1 M NaCl, followed by a wash with buffer A. Protein kinase activity of the immunoprecipitated HA-Tpk1 was determined under the standard assay conditions. This HA-Tpk1 preparation displayed a −/+ cAMP activity ratio of 0.9 and a K_m value of 50 μM for Kemptide (data not shown; comparable to value reported in Ref. 28). The *in vitro* phosphorylation assays were performed in a final volume of 40 μl using partially purified HA-Tpk1 (as described above) or C_b (of commercial origin) as PKA source. The assay was started by mixing the substrates GST-Pyk1 and GST-Pyk2 (amount indicated in each case) with 0.1 mM [γ-^{32}P]ATP (1000–1300 dpm/pmol) in buffer PA and 15 μg/ml protein kinase inhibitor amide fragment 6-22, when added. The reactions were incubated 15 min at 30 °C. The incorporation of phosphate into GST-Pyk1 and GST-Pyk2 was determined by scintillation counting of phosphorylated enzyme excised from dried SDS-PAGE gels. Alternatively, SDS-PAGE-dried gels were exposed to autoradiography or subjected to digital imaging analysis (Bio-Imaging Analyzer Bas-1800II and Image Gauge 3.12, Fujifilm).

In the experiment in Fig. 4, yeast crude extracts derived from strains *1115* and *tpk1^{w1}*, grown on YPG medium during 16 h at 30 °C, were used as the source of PKA activity. The crude extracts were prepared as described above. Samples of crude extract (100 μg/ml) were mixed with semi-purified GST-Pyk1 or GST-Pyk2 (10–20 μg) in buffer PA plus 0.1 mM [γ-^{32}P]ATP (2200 dpm/pmol) and 10 μM cAMP, when added, in a final volume of 40 μl. After 30 min at 30 °C, aliquots were subjected to SDS-PAGE and autoradiography. The total amount of catalytic activity in the crude extract preparations was determined under the standard PKA assay conditions.

Immunological Analysis—Samples of crude extract or purified preparations of GST-Pyk1, GST-Pyk2, and GST were analyzed by SDS-PAGE, blotted onto nitrocellulose membranes using 25 mM Tris, 192 mM glycine, 20% (v/v) methanol buffer in a Transphor apparatus. Blots were blocked with 5% nonfat milk, 0.05% Tween 20 in Tris-buffered saline. Primary antibodies were used at a dilution of 1/500 for anti-GST antibody and 1/1000 for anti-pyruvate kinase antibody. Secondary antibodies were used at 1/10,000 (anti-rabbit and anti-goat IgG-peroxidase-conjugated). After three washes the blots were developed with Chemiluminescence Luminol reagent, and immunoreactive bands were visualized by autoradiography and analyzed by digital imaging.

In Vivo Phosphorylation of GST-Pyk1 and GST-Pyk2—Yeast strains EJ758+*GST-PYK2*, EJ758+*GST-PYK1*, *tpk1^{w1}+GST-PYK2*, and *tpk1^{w1}+GST-PYK1* were grown in SD-Ura medium during 16 h at 30 °C. The cultures (20 ml) were centrifuged, and the cell pellets were washed with phosphate-free YPD medium, resuspended in the same medium (30 ml), grown until exponential phase (A_{600} = 0.8), concentrated to 3 ml, and further incubated with 0.5 mCi of [^{32}P]orthophosphate. After 2 h the induction of GST fusion protein was performed as described above. The cells were washed with cold saline buffer and disrupted with glass beads in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 5 mM dithiothreitol, 10% glycerol, 0.5 M NaCl plus 2 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 3 μg/ml antipain, 10 mM NaF, 5 mM β-glycerophosphate, and 1 mM $NaVO_4$. ^{32}P uptake was measured and was similar for both wild type and *tpk1^{w1}* strains. GST-Pyk1 and GST-Pyk2 were purified by glutathione-agarose chromatography and subjected to SDS-PAGE. The gel was dried and analyzed by digital imaging.

Determination of Pyruvate Kinase Activity—The pyruvate kinase activity was determined in 50 mM imidazole buffer, pH 7.0, containing 62 mM $MgCl_2$ and 100 mM KCl. Under standard conditions, 1.5 mM ADP, 1.5 mM FBP as activator, and different amounts of PEP were used. The reaction was coupled to NADH oxidation by addition of 1 unit of lactate dehydrogenase per milliliter and 0.22 mM NADH. The time course of the reaction was monitored at 30 °C by measuring the decrease in absorbance at 340 nm. For the determination of kinetic parameters, the pyruvate kinase preparation used came from a partial purification by precipitation from crude extracts with 60% $(NH_4)_2SO_4$. Pellets were stored at −20 °C and desalted through Sephadex G-25 in imidazole buffer, immediately before the assay. Pyruvate kinase activity is expressed in enzyme units: 1 enzyme unit causes the transphosphorylation of 1 μmol of phosphate from PEP to ADP per minute at 37 °C, under standard assay conditions.

Alkaline Phosphatase Treatment of Pyk1—Alkaline phosphatase was used to dephosphorylate the endogenous pyruvate kinase (Pyk1) present in crude extracts of strain *1451*. The dephosphorylation reactions were performed for 30 min at 37 °C in a total volume of 80 μl. 300 μg of crude Pyk1 preparations, obtained through precipitation with 60% $(NH_4)_2SO_4$ and desalting, as described above, were incubated with 0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl, and 100 units of phosphatase alkaline-biotinamidocaproyl. The reaction was terminated by the addition of 150 μl of streptavidin immobilized on iron oxide. The alkaline phosphatase biotinamidocaproyl-streptavidine iron oxide complex was incubated for 30 min at room temperature and then magnetically separated for 15 min. The supernatant was used as a source of dephosphorylated enzyme for the measurement of Pyk1 activity.

Protein Determination and Analysis of Kinetic Data—Protein was determined by the Bradford assay (29) with bovine serum albumin as standard. Kinetic data of Pyk phosphorylation by PKA were analyzed according to the Michaelis-Menten equation. The substrate (Pyk) concentration used in the assays of Fig. 5 was found to be very low. Under these conditions the combination of the substrate with the enzyme becomes rate-limiting, and the Michaelis-Menten equation becomes $v = V_{max}/K_m$ [S], where S is the substrate concentration. The tangent of the curve at the origin gives the specificity constant value V_{max}/K_m, which is the more critical parameter in determining the specificity of an enzyme for a substrate.

Kinetic analysis of pyruvate kinase activity was performed using Hill equation (Fig. 7). The k_{cat} presented in Fig. 7*B* was calculated normalizing the k_{cat} expressed in units per milligram of total protein in the preparation (units/milligram of protein) to the Pyk relative abundance. This parameter was estimated as a ratio between the immunoreactive band of Pyk in the Western blots relative to total Coomassie Blue-stained protein, quantified by bio-imaging analysis.

Reproducibility of Results—All experiments were repeated at least twice with independent transformants, cultures, and enzymatic preparations. The results always showed consistent trends, *i.e.* different transformants and extracts gave highly reproducible results. In all cases, results from representative experiments are shown.

