Glucose-Induced Stimulation of the Ras-cAMP Pathway in Yeast Leads to Multiple Phosphorylations and Activation of 6-Phosphofructo-2-kinase

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ABSTRACT: Yeast cells respond to changes of the environment by complex modifications of the metabolism. An increase of the extracellular glucose concentration activates the Ras-cAMP pathway. Via a production of cAMP this pathway stimulates the cAMP-dependent protein kinase (PKA) which is involved in the posttranslational regulation of the key enzymes of gluconeogenesis and glycolysis. 6-Phosphofructo-2-kinase (PFK2) catalyzes the synthesis of fructose 2,6-bisphosphate, the most potent activator of the glycolytic key enzyme 6-phosphofructo-1-kinase. We investigated the molecular mechanism of the glucose-induced phosphorylation and activation of PFK2 in *Saccharomyces cerevisiae*. After an incubation of PFK2 with ATP and PKA in vitro, two amino acid residues, Thr157 and Ser644, are phosphorylated and the enzyme is activated. A stimulation of the Ras-cAMP pathway by glucose addition to cultivated yeast cells leads to an in vivo activation of PFK2 which is accompanied by a more complex phosphorylation pattern of the enzyme. The phosphorylation of the protein on Ser644 is the result of PKA stimulation while the protein kinase(s) catalyzing the 5-fold phosphorylation of the peptide fragment T_{67-101} is (are) still unknown. The functional significance of T_{67-101} and its phosphorylation is supported by the finding that PFK2 lacking this peptide is inactive.

The yeast *Saccharomyces cerevisiae* possesses rapidly responding, highly complex signaling pathways which allow it to adapt to changing environments (*1*). Among the yeast signaling pathways the Ras-cAMP pathway is the most important one, which has been studied in great detail (*2*−*4*). This pathway regulates the production of cAMP which is synthesized by adenylate cyclase (*5*, *6*). Its activity is dependent on the Ras proteins and the glucose receptor system Gpr1-Gpa2 (*7*). cAMP activates the cAMP-dependent protein kinase (PKA)[1] by binding to the regulatory subunits, resulting in a dissociation and concomitant activation of the catalytic subunits (*8*, *9*). Activated PKA is involved in the posttranslational modification and activity regulation of a variety of proteins. Among them are the key enzymes of glycolysis and gluconeogenesis, 6-phosphofructo-1-kinase and fructose-1,6-bisphosphatase. Their activity is mainly regulated by fructose 2,6-bisphosphate (Fru-2,6-P_2), the most potent allosteric activator of 6-phosphofructo-1-kinase and inhibitor of fructose-1,6-bisphosphatase (*10*−*12*). An elevated concentration of Fru-2,6-P_2 increases the glycolytic flux whereas a lowered concentration of Fru-2,6-P_2 may result in a switch toward a net gluconeogenesis.

Fru-2,6-P_2 is a signal molecule which connects the Ras-cAMP pathway with glycolysis (*13*). The enzyme 6-phosphofructo-2-kinase (PFK2) catalyzes the synthesis of Fru-2,6-P_2 (*10*). Its activity can be modulated by phosphorylation and dephosphorylation of the protein. In mammals PFK2 activity resides on the N-terminus of a bifunctional protein which also exerts fructose-2,6-bisphosphatase (FBPase2) activity at its C-terminus (*10*). The mechanism of the regulation of PFK2/FBPase2 was intensively studied for the different isozymes of the bifunctional enzyme. The rat liver PFK2/FBPase2 is phosphorylated at Ser32 within the N-terminal domain by PKA (*14*). This inhibits the kinase and activates the phosphatase activity. In contrast to the liver isozyme the bovine heart PFK2/FBPase2 is phosphorylated at Ser466 and Thr475 within the C-terminal domain by PKA and protein kinase C (PKC), respectively, resulting in an activation of the kinase activity (*15*). In rat heart the increase in Fru-2,6-P_2 concentration is a result of a double phosphorylation of the phosphatase domain of PFK2/FBPase2 at Ser466 and Ser483 (*16*). Also, the human placental isozyme is activated by PKA and PKC, and the two kinases share the same phosphorylation site (Ser460) (*17*).

