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

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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-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 (tpk1Δ/Δ). 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) 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 adenylyl 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 glycerol synthesis and degradation, such as glycerol 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 the 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 (11–14). We have found that the PKA catalytic subunit (Pyk1) is phosphorylated in vivo by PKA when expressing the catalytic subunit in yeast cell extracts or in a strain overexpressing the catalytic subunit. We have also shown that the yeast pyruvate kinase, one of the main targets of PKA phosphorylation in vivo, is phosphorylated in yeast extracts by the PKA catalytic subunit, both in vitro and in vivo. The phosphorylation of the enzyme is not only dependent on the cAMP level but also on the carbon source, glucose or galactose, indicating that the PKA pathway is involved in the regulation of the metabolic flux between glycolysis and gluconeogenesis. These results suggest that the PKA pathway plays a role in the coordination of the metabolic fluxes in yeast cells, which is consistent with the role of PKA in other organisms, where this pathway is involved in the regulation of the metabolic fluxes between glycolysis and gluconeogenesis.
Table I
List of strains and nomenclature

| Strain         | Genotype        | Genetic nomenclature |
|----------------|-----------------|----------------------|
| EJ758-YOR347C  | pYEX4T1-GSTYOR347C | EJ758 + GST-PYK2    |
| EJ758-YAL038W  | pYEX4T1-GSTYAL038W | EJ758 + GST-PYK1    |
| KT 1115<sup>a</sup> | Mata leu2 ura3 his3 pep4Δ | 1115 + HA-TPK1  |
| KT 1115<sup>a</sup> | Mata leu2 ura3 his3 pep4Δ + Ycp50-HATPK1 | 1115 + HA-TPK1 + Yepl51-BCY1 |
| KT 1115<sup>a</sup> | Mata leu2 ura3 his3 pep4Δ + Ycp50-HATPK1 + Yepl51-BCY1 | 1115 + GST |
| KT 1451<sup>a</sup> | Mata leu2 ura3 his3 pep4Δ bcy1-14 | 1451 |
| RS13-58<sup>b</sup> - 1<sup>d</sup> | Mata his3 leu2 ura3 trpl ade8 tpk1<sup>−</sup> tpk2<sup>−</sup> tpk3<sup>−</sup> trp1<sup>−</sup> bcy1::LEU2 | t pk1<sup>−</sup> + GST-PYK2<sup>d</sup> |
| RS13-58<sup>b</sup> - 1 | Mata his3 leu2 ura3 trpl ade8 tpk1<sup>−</sup> tpk2<sup>−</sup> tpk3<sup>−</sup> trp1<sup>−</sup> bcy1::LEU2 | t pk1<sup>−</sup> + GST-PYK2<sup>d</sup> |
| RS13-58<sup>b</sup> - 1<sup>d</sup> | Mata his3 leu2 ura3 trpl ade8 tpk1<sup>−</sup> tpk2<sup>−</sup> tpk3<sup>−</sup> trp1<sup>−</sup> bcy1::LEU2 | t pk1<sup>−</sup> + GST-PYK2<sup>d</sup> |

These strains have been previously described in the literature: *<sup>c</sup>(18), *<sup>c</sup>(13), *<sup>c</sup>(19).<sup>d</sup> Strains developed in this report.

14. While looking for PKA substrates in *Saccharomyces 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 glyogen 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(TZ-5), and anti-goat IgG-peroxidase-conjugated, Chemiluminescence Luminol reagent were from Santa Cruz Biotechnology, Inc. [32P]orthophosphate were from PerkinElmer Life Sciences. Phosphocellulose paper (P81) was from Whatman. Colloidal Coomassie Blue stain was from Bio-Rad Laboratories, Inc. 

**Protein Kinase A Phosphorylation of Yeast Pyruvate Kinases**

**Strains**—Standard yeast strains were from the laboratory of R. Schekman. Strains EJ758-YOR347C and EJ758-YAL038W 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'-GAATTCAGCTGACCAC-3', and primer R, 5'-GATCCCCGGGAATTGGCATC-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).

**Expression of GST fusion proteins**—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 YEp50-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 CPU1 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 prepa-
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 357-nm nitrogen laser and a Scout-384 probe, was used to obtain positive ion mass spectra of different purified protein preparations. Deposition of the samples on the surface of the detector was performed by direct injection in reflection 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 wash buffer A. In these conditions the combination of the substrate with the enzyme becomes rate-limiting, and the Michaelis-Menten equation becomes 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 medium, grown overnight, and re-inoculated into SD-Ura-Leu, grown to an OD600 of 0.8, and induced with 0.5 mM Cu2SO4 for 2 h before harvest. Cells were homogenized with glass beads in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM MgCl2, 5 mM dithiothreitol, 10% glycerol, and 0.5 mM NaCl plus 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, and 3 μg/ml aprotinin (buffer B). The GST protein was purified from crude extract of 1115+GST cultures induced by 0.5 mM Cu2SO4 and diluted different amounts of the protein and GST-Pyk1 and GST-Pyk2 fusion proteins were purified by glutathione agarose chromatography in buffer B plus 1 μM NaCl. Proteins were eluted by 1 h incubation at 37 °C with buffer B, 1 μM NaCl, 40 mM glutathione, 20 mM dithiothreitol. After dialysis in buffer, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 4 mM MgCl2, 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 μM 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 kcat/Km ratio of 0.9 and a kcat/Km 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 Cα (of commercial origin) as PKA source. The assay was started by mixing the substrates GST-Pyk1 and GST-Pyk2 indicated in each experiment and 0.1 μM γ32P-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 tpk1Δ+Δ, 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 γ32P-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 Luminal reagent, and immunoreactive bands were visualized by autoradiography and analyzed by digital imaging.