RESULTS

Identification of Substrates of Yeast PKA—To identify possible targets of *S. cerevisiae* PKA our initial approach was to immunoprecipitate PKA holoenzyme and to further analyze

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015

the co-immunoprecipitated proteins. The rationale was to overexpress a tagged Tpk1 protein together with an excess of the regulatory subunit Bcy1 to form a tagged holoenzyme containing Tpk1. Extracts from a strain cotransformed with Ycp50-*HATPK1* and Yep51-*BCY1* were subjected to immunoprecipitation with anti-HA antibody as described under "Experimental Procedures." Controls of immunoprecipitation were done using nonspecific antibody. The immunoprecipitates were washed with 0.2 M NaCl and assayed for PKA activity measuring the ratio of activity −/+ cAMP. For anti-HA immunoprecipitates, the ratio was 0.2, indicating a good holoenzyme preparation; no PKA activity was detected in nonspecific immunoprecipitates. The immunoprecipitates were subjected to phosphorylation by incubation with [γ-^{32}P]ATP in the absence or presence of cAMP, finally resolved by SDS-PAGE, and autoradiography. We looked for those proteins that had a phosphorylation pattern dependent on the specific anti-HA antibody and with a high relative radioactivity to protein ratio. The results in Fig. 1 show the Coomassie Blue staining and phosphorylation pattern of the immunoprecipitated proteins. A phosphoprotein of 55 kDa was selected as a good candidate, because, in addition, it showed a moderate increase of its phosphorylation in the presence of cAMP. The protein band was cut from the gel and submitted to tryptic digestion and mass spectrometric analysis (MALDI). A MASCOT (27) search of the *S. cerevisiae* subset of the NCBI non-redundant data base was performed using the MALDI mass spectrum of this band. 21/22 peptide masses matched two almost identical pyruvate kinase sequences (GenBank™ accession numbers 4180 and 6319279), with identical MASCOT probability scores of 274. This analysis revealed that the 55-kDa band was pyruvate kinase 1 (Pyk1). Because Pyk1 and the recently identified isoform Pyk2 (17) have a high degree of similarity in their protein sequence (68% identity), we decided to follow this work with both enzymes, although Pyk2 was not detected in the immunoprecipitates.

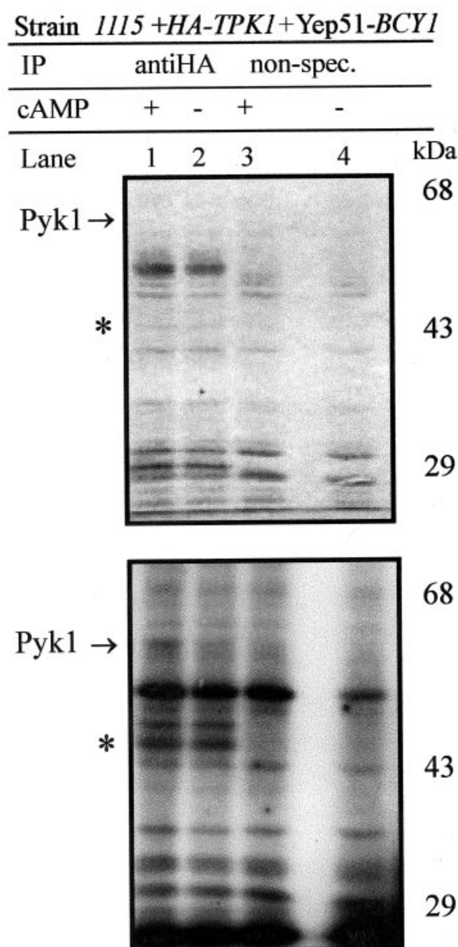


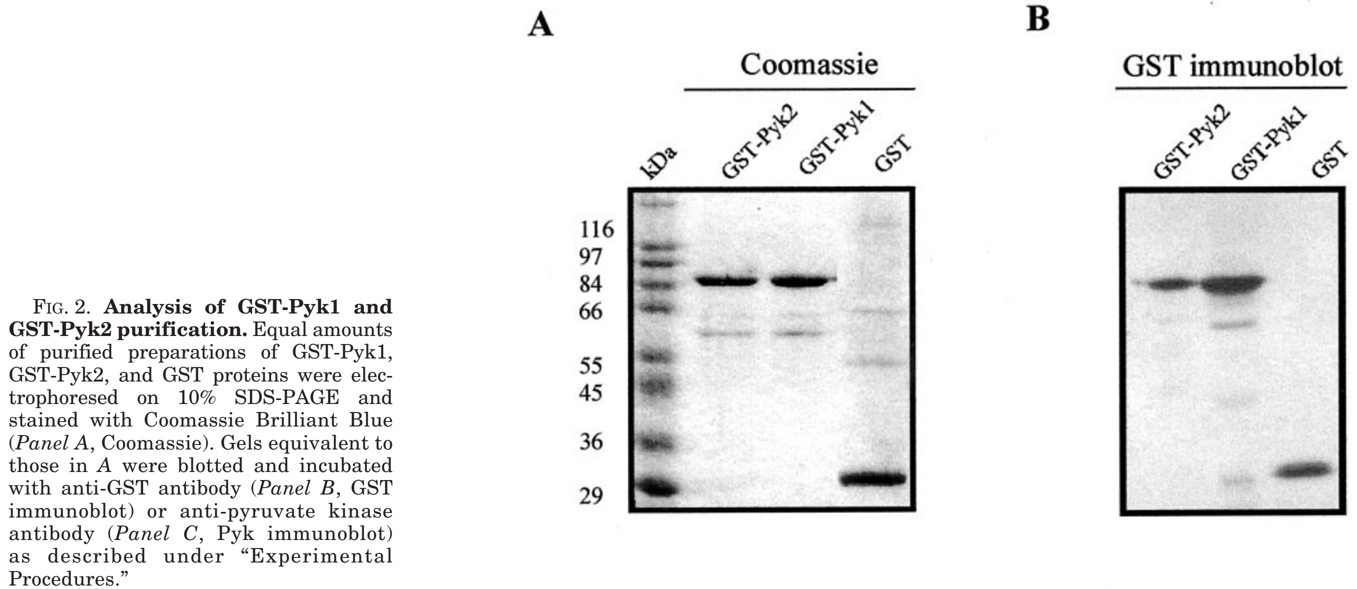
FIG. 1. **Co-immunoprecipitation of Pyk1 with yeast PKA holoenzyme.** Crude extract from 1115+*HA-TPK1*+YEp51-*BCY1* cells, overexpressing the Bcy1 protein and expressing HA-Tpk1 fusion protein, was subjected to immunoprecipitation (*IP*) with either anti-HA antibodies (*lanes 1* and *2*) or nonspecific antibody (*lanes 3* and *4*). The resulting immune complexes were subjected to phosphorylation (as described under "Experimental Procedures") without cAMP (*lanes 2* and *4*) or with 10 μM of cAMP (*lanes 1* and *3*). The samples were separated in SDS-PAGE. The gel was stained with Coomassie Blue colloidal (*upper gel*) and autoradiographed (*lower gel*). The protein band at 55 kDa showing specific cAMP-dependent phosphorylation is indicated with an *arrow*. The *asterisk* shows for comparison a band with specific phosphorylation, high radioactivity/protein ratio, but no cAMP dependence. The selected protein band was excised and analyzed with MALDI.

Phosphorylation of Pyk1 and Pyk2 by PKA in Vitro—*In vitro* phosphorylation of Pyk1 and Pyk2 was assayed using either Tpk1 or the catalytic subunit from bovine heart PKA (C_b) as enzyme sources. GST-Pyk1 and GST-Pyk2 were purified from strains containing plasmids coding for fusion proteins GST-YAL038W and GST-YOR347C, respectively, under a copper-dependent promoter. ORF sequences were verified by DNA sequencing, because the commercially available clones were not checked. Samples of the purified proteins were analyzed by SDS-PAGE and Western blotting using either anti-GST or anti-Pyk antibodies (Fig. 2). A band of ~84 kDa, as expected for the molecular mass of both fusion proteins, was detected both by Coomassie Blue staining (Fig. 2*A*) or by Western blot using anti-GST antibody (Fig. 2*B*). Fig. 2*C* shows that an antibody raised against rabbit muscle pyruvate kinase whole molecule cross-reacted with Pyk1 but did not recognize Pyk2. This result was unpredictable, because both yeast pyruvate kinases are ~45% identical to rabbit muscle pyruvate kinases M1 and M2. However, upon manual comparison of the sequences from the very few regions where higher eukaryotic pyruvate kinases are different, with the *S. cerevisiae* Pyk sequences, we could find higher identity for Pyk1 than for Pyk2 with the rabbit Pyks.