The monofunctional yeast PFK2 can be both activated or inactivated by in vivo phosphorylation. We have shown earlier that phosphorylation at Ser652 by PKC under hypoosmotic stress inactivates PFK2 (*18*). On the other hand, glucose induction of yeast cells is long known to activate PFK2 (*19*). The molecular mechanism of this activation is not yet clear. The activity of the purified PFK2 can be increased about 10-fold upon incubation with ATP and the catalytic subunit of PKA (*20*). The in vitro phosphorylated Ser644 of PFK2 was suggested to represent the potential in vivo phosphorylation site for protein kinase A (*21*).

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[1] Abbreviations: ACN, acetonitrile; DTT, dithiothreitol; FBPase2, fructose-2,6-bisphosphatase; 4HCCA, α-cyano-4-hydroxycinnamic acid; MALDI-TOF MS, matrix-assisted laser desorption ionization−time of flight mass spectrometry; ME, mercaptoethanol; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; PFK2, 6-phosphofructo-2-kinase; PP-2A, phosphoserine/phosphothreonine-specific protein phosphatase 2A; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; PKA, cyclic AMP-dependent protein kinase; Thr157,Ser644/Ala, threonine to alanine and serine to alanine double mutant.

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MATERIALS AND METHODS

Materials. Yeast nitrogen base and casamino acids were from Difco. The expression vector pMK11PFK2 was a gift from M. Kretschmer, Boston, MA. [γ-^{32}P]ATP, [^{32}P]orthophosphoric acid, HiTrap affinity columns, and acetonitrile were from Amersham Pharmacia Biotech. The expand high-fidelity PCR system was from Roche Diagnostics. Restriction endonucleases were purchased from MBI Fermentas. T4 ligase and phosphoserine/phosphothreonine-specific protein phosphatase 2A (PP-2A) were obtained from Promega. The BigDye terminator cycle sequencing kit was from PE Applied Biosystems. The catalytic subunit of PKA (bovine heart), trypsin (TPCK treated), and HPLC-grade water were from Sigma. α-Cyano-4-hydroxycinnamic acid was from Fluka, and trifluoroacetic acid was from J. T. Baker.

Yeast Expression Vector. The plasmid pMK11PFK2 using uracil as selection marker contains the open reading frame of yeast PFK2 fused to the Gal1 promoter. It was modified with a His_6-tag linker at the N-terminus of the PFK2 cDNA. The resulting plasmid pMK11PFK2His was used for transformation of yeast strain DFY658. The expression of the recombinant soluble His-tagged PFK2 (PFK2His) was induced by 2% galactose. The introduced histidine residues are excluded from the numbering of amino acid residues in the primary structure of PFK2His.

Yeast Strains and Culture Media. The *S. cerevisiae* strains DFY658 (*pfk26*::*LEU2*, *fbp26*::*HIS3*, *leu2*, *his3*, *ura3*) (*22*), T162-1A (*Mat*α *his3 leu2 ura3 trp1 ade8 tpk2*::*HIS3 tpk3*::*TRP1*) (*23*), and RS13-58A (*Mata his3 leu2 ura3 trp1 ade8 tpk1^{w11} tpk2*::*HIS3 tpk3*::*TRP1 bcy1*::*LEU2*) (*24*) were used as hosts for pMK11PFK2His. The yeast cells were grown on minimal medium (YNB-P) (0.67% yeast nitrogen base without amino acids, 0.2% casamino acids, 50 μg/mL tryptophan, and 50 μg/mL adenine) containing no uracil. The low-phosphate culture medium (YNB-LP) was prepared according to Rubin (*25*) and supplied with the same additives as the YNB-P. The carbon source (2% galactose or 2% glucose) was used as indicated.