Protein Kinase A Phosphorylation of Yeast Pyruvate Kinases—Yeast strains EJ758+GST-PYK2, EJ758+GST-PYK1, tpk1Δ+Δ+GST-PYK2, and tpk1Δ+Δ+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 (A600 = 0.8), concentrated to 3 ml, and further incubated with 0.5 ml of [32P]orthophosphate. The radioactive 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 MgCl2, 5 mM dithiothreitol, 10% glycerol, 0.5 mM NaCl plus 2 μl μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 3 μg/ml aprotinin, 10 mM NaF, 5 μM β-glycerophosphate, and 1 μM NaVO4. [32P]uptake was measured and was similar for both wild type and tpk1Δ+Δ 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 ml imidazole buffer, pH 7.0, containing 62 mM MgCl2 and 100 mM KCl. Under standard conditions, 1.5 μM of lactate dehydrogenase was used as the source of PKA activity. The crude extracts were prepared as described above. Samples of crude extract (100 μg/ml) were incubated at 30 °C for 2 h, the formation of lactate dehydrogenase per milliliter and 0.22 mM NaDH. The time course of the reaction was monitored at 37 °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% (NH4)2SO4 and desalting, as described above, were used with 0.1 μM Tris-HCl, pH 9.5, 50 mM MgCl2, 0.1 mM NaCl, and 100 μM of phosphates—alkaline-biokinamidocaproyl. The reaction was terminated by the addition of 100 μl of 0.01 M of potassium hexacyanoferrate III (0.5%) and 10 μl of streptavidin immobilized on iron oxide. The alkaline phosphatase biokinamidocaproyl-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. PKA phosphorylation by PKA was 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 = VmaxKm/(Km + [S]), where S is the substrate concentration. The tangent of the slope at the origin gives the specificity constant value VmaxKm, 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 kcat presented in Fig. 7B was calculated normalizing the kcat 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...
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-32P]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.

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 Bcy1 from bovine heart PKA (C₅) 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. 2A) or by Western blot using anti-GST antibody (Fig. 2B). Fig. 2C 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 ~5% 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-32P]ATP and either C₅ 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 C₅ 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 (tpk1Δ). The mutant allele tpk1Δ 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-
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 tpk1w1 strain (data not shown). Equal amounts of GST-Pyk1 and GST-Pyk2 were submitted to phosphorylation by either of

![Fig. 2. Analysis of GST-Pyk1 and GST-Pyk2 purification.](Image)

![Fig. 3. In vitro phosphorylation of GST-Pyk1 and GST-Pyk2.](Image)
the two extracts and \( \gamma^{32P} \)ATP, and the reactions were analyzed by SDS-PAGE and autoradiography (Fig. 4A). Both proteins were phosphorylated by WT extracts and not by \( tpk1^{\mu} \) 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. 4B. 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 Cytryńska 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 Cb 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. 5A shows the dependence of phosphorylation of GST-Pyk1 and GST-Pyk2 by Cb on enzyme concentration. At the point of maximum phosphorylation, under these experimental conditions, Cb 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 Cb, experiments were carried out using variable concentrations of the two immobilized protein substrates in phosphorylation assays using 2 units of Cb subunit (Fig. 5B). The upper panel of Fig. 5B 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. 5B. Experimentally we could not attain the \( V_{\text{max}} \) due to the limitation in increasing the substrate concentration. However, some information can be obtained from the data of Fig. 5B by comparing the velocities of phosphorylation of Kemptide and Pyk-GST substrates using the 2 units of Cb 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
Previous to the study of the relationship between phosphorylation and Pyk activity, our rationale was to use a classic genetic approach and determine the kinetic parameters $V_{\text{max}}/K_m$ 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 J451, with a mutation in the BCY1 gene (bcy1-14) consisting of a small deletion in the $\alpha$-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 J451 strain. Finally the $\text{tpk}^{1\text{w}1}$ 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 J451 strain, and the lower in the $\text{tpk}^{1\text{w}1}$ 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 J451, whereas $\text{tpk}^{1\text{w}1}$ strains display no activity either in the absence or presence of cAMP. Previous work from our laboratory (11, 13) has shown that strain J451, 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.
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 tpk1Δ. 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 C6 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 states 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 tpk1Δ. The opposite treatment, the phosphorylation of Pyk1 with C6, 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, *in vitro* phosphorylation of Pyk1 and Pyk2 was demonstrated using either Tpk1 or Cb 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 sites for PKA phosphorylation, we examined the effect of protein kinase A and alkaline phosphatase on Pyk1 and Pyk2. We have demonstrated both in *vivo* and in *vitro* phosphorylation of these substrates. In this work, *in vitro* phosphorylation of Pyk1 and Pyk2 was demonstrated using either Tpk1 or C6 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 RRXS 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-
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 tpk1Δ; 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 Cb 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.

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 determined assaying the phosphorylation of GST-Pyk1 and GST-Pyk2 by Cb. 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 Cb. 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 Cb for GST-Pyk2 is greater than for GST-Pyk1 (0.1 versus 0.05) (Fig. 5C). A similar substrate preference is shown for yeast PKA, because the experiments of Fig. 4B 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 Cb 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.
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 performed 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 (tpk1Δ) the following differences can be found: a discrete shift of the titration curve for PEP to the left (Fig. 7A) 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 nH of 2 for the less phosphorylated enzyme.

As demonstrated by the results of phosphorylation by Ca2+ 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 Ca2+, 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 nH 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 non-fermentable 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.

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Protein Kinase A Phosphorylation of Yeast Pyruvate Kinases

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