Different aliquots of the purified preparations of GST-Pyk1 and GST-Pyk2 were submitted to phosphorylation conditions, using [γ-^{32}P]ATP and either C_b or HA-Tpk1 preparation. The samples were subjected to SDS-PAGE and autoradiographed. Fig. 3 shows that Pyk1 and Pyk2 were phosphorylated by both bovine and yeast PKA catalytic subunits, with an increase in phosphorylation accompanying the increase in substrate concentration. Heterologous PKA phosphorylation was completely inhibited by the specific protein kinase peptide inhibitor, as expected. Control phosphorylation reactions were assayed for the yeast HA-Tpk1 by using immunoprecipitates prepared using nonspecific antibody as source of PKA. Control GST protein was neither phosphorylated by C_b nor Tpk1 (not shown).

To corroborate by a different approach the phosphorylation of Pyk1 and Pyk2 by yeast PKA, we assayed the phosphorylation *in vitro* using as a source of PKA crude extracts from a wild type (WT) strain (*1115*) containing normal levels of holoenzyme and from a strain with a deletion of *TPK2* and *TPK3* genes and containing and attenuated form of tpk1 (*tpk1*^{w1}). The mutant allele *tpk1*^{w1} contains a single missense mutation in the codon for an amino acid very conserved among PKA catalytic subunits. This mutant PKA catalytic subunit gene encodes a func-

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015



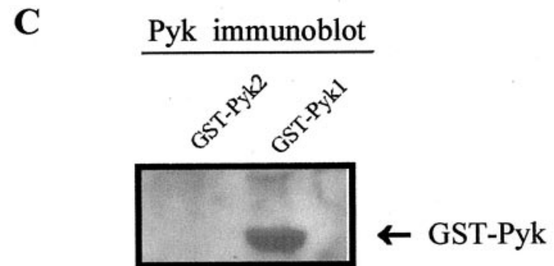


FIG. 2. **Analysis of GST-Pyk1 and GST-Pyk2 purification.** Equal amounts of purified preparations of GST-Pyk1, GST-Pyk2, and GST proteins were electrophoresed on 10% SDS-PAGE and stained with Coomassie Brilliant Blue (*Panel A*, Coomassie). Gels equivalent to those in *A* were blotted and incubated with anti-GST antibody (*Panel B*, GST immunoblot) or anti-pyruvate kinase antibody (*Panel C*, Pyk immunoblot) as described under "Experimental Procedures."

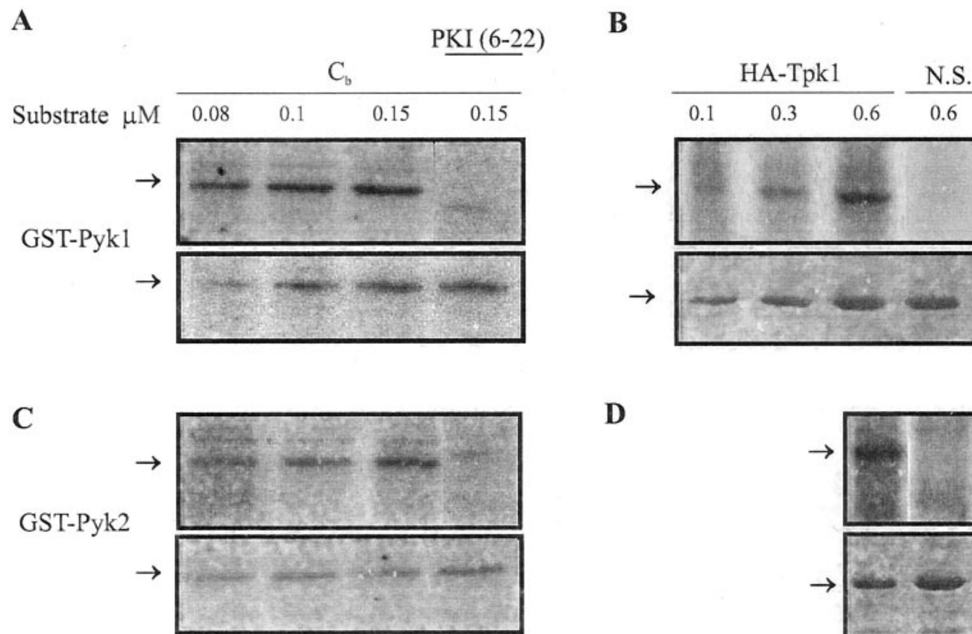
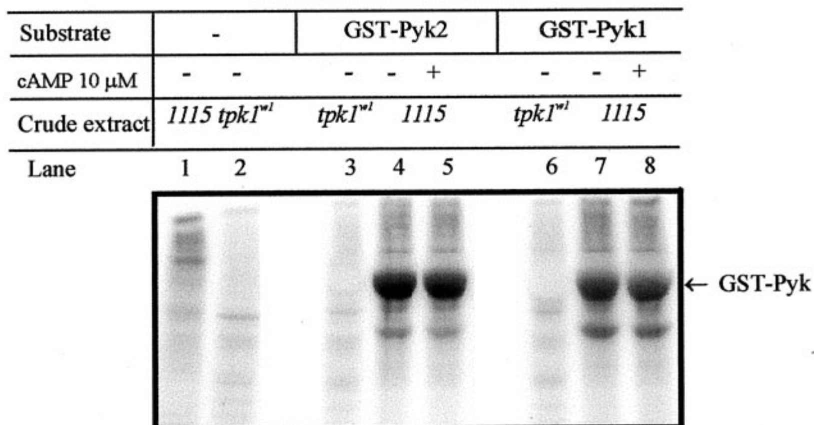


FIG. 3. ***In vitro* phosphorylation of GST-Pyk1 and GST-Pyk2.** Different amounts of purified GST-Pyk1 (*A* and *B*) or GST-Pyk2 (*C* and *D*) were incubated with 2 units of C_b (*A* and *C*) or with 5 units of HA-Tpk1 (*B* and *D*) and [γ-^{32}P]ATP, in a final volume of 40 μl. Radiolabeled products were resolved by SDS-PAGE, stained with Coomassie Brilliant Blue (*lower part of each panel*) and examined by autoradiography (*upper part of each panel*). Bands corresponding to GST-Pyk1 and GST-Pyk2 are indicated by *arrows*. *PKI (6-22)*, protein kinase inhibitor amide fragment; *N.S.*, control assay performed with the immunoprecipitate obtained with nonspecific preimmune serum instead of anti-HA antibody.

tionally attenuated protein (19). The WT extract displayed a −/+ cAMP activity ratio of 0.2, indicating it was a good source of holoenzyme; on the contrary we could not measure PKA activity in either permeabilized cells or crude extracts from the *tpk1^{w1}* strain (data not shown). Equal amounts of GST-Pyk1 and GST-Pyk2 were submitted to phosphorylation by either of