Site-Directed Mutagenesis of PFK2 and Deletion Mutation. The point mutations were introduced in the plasmid pMK11PFK2 by PCR-enabled site-directed mutagenesis according to Nelson and Long (*26*). The Ser644/Ala mutant was constructed as described in ref *27*. Thr157 was changed to alanine using the oligonucleotides 5′-CAT AAA AGA AGA CCT **G**CC ACC ATC G-3′, 5′-CGT CAC TTG AGT TAC AGC ATT TTC ATT TGT TTG ATA GG-3′, CGT CAC TTG AGT TAC AGC-3′, and CGT CAC TTG AGT TAC AGC-3′. The presence of the desired mutations was confirmed by DNA sequencing and MALDI-TOF MS analysis of the mutant proteins.

The deletion mutant for the peptide T_{67-101} was constructed by PCR-enabled mutation using the oligonucleotides 5′-CGA TGT AGC GAT TTC TGA AAA AG-3′, 5′-CTT TTT CAG AAA TCG CTA CAT CG-3′, 5′-ACC ATA GGA ATG ATA ATG CGA TTA G-3′, and 5′-AAA TGA CTT TCC CGT AGC TGG-3′.

Protein Expression and Purification. To facilitate the purification of recombinant yeast PFK2, a polyhistidine tag was added to the N-terminus of the wild-type PFK2 and the mutant proteins as described in ref *28*. The *S. cerevisiae* strain DFY658 was transformed with pMK11PFK2His. Fresh culture medium (YNB-P) supplemented with 2% galactose was inoculated from overnight cultures and incubated at 30 °C for 48 h. Yeast cells were harvested by centrifugation and disrupted with glass beads. The purification procedure of the wild-type PFK2 and the mutant proteins was performed using a HiTrap affinity column according to ref *28*. The in vivo phosphorylated proteins were purified at 4 °C.

Assay of Enzyme Activity. PFK2 activity was measured at 30 °C in 50 mM Tris-HCl, pH 7.4, 6 mM ATP, 2 mM fructose 6-phosphate, 6.5 mM glucose 6-phosphate, 20 mM $MgCl_2$, 2 mM K_2HPO_4, and 5 mM ME. Samples were withdrawn at 0, 2, and 5 min and assayed for fructose 2,6-bisphosphate as described in ref *19*. Protein concentration was determined by the method of Bradford (*29*).

In Vitro Protein Phosphorylation of PFK2 by PKA. Wild-type and mutant PFK2 proteins were incubated at 30 °C for 2−20 min in phosphorylation buffer containing 20 mM HEPES, pH 7.4, 1 mM DTT, 0.34 mM EDTA, 0.34 mM EGTA, 44 mM $MgCl_2$, 2 mM ATP containing 100 μCi of [γ-^{32}P]ATP, and 0.2 unit/mL catalytic subunit of PKA. A control sample was prepared without PKA. Withdrawn samples (10 μL) were treated with loading buffer [65 mM Tris-HCl, pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 0.2% (w/v) bromphenol blue, 100 mM DTT] at 95 °C for 5 min. The proteins were visualized by silver staining (Bio-Rad). Dried gels were analyzed by phosphorimager (Molecular Dynamics) scanning. The incorporation of ^{32}P was quantified using the ImageQuant software (Molecular Dynamics) using a dilution series of known ^{32}P concentrations for calibration. For MALDI-TOF MS analysis the wild-type PFK2 and the Thr157,Ser644/Ala double mutant were incubated with PKA as described above using unlabeled ATP. After SDS−PAGE the PFK2 was subjected to an in-gel digestion with trypsin followed by MALDI-TOF MS analysis as described below.

In Vivo Phosphorylation of PFK2 Induced by Glucose. The in vivo labeling of PFK2 was performed according to ref *30* with some modifications. Cells carrying plasmid-encoded wild-type or mutant PFK2 were grown on YNB-LP (250 mL) medium with 2% galactose at 30 °C to an OD_{600} of 2. Cells were collected by centrifugation for 10 min at 3000*g* and room temperature, resuspended in 250 mL of fresh prewarmed YNB-LP medium containing 100 μCi of [^{32}P]orthophosphoric acid and 2% glucose, and incubated for 30 min at 30 °C. Cells were chilled quickly and harvested by centrifugation at 4 °C. Threefold the weight of the cell pellet ice-cold potassium phosphate buffer (100 mM, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM ME) containing a mixture of protease and phosphatase inhibitors (50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM sodium vanadate, 1 mM phenylmethanesulfonyl fluoride, 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin) was added to the cell pellet. The cells were disrupted using glass bead agitation followed by centrifugation at 20000*g* and 4 °C for 10 min. PFK2 was immunoprecipitated from the supernatant according to ref *27*, resuspended in loading buffer, and electrophoresed (SDS−PAGE). After the gels were stained and dried, the sample analysis was carried out on the phosphorimager.

For MALDI-TOF MS analysis the in vivo phosphorylation of PFK2 was performed as described above in the presence of 2% glucose and 2 mM unlabeled inorganic phosphate. Cells were homogenized in 100 mM sodium phosphate buffer, pH 7.4, containing 600 mM NaCl, 10 mM imidazole,

50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM sodium vanadate, 1 mM phenylmethanesulfonyl fluoride, 0.01 mg/mL leupeptin, and 0.01 mg/mL pepstatin. The PFK2 was purified by metal chelate affinity chromatography as described (*28*).

Dephosphorylation by PP-2A. After each phosphorylation experiment an aliquot of the modified protein was dephosphorylated to ensure the localization of the phosphorylation sites. The protein was equilibrated with 50 mM Tris-HCl buffer, pH 7.4, 18 mM $MgCl_2$, 1 mM DTT, 0.01 mM EGTA, 0.05% ME, and 0.1 mg/mL BSA. Treatment with phosphoserine/phosphothreonine-specific protein phosphatase 2A (2.5 units/mL) was carried out for 60 min at 37 °C according to ref *31*. The reaction was terminated by addition of loading buffer. After SDS−PAGE the PFK2 was prepared for MALDI-TOF MS as described below.

In-Gel Digestion and MALDI-TOF MS Analysis. The in-gel digestion of PFK2 was performed according to ref *28*. The extraction of the tryptic digest from the gel pieces was carried out with 50% acetonitrile and 0.1% trifluoroacetic acid. For MALDI-TOF MS analysis the tryptic digest was crystallized with a matrix solution containing α-cyano-4-hydroxycinnamic acid. The matrix solution was prepared according to ref *32*; 20 mM diammonium citrate was added to increase the detection efficiency of the phosphopeptide fragments. The mixture was thoroughly vortexed and centrifuged, leaving a clear matrix solution. The sample−matrix solution was deposited onto the target as a two-layer sample preparation (*33*). All mass spectra were obtained on a Bruker BiflexIII mass spectrometer (Bruker Daltonik) in the linear- or reflector-mode operation. System calibration was carried out according to ref *28*.

RESULTS

In Vitro Phosphorylation of Wild-Type PFK2 and PFK2 Mutants by PKA. We studied whether the already known Ser644 (*27*) is the only in vitro PKA phosphorylation site of PFK2. An analysis of the primary structure of PFK2 with the help of the Internet tool "NetPhos" (http://www.cbs.dtu.dk/services/NetPhos/) revealed Thr157 near the N-terminus and Ser667 near the C-terminus of PFK2 as additional potential phosphorylation sites of PKA. With the help of site-directed mutagenesis the following different amino acid substitutions were carried out: single mutations (Ser644/Ala and Thr157/Ala), double mutation (Thr157, Ser644/Ala), and 3-fold mutation (Thr157,Ser644,Ser667/Ala). The wild-type PFK2 and the different mutants were incubated with [^{32}P]ATP and the catalytic subunit of the PKA as described in Materials and Methods. Both the wild-type PFK2 and the single mutants were in vitro phosphorylated and activated while the double mutant was not phosphorylated (Figure 1A). This result suggests that the in vitro phosphorylation of PFK2 affects only Thr157 and Ser644. A quantification of the phosphate incorporation showed that per mole of PFK2 twice as much radioactivity was incorporated into the wild-type enzyme as into the two single mutants (Figure 1B). For the wild-type PFK2 most of the phosphate was incorporated within less than 5 min, confirming that the enzyme is a good substrate for PKA in vitro. The results of the radioactive labeling experiments were verified by MALDI-TOF MS analysis of the tryptic digests