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015



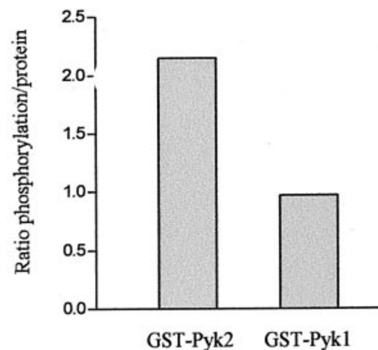


FIG. 4. ***In vitro* phosphorylation of GST-Pyk1 and GST-Pyk2 by WT and *tpk1^{w1}* crude extracts.** Samples containing 10–20 μg of immobilized GST-Pyk1 (*lanes 6–8*), GST-Pyk2 (*lanes 3–5*), or no protein (*lanes 1* and *2*) were incubated with crude extracts from *tpk1^{w1}* strain (*panel A*, *lanes 2*, *3*, and *6*) or crude extract from *1115* strain in the absence (*panel A*, *lanes 1*, *4*, and *7*) or in the presence (*panel A*, *lanes 5* and *8*) of 10 μM cAMP as described under "Experimental Procedures" and subjected to SDS-PAGE and autoradiography. The ratio phosphorylation/protein (*panel B*) was determined by densitometric analysis of Coomassie Blue-stained bands and the corresponding autoradiography of *lanes 5* and *8* of *panel A*.

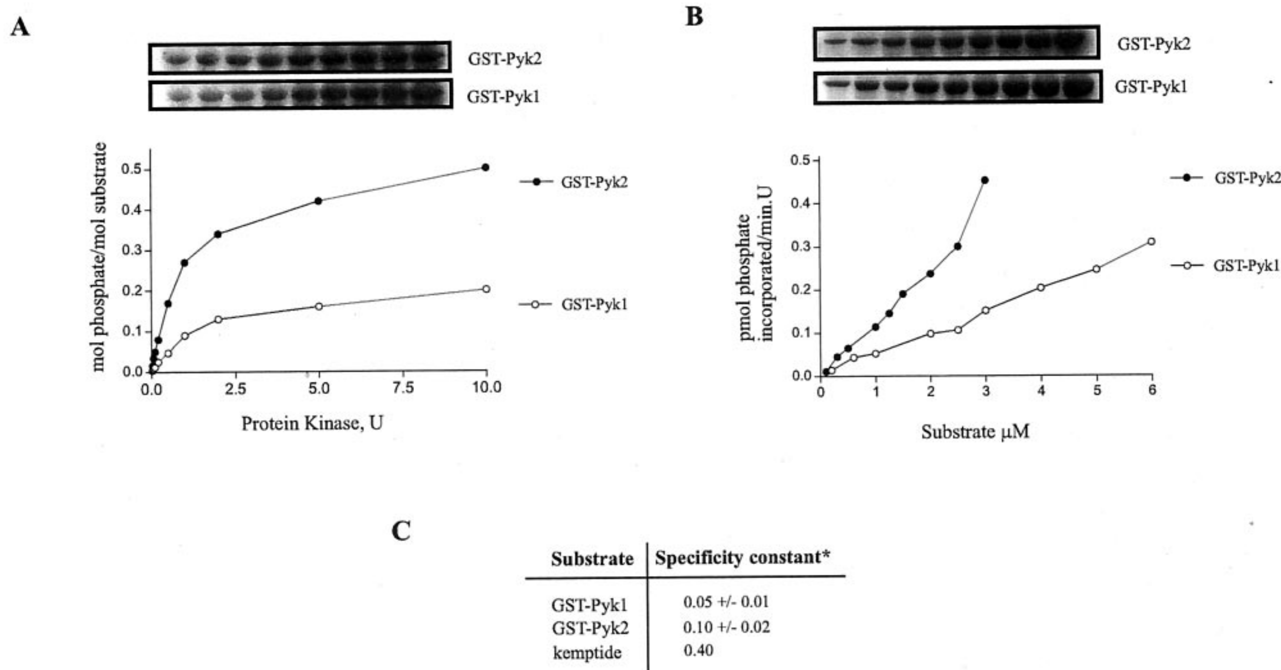
the two extracts and [γ-^{32}P]ATP, and the reactions were analyzed by SDS-PAGE and autoradiography (Fig. 4*A*). Both proteins were phosphorylated by WT extracts and not by *tpk1^{w1}* extracts, indicating that the phosphorylation was PKA-dependent. GST-Pyk2 was more efficiently phosphorylated by the WT extract than GST-Pyk1, as shown in Fig. 4*B*. Under these assay conditions phosphorylation of either GST-Pyk1 or GST-Pyk2 was not dependent on cAMP, even though the WT extracts showed cAMP-dependent activity when using Kemptide as substrate.

Preliminary results from Cytryñska *et al.* (8) and Rayner *et al.* (9) showed that Pyk1 could be phosphorylated *in vitro* by Tpk2 and Tpk1, respectively. From our last results and from the results of the immunoprecipitation (Figs. 1 and 3) we can say that both Pyk1 and Pyk2 are phosphorylated by Tpk1 *in vitro*.

Kinetic Constants for Pyk1 and Pyk2 Phosphorylation—To analyze whether the kinetics of phosphorylation of both Pyk1 and Pyk2 corresponded to those of a PKA substrate, we measured the kinetic parameters of phosphorylation using C_b as enzyme source. GST-Pyk1 and GST-Pyk2 were used as immobilized proteins, because the concentration of the eluted proteins was not sufficient to be assayed at high concentrations. It has been well described that protein affinity chromatography can be used as a method to estimate binding constants (18, 30). It has been assumed that the bound ligand (in this case the GST-Pyk) is always in equilibrium with the solution ligand (in this case the C_b) and that interactions of solid phase-bound protein with liquid-phase ligand are the same as interactions in liquid state. The results for interactions, which have been measured in the literature by more than one method, agree well (30).

Fig. 5*A* shows the dependence of phosphorylation of GST-Pyk1 and GST-Pyk2 by C_b on enzyme concentration. At the point of maximum phosphorylation, under these experimental conditions, C_b catalyzed the incorporation of 0.5 mol of phosphate/mol of GST-Pyk2 and 0.15 mol of phosphate/mol of GST-Pyk1. To find the kinetic parameters for the phosphorylation of GST-Pyk1 and GST-Pyk2 by C_b, experiments were carried out using variable concentrations of the two immobilized protein substrates in phosphorylation assays using 2 units of C_b subunit (Fig. 5*B*). The *upper panel* of Fig. 5*B* shows the autoradiography of phosphorylated proteins; quantification of incorporated phosphate was measured by liquid scintillation counting of the sliced bands and is shown in the *lower panel* of Fig. 5*B*. Experimentally we could not attain the V_{max} due to the limitation in increasing the substrate concentration. However, some information can be obtained from the data of Fig. 5*B* by comparing the velocities of phosphorylation of Kemptide and Pyk-GST substrates using the 2 units of C_b during 15 min. The phosphorylation of this synthetic peptide would have reached a total of 30 pmol in the 15-min period, according to the manufacturer's indication; the phosphorylation of Pyk1-GST and Pyk2-GST reached 9 and 15 pmol, respectively, in the 15-min period. These results indicate that the velocities of phosphorylation for the three substrates are quite similar. In this estimation we have not taken into account the K_m values for the two Pyk substrates. This parameter can not be calculated, because

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015



Substrate	Specificity constant*
GST-Pyk1	0.05 +/- 0.01
GST-Pyk2	0.10 +/- 0.02
kemptide	0.40

FIG. 5. **Kinetic behavior of *in vitro* phosphorylation of GST-Pyk1 and GST-Pyk2 by catalytic subunit from bovine heart PKA.** Immobilized GST-Pyk1 (5 μM) and GST-Pyk2 (3 μM) were incubated with the indicated amounts (units/40 μl) of C_b subunit and 0.1 mM [γ-^{32}P]ATP (1000 dpm/pmol) for 15 min at 30 °C (*panel A*). In *panel B*, 2 units/40 μl of C_b and 0.1 mM [γ-^{32}P]ATP (1000 dpm/pmol) were incubated with the indicated concentration of GST-Pyk1 or GST-Pyk2. After incubation, samples were subjected to SDS-PAGE and autoradiography. The incorporation of phosphate into the substrate was determined by scintillation counting of the phosphorylated enzyme band excised from SDS-PAGE gels. Phosphorylation of Kemptide was determined by the phosphocellulose paper method. A portion of the autoradiograms, including the position of pyruvate kinase, is shown *above each panel*. *Panel C*, the specificity constants (k_{cat}/K_m) expressed as pmol·(min·unit)$^{-1}\mu$M^{-1}, calculated for GST-Pyk1 and GST-Pyk2 as described under "Experimental Procedures." The estimated parameters used for Kemptide were K_m = 45 μM and k_{cat} = 17 pmol/min·unit. Values are expressed as means ± S.E. from two separate experiments.

the concentrations of protein substrate used turned out to be very low; however, the parameter V_{max}/K_m, which can be obtained from the tangents to the origin of the curves of Fig. 5*B* (see "Experimental Procedures") is the most critical parameter in determining the specificity of an enzyme for a substrate. The data of Fig. 5*C* show the specificity constants for the two substrates as compared with Kemptide, experimentally measured using C_b. When comparing both GST-Pyk substrates, it can be seen that GST-Pyk2 is a better substrate for C_b than GST-Pyk1. The efficiency for *in vitro* phosphorylation of GST-Pyk1 and GST-Pyk2 by C_b was 8- and 4-fold lower than for Kemptide under the same conditions. However, it is known that natural PKA substrates are not always better than the corresponding peptides derived from it or peptides used as models, because even in the case of sharing the same substrate determinants, the phosphorylation site structure in the protein substrate is likely to be more constrained (31).

Phosphorylation of Pyk1 and Pyk2 by Endogenous PKA in Vivo—To demonstrate the *in vivo* phosphorylation of Pyk1 and Pyk2, yeast cells from WT and *tpk1^{w1}* strains expressing GST-Pyk1 and GST-Pyk2 were metabolically labeled with $^{32}P_i$; GST-Pyk1 and GST-Pyk2 from those cells were purified through glutathione-agarose affinity chromatography. The purified proteins were analyzed by SDS-PAGE and digital imaging analysis. The results indicate that GST-Pyk1 (Fig. 6*A*) and GST-Pyk2 (Fig. 6*B*) were in fact phosphorylated *in vivo* and that phosphorylation was PKA-dependent, because there was no radiolabeled phosphoprotein that comigrated with GST-Pyk1 and GST-Pyk2 in the purification from *tpk1^{w1}*-radiolabeled strains.

Effect of Phosphorylation on Pyruvate Kinase Activity—To analyze whether phosphorylation by PKA had an effect on pyruvate kinase activity, our rationale was to use a classic genetic approach and determine the kinetic parameters V_{max} and K_m of endogenous, partially purified pyruvate kinase, derived from strains with different degree of PKA activity. The aim was to find a correlation between pyruvate kinase activity and PKA activity. On the top scale with high PKA activity, we used strain *1451*, with a mutation in the *BCY1* gene (*bcy1–14)* consisting of a small deletion in the α-helix C of cAMP binding domain A, which confers to the holoenzyme high constitutive activity, almost independent of cAMP (11). Following in the order of PKA activity we used a WT strain (*1115*), with the same genetic background as the *1451* strain. Finally the *tpk1^{w1}* strain was used as a source of poor PKA activity (see above). Using this approach we expected to obtain a pyruvate kinase with a variable degree of modification by PKA phosphorylation, the higher modification being expected in the *1451* strain, and the lower in the *tpk1^{w1}* strain. In fact the ratio of −/+ cAMP PKA activity in extracts from the cells in logarithmic stage of growth in YPD, measured in permeabilized cells, reflects a ratio of 0.2 for strain *1115* and 0.9 for strain *1451*, whereas *tpk1^{w1}* strains display no activity either in the absence or presence of cAMP. Previous work from our laboratory (11, 13) has shown that strain *1451*, when compared with the wild type strain *1115*, shows a higher severity in classic PKA-dependent phenotypes such as glycogen accumulation, respiratory capacity, and growth at 37 °C.

It has been reported that the expression of Pyk1 mRNA is dependent on the presence of glucose in the growth medium, with a decrease in expression along the diauxic shift (32, 33). Previous to the study of the relationship between phosphorylation and Pyk activity, we decided to investigate the level of expression of Pyk in two different stages of growth in YPD

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015

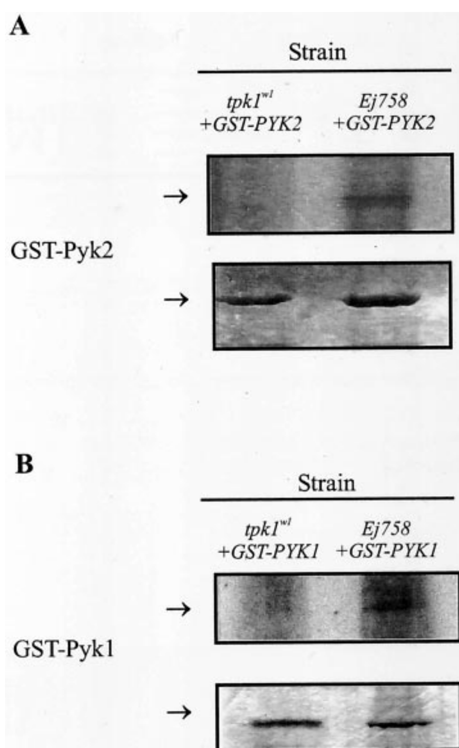


FIG. 6. ***In vivo*** **phosphorylation of GST-Pyk1 and GST-Pyk2 by yeast PKA.** Wild type cells (*EJ758*) and mutant *tpk1^{w1} tpk2Δ tpk3Δ* cells (*tpk1^{w1}*) expressing GST-Pyk2 (*panel A*) or GST-Pyk1 (*panel B*) were metabolically labeled with $^{32}P_i$. The GST fusion proteins were purified by glutathione-agarose chromatography and subjected to SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (*lower part of each panel*) and subjected to digital imaging (*upper part of each panel*).

medium: before and after diauxic shift (data not shown). We determined the specific activity (units per milligram of total protein) of Pyk in both stages by measuring the catalytic activity in the absence and in the presence of FBP and the levels of Pyk by Western blot using anti-Pyk antibody. The diauxic shift was followed by the change in A_{600} and by measurement of glucose consumption. As expected, according to the reports on the Pyk mRNA levels and glucose concentration (32, 33), the specific activity of Pyk was glucose-dependent and decreased during the post-diauxic shift; this phenomenon was observed for the three strains: *1451*, *1115*, and *tpk1^{w1}*. The decrease in specific activity correlated with the levels of Pyk1 followed by Western blot. According to these results the kinetic experiments that follow were performed on partially purified preparations of Pyk from cells in a pre-diauxic shift stage, in which the specific activity of Pyk1 is maximal and there is no expression of Pyk2 (17).

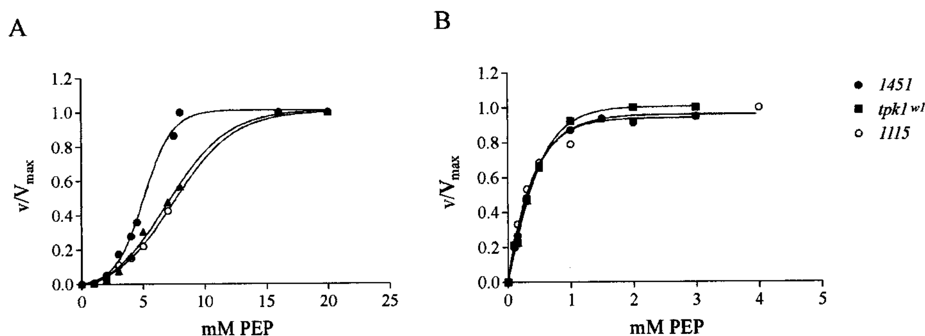
We analyzed the kinetic behavior of semi-purified Pyk from each strain toward PEP in the presence or absence of FBP (Fig. 7*A*); kinetic parameters derived from the Hill plot of the *curves* shown in Fig. 7*A* are shown in Fig. 7*B*. In the presence of the allosteric activator FBP (Fig. 7*A*, *right panel*), the $K_{0.5}$ value for PEP and the k_{cat} value for the reaction in the three strains were quite similar; the n_H value for Pyk1 from strain *tpk1^{w1}* was a little higher, indicating that a complete shift toward a Michaelis-Menten behavior had not been attained at 1.5 mM FBP. The $K_{0.5}$ for PEP and n_H values for the Pyk1 activity measured in the absence of the allosteric activator, FBP, differed among the three strains. The most remarkable result is the increase in cooperativity displayed by the Pyk1 derived from the *1451* strain, with an increase in the n_H and a decrease in the $K_{0.5}$ for PEP (Fig. 7*A*, *left panel*, and Fig. 7*B*). Taken as a whole, the parameters of the enzyme derived from the *1451* strain, which presumably is more phosphorylated, reflect a more active pyruvate kinase. No significant changes in the kinetic parameters measured in the absence of FBP have been observed between Pyk preparations derived from strains *1115* and *tpk1^{w1}*. This lack of difference is not surprising, because one would expect that phosphorylation events in a wild type strain would be only transient, and the degree of phosphorylated molecules would be very low when taking samples from logarithmically growing cells, as compared with extracts from the *1451* strain, where PKA is constitutively activated and the degree of phosphorylation of its target substrates might be more sustained.

To confirm whether the kinetic parameters for the Pyk1 derived from the *1451* strain, suggesting a more active pyruvate kinase in this strain with higher PKA activity, were due to enzyme phosphorylation, we examined the effect of protein kinase A and alkaline phosphatase on Pyk1 activity. Pyk1 preparations from *1451* strain were incubated either with C_b or with alkaline phosphatase. The phosphatase used could be removed from the reaction with magnetic beads to avoid interference in the ulterior pyruvate kinase assay. Pyk1 activity was measured in the more highly phosphorylated or in the dephosphorylated enzymatic preparation using a concentration of PEP of 8 mM, the concentration at which the differences of Pyk1 activity were maximum among the different phosphorylation sates of the Pyk1 (see Fig. 7). The results, shown in Fig. 8, indicate that the alkaline phosphatase treatment resulted in a decrease in Pyk activity of around 35%, as compared with a control in which the preparation was maintained in the presence of phosphatase inhibitors throughout. This result indicated that Pyk1 from strain *1451* was phosphorylated and that the phosphatase treatment produced the loss of phosphates with the concomitant loss of activity as in the case of Pyk1 from strain *tpk^{w1}*. The opposite treatment, the phosphorylation of Pyk1 with C_b, resulted in a stimulatory effect on Pyk1 activity with an increase of 1.6-fold on the activity at 8 mM PEP in the absence of FBP (Fig. 8) as compared with the control. This result indicates that Pyk1 preparations of *1451* strain were not fully phosphorylated. As a whole, if we estimate the change in activity from the less phosphorylated to the more phosphorylated Pyk1, we have an overall change of activity of almost 2.5-fold produced by PKA.

DISCUSSION

In the present study, we identify and characterize two substrates of PKA from *S. cerevisiae*: Pyk1 and Pyk2. We have demonstrated both *in vivo* and *in vitro* phosphorylation of these substrates. In this work, i*n vitro* phosphorylation of Pyk1 and Pyk2 was demonstrated using either Tpk1 or C_b as enzyme source, and the results show that both Pyks were substrates of both types of PKA. It has been reported that yeast and mammalian PKAs recognize similar specific features of peptide substrates (28). Analyzing Pyk1 and Pyk2 sequences for putative PKA phosphorylation sites, only one canonical RR*X*S consensus sequence for PKA was found, corresponding to RRTS in position 19–22 for Pyk1 and 21–24 for Pyk2, conveniently located in the interface of catalytic domain A and regulatory domain C of the protein, according to its crystal structure (15). These sequences are conserved in all the pyruvate kinases from fungal origin sequenced at present, such as the ones from *Schizosaccharomyces pombe*, *Aspergillus niger*, *Emericella nidulans*, and *Yarrowia lipolytica*, but not in pyruvate kinase isoforms from higher eukaryotes. Other putative sites for PKA phosphorylation are far from the ideal consensus sequence, although they cannot be discarded and they are present either in the catalytic or regulatory domain of Pyk1. The stoichiome-

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015



Strain	− FBP			+ FBP [a]		
	$K_{0.5}$(PEP) (mM)	k_{cat} [b]	n_H	$K_{0.5}$(PEP) (mM)	k_{cat} [b]	n_H
tpk1^w1	8.0	14	2.5	0.31	16.4	2.0
1115	7.5	11	2.6	0.26	11.0	1.2
1451	5.4	13	3.1	0.32	14.4	1.4

FIG. 7. **Activity of partially purified pyruvate kinase from different PKA background strains.** Pyk activities were measured as a function of the concentration of PEP in the absence (*Panel A*, *left*) or in the presence (*Panel A*, *right*) of 1.5 mM FBP in partially purified samples from $tpk1^{w1}$, *1115* and *1451* strains, as described under "Experimental Procedures." The figure shows the data from representative experiments. *Panel B* shows the pyruvate kinase activities and kinetic parameters derived from the experiments in *Panel A*. *a*, kinetic constants determined in the presence of 1.5 mM FBP. *b*, expressed in units/relative amount of Pyk as described under "Experimental Procedures."

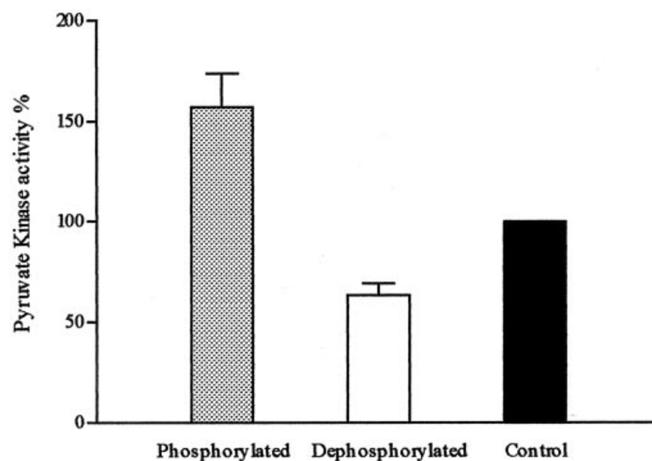


FIG. 8. **Effect of phosphorylation and dephosphorylation on Pyk1 activity.** Pyk activity was measured at 8 mM PEP in partially purified samples from *1451* strains phosphorylated with C_b or dephosphorylated with alkaline phosphatase as described under "Experimental Procedures." Data are expressed as the percentage of activity of Pyk (units/milligram of total protein) taking as 100% the activity of an aliquot incubated in the absence of additional protein kinase or alkaline phosphatase and in the presence of phosphatase inhibitors. Results represent mean values ± S.E. with $n = 3$ for each condition.

tries of phosphorylation attained with C_b *in vitro* are compatible with one phosphorylation site per molecule of pyruvate kinase; however, optimization of the phosphorylation conditions, so as to compensate for the low efficiency usually displayed by PKAs, was not assayed. It has been reported that Pyk1 could be visualized by isoelectric focusing as four different forms with different isoelectric points, suggesting multiple phosphorylation sites per monomer, either under *in vitro* phosphorylation conditions (8) or analyzing the protein profile from exponentially growing cells (35). In higher eukaryotes at least, several kinases have been reported to be responsible for the multiphosphorylation of pyruvate kinase (36).

The phosphorylation of Pyk1 and Pyk2 by yeast PKA was also assayed *in vitro* using crude extracts from strains WT and tpk^{w1} as source of protein kinase. A clear dependence on PKA activity was evident, because extracts from the PKA weak strain (tpk^{w1}) phosphorylated neither GST-Pyk1 nor GST-Pyk2. However, even though the WT extracts showed cAMP-dependent phosphorylation of Kemptide, the phosphorylation of the Pyk recombinant proteins was cAMP-independent. This lack of dependence on cAMP could be explained if PKA from *S. cerevisiae* behaves as PKA from the fungus *Mucor rouxii*, where we have demonstrated (12) that the protein substrate is involved in the activation of PKA by cAMP and that, depending on the incubation conditions, different degrees of activation by cAMP are obtained using different substrates. In fact, under the phosphorylation conditions used for Fig. 1, where the source of holoenzyme is an immunoprecipitate of tagged Tpk1 bound to Bcy1, dependence on cAMP for the phosphorylation of endogenous Pyk1 was demonstrated.

The kinetic parameters for the phosphorylation reaction were defined assaying the phosphorylation of GST-Pyk1 and GST-Pyk2 by C_b. The specificity constant for both protein substrates, as a measure for the efficiency in phosphorylation, indicates that they both fall in the range of the specificity constant attained for Kemptide in a parallel experiment, suggesting that both Pyks are genuine substrates for C_b. Although we did not measure the kinetic parameters for phosphorylation by yeast PKA, comparative studies between yeast and mammalian PKAs (28) indicate that the yeast PKA is around 20- to 30-fold less efficient than the mammalian enzymes, due almost exclusively to a decrease in substrate binding for the yeast enzyme with a consequent increase in the K_m value. The specificity constant of C_b for GST-Pyk2 is greater than for GST-Pyk1 (0.1 *versus* 0.05) (Fig. 5*C*). A similar substrate preference is shown for yeast PKA, because the experiments of Fig. 4*B* show a higher phosphorylation/protein ratio for GST-Pyk2 protein. A physiological relevance for Pyk phosphorylation can be expected, because we could demonstrate *in vivo* phosphorylation of the recombinant GST-Pyks (Fig. 6), absolutely dependent on the presence of active PKA in the cell. We could not measure the affinity of either Pyk for C_b or for yeast PKA to attempt a comparison with the intracellular concentration of Pyk, which in principle could be estimated from data obtained from the literature. However, it has been reviewed (32) that it is difficult to draw physiological relevance of protein phosphorylation from the comparison of K_m values with endogenous concentration, because, from the heterogeneity of substrate sequences that are known and from the detailed knowledge of the binding of the protein kinase inhibitor to the multi-substrate protein kinase A catalytic site, it seems that natural substrates have not evolved to be "optimum substrates" according to the *in vitro* definition. It has therefore been highlighted

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015

that not only kinetic parameters define “physiological” optimum. In fact, it would be difficult to estimate an endogenous concentration for Pyk1 in yeast, because it has been described to have a granular cytoplasmic distribution (37).

Some preliminary experiments were preformed to find a correlation between phosphorylation and Pyk activity. With this aim we determined pyruvate kinase activity from partially purified preparations derived from strains with different levels of PKA activity. If one compares the behavior of the Pyk derived from the strain in which greater phosphorylation might be expected (*1451*) with the one with lower PKA-mediated phosphorylation ($tpk1^{w1}$) the following differences can be found: a discrete shift of the titration curve for PEP to the left (Fig. 7*A*) together with an increase in the cooperativity for PEP in the absence of FBP, for the Pyk derived from strain *1451*, and a Hill coefficient of 1.4 in the presence of FBP, as compared with an n_H of 2 for the less phosphorylated enzyme.

As demonstrated by the results of phosphorylation by C_b and dephosphorylation by alkaline phosphatase of the Pyk1 derived from the *1451* extracts (Fig. 8), it is apparent that the species obtained from this strain, already partially phosphorylated, when compared with a strain with low PKA activity, can be further phosphorylated by additional C_b, with a further increase in enzymatic activity and dephosphorylated by phosphatase with a decrease in the activity of 2.5-fold as compared with the highly phosphorylated preparation. These preliminary results suggest that phosphorylation of Pyk1 by PKA is a positive regulator of enzyme activity, leading to a bias in the allosteric equilibrium toward the R-state, reflected in the shift of the saturation curve for PEP and an increase in cooperativity. This shift in the equilibrium between R and T states could have as a consequence an increase in the affinity for the activator ligand FBP, as predicted by the concerted model of allosterism. Although affinity for FBP has not been measured, a suggestion of increase in affinity can be speculated from the n_H of 2 displayed by the less phosphorylated Pyk1 in the presence of 1.5 mM FBP as compared with 1.4 for the more phosphorylated enzyme. A similar modification of the kinetic parameters has been described for a point mutation in the *Escherichia coli* pyruvate kinase (38) precisely in a residue (Arg-271) located in the interface between the catalytic domain A and the regulatory domain C of FBP binding, suggesting that Ser-22 in Pyk1, located structurally in the same interface, might be the target for PKA phosphorylation. It is interesting to mention that yeast cells, containing a point mutation in a residue (R19Q) located in the vicinity of Ser-22 (Arg-19, contained in the consensus PKA phosphorylation sequence RRTS) from yeast Pyk1, do not grow in glucose as carbon source (39), indicating null or low catalytic activity for the Pyk1 from this mutant. The modification in the kinetic parameters observed, taken as a whole, suggest that the phosphorylated enzyme is more active; phosphorylation of rat liver pyruvate kinase by PKA, on the contrary, shifts the equilibrium between the active and inactive forms of the enzyme to favor the inactive form (40). An activation of Pyk by PKA could have been predicted from genetic results (16), which indicate that the phenotypes of cells overexpressing Pyk1 are similar to the classic phenotypes observed upon activation of the cAMP/PKA pathway in yeast; namely, reduction in glycogen accumulation, reduction of sporulation proficiency, and impairment in growth at 37 °C and in nonfermentable carbon sources. Very recent results from the same group (9) also suggest that PKA positively regulates yeast pyruvate kinase *in vivo*.

Pyruvate kinase catalyzes the final step in glycolysis, producing the second of two ATP molecules generated in the glycolytic pathway. The enzyme converts phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP. This reaction is a committed step leading to either anaerobic fermentation or oxidative phosphorylation of pyruvate. In most cells the reaction is essentially irreversible and is one of the major control points of glycolysis. The regulation of Pyk is important for controlling the levels of ATP, GTP, and glycolytic intermediates in the cell. Allosteric regulation of enzyme activity is a mechanism for finely tuning biochemical reaction pathways to maintain an appropriate balance of intracellular substrate and product concentrations; it also allows an enzyme coordinate its activity with other cellular reactions and signaling pathways.

The rate of yeast glycolysis was generally assumed to be determined by the rate of the first irreversible step of the pathway, which is catalyzed by phosphofructokinase. The phosphofructokinase activity increases as a result of rising concentration of its substrate, fructose 6-phosphate, and its main allosteric activator, fructose 2,6-bisphosphate, which in turn is positively regulated at the transcriptional and post-translational levels by PKA. Activation of phosphofructokinase increases the concentration of fructose 1,6-bisphosphate, which is an allosteric activator of pyruvate kinase, the second specific glycolytic enzyme. In this way the glycolysis is activated progressively by greater concentrations of enzyme substrates and allosteric activators (34). The results of this work suggest a second point of regulation by PKA in the glycolysis through the activation of Pyk by phosphorylation and its consequent increase in sensitivity to small changes in the levels of its substrate PEP or its activator FBP that occur, for example, during the shift from non-fermentable to fermentable carbon sources.

REFERENCES

1. Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987) *Cell* **50,** 227–287
2. Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D., McBullen, B., Hurwitz, M., Krebs, E. G., and Wigler, M. (1987) *Mol. Cell. Biol.* **7,** 1371–1377
3. Thevelein, J. M., and de Winde, J. H. (1999) *Mol. Microbiol.* **33,** 904–918
4. Rolland, F., Winderickx, J., and Thevelein, J. M. (2001) *Trends Biochem. Sci.* **26,** 310–317
5. Entian, K. D., and Barnett, J. A. (1992) *Trends Biochem. Sci.* **17,** 506–510
6. Navas, M. A., and Gancedo, J. M. (1996) *J. Bacteriol.* **178,** 1809–1812
7. Dickinson, J. R., and Schweizer, M. (1999) in *The Metabolism and Molecular Physiology of Saccharomyces cerevisiae* (Dickinson, J. R., and Schweizer, M., eds) pp. 209–275, Taylor & Francis Ltd, London
8. Cytryñska, M., Frajnt, M., and Jakubowicz, T. (2001) *FEMS Microb. Lett.* **203,** 223–227
9. Rayner, T. F., Gray, J. V., and Thorner, J. W. (2002) *J. Biol. Chem.* **277,** 16814–16822
10. Gonçalves, P. M., Griffioen, G., Bebelman, J. P., and Planta, R. J. (1997) *Mol. Microbiol.* **25,** 483–493
11. Zaremberg, V., and Moreno, S. (1996) *Eur. J. Biochem.* **237,** 136–142
12. Zaremberg, V., Donnella-Deana, A., and Moreno, S. (2000) *Arch. Biochem. Biophys.* **381,** 74–82
13. Portela, P., Zaremberg, V., and Moreno, S. (2001) *Microbiology* **147,** 1149–1159
14. Sorol, M. R., Pereyra, E., Mizyrycki, C., Rossi, S., and Moreno, S. (2001) *Exp. Cell Res.* **271,** 337–342
15. Jurica, M. S., Mesecar, A., Heath, P. J., Shi, W., Nowak, T., and Stoddard, B. L. (1998) *Structure* **6,** 195–210
16. Brazill, D. T., Thorner, J., and Martin, G. S. (1997) *J. Bacteriol.* **179,** 4415–4418
17. Boles, E., Schulte, F., Miosga, T., Freidel, K., Schlüter, E., Zimmermann, F. K., Hollenberg, C. P., and Heinisch, J. J. (1997) *J. Bacteriol.* **179,** 2987–2993
18. Martzen, M. R., McCraith, S. M., Spinelli, S. L., Torres, F. M., Fields, S., Grayhack, E. J., and Phizicky, E. M. (1999) *Science* **286,** 1153–1155
19. Cameron, S., Levin, L., Zoller, M., and Wigler, M. (1988) *Cell* **53,** 555–566
20. Hoffman, C. S., and Winston, F. (1987) *Gene* **57,** 267–272
21. Mitchell, D. A., Marshall, T. K., and Deschenes, R. J. (1993) *Yeast* **9,** 715–723
22. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
23. Sherman, F., Fink, G., and Hicks, J. B. (1981) in *Methods in Yeast Genetics: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Roskoski, R., Jr. (1983) *Methods Enzymol.* **99,** 3–6
25. Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fostsis, T., and Mann, M. (1996) *Nature* **379,** 466–469
26. Shevchenko, A., Jensen, O., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Boucherie, H., and Mann, M. (1996) *Proc. Natl.*

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015

Acad. Sci. U. S. A. **93,** 14440–14445
27. Perkins, D. N., Pappin, D. J. C., Creasy, D. M., and Cottrell, J. S. (1999) *Electrophoresis* **20,** 3551–3567
28. Denis, C. L., Kemp, B. E., and Zoller, M. J. (1991) *J. Biol. Chem.* **266,** 17932–17935
29. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254
30. Formosa, T., Barry, J., Alberts, B. M., and Greenblatt, J. (1991) *Methods Enzymol.* **208,** 24–45
31. Walsh, D. A., Glass, D. B., and Mitchell, R. D. (1992) *Curr. Opin. Cell Biol.* **4,** 241–251
32. Moore, P. A., Sagliocco, F. A., Wood, R. M., and Brown, A. J. (1991) *Mol. Cell. Biol.* **11,** 5330–5337
33. DeRisi, J. L., Iyer, V. R., and Brown, P. O. (1997) *Science* **278,** 680–686
34. Gonçalves, P., and Planta, R. J. (1998) *Trends Microbiol.* **6,** 314–319
35. Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999) *Mol. Cell. Biol.* **19,** 1720–1730
36. Kumar, A., Agarwal, S., Heyman, J., Matson, S., Heitman, M., Piccirillo, S., Unansky, L., Drawid, A., Jansen, R., Liu, Y., Cheung, K., Miler, P., Gerstein, M., Roder, S., and Snyder, M. (2002) *Genes Dev.* **16,** 707–719
37. Pilkis, S. J., Claus, T. H., and el-Maghrabi, M. R. (1988) *Adv. Second Messenger Phosphoprotein Res.* **22,** 175–191
38. Valentini, G., Chiarelli, L., Fortin, R., Speranza, M. L., Galizzi, A., and Mattevi, A. (2000) *J. Biol. Chem.* **275,** 18145–18152
39. Fenton, A. W., and Blair, J. B. (2002) *Arch. Biochem. Biophys.* **397,** 28–39
40. El-Maghrabi, M. R., Claus, T. H., McGrane, M. M., and Pilkis, S. J. (1982) *J. Biol. Chem.* **257,** 233–240

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015

jbc The Journal of Biological Chemistry
AFFINITY SITES
ASBMB
American Society for Biochemistry and Molecular Biology

ENZYME CATALYSIS AND REGULATION:

In Vivo and _in Vitro_ Phosphorylation of Two Isoforms of Yeast Pyruvate Kinase by Protein Kinase A

Paula Portela, Steven Howell, Silvia Moreno and Silvia Rossi

J. Biol. Chem. 2002, 277:30477-30487.
doi: 10.1074/jbc.M201094200 originally published online June 12, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201094200

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 16 of which can be accessed free at
http://www.jbc.org/content/277/34/30477.full.html#ref-list-1

Downloaded from http://www.jbc.org/ at SWETS SUBSCRIPTION SERVIC on February 6, 2015