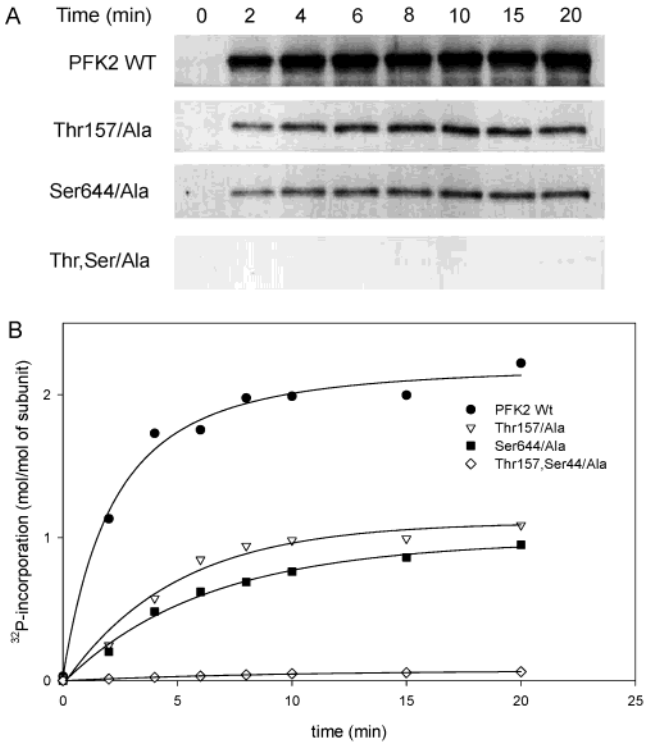

FIGURE 1: Time-dependent phosphorylation of wild-type PFK2 and mutants by PKA. (A) Purified PFK2 was incubated with [γ-^{32}P]-ATP and the catalytic subunit of PKA. At the indicated times aliquots (10 μL) were removed for SDS−PAGE analysis and quantified by phosphorimaging. (B) Time-dependent phosphate incorporation into wild-type PFK2 and mutants.

of in vitro phosphorylated wild-type and mutant PFK2s. The mass spectral analysis showed that the peaks with *m*/*z* 1414.7 and *m*/*z* 1452.0, corresponding to the tryptic peptides $T_{642-654}$ carrying Ser644 and $T_{154-166}$ carrying Thr157, respectively, disappeared after phosphorylation of the enzyme (Figure 2A). Instead, two new peaks, each 80 Da larger than the corresponding wild-type peak (*m*/*z* 1494.7 and *m*/*z* 1532.0, respectively), appear in the spectrum of the tryptic digest of the phosphorylated enzyme. In the mass spectrum of the tryptic digest of the unphosphorylated control sample the peaks with *m*/*z* 1414.7 and *m*/*z* 1452.0 were both detected (Figure 2B). The absence of the peaks of the two unphosphorylated peptides in the spectrum of phosphorylated PFK2 proves the completeness of the in vitro phosphorylation of the enzyme by PKA. The MALDI-TOF MS analysis of the PKA-treated Thr157,Ser644/Ala mutant of PFK2 also confirmed the results obtained earlier with the radioactivity assays. The peaks corresponding to the peptide fragments carrying Thr157/Ala and Ser644/Ala mutations were detected without carrying a phosphate residue and being 30 (*m*/*z* 1422.1) and 16 (*m*/*z* 1398.9) Da smaller than the correspondng fragments of the wild-type PFK2 (Figure 3). These mass differences result from the difference between threonine (*m*/*z* 101.1) or serine (*m*/*z* 87.1) to alanine (*m*/*z* 71.1), respectively.

Glucose-Induced in Vivo Phosphorylation and Activation of PFK2. PFK2 is activated after glucose addition to yeast cells (*19*). PKA was assumed to catalyze the in vivo phosphorylation of Ser644 in PFK2 representing the hypothetical phosphorylation site (*27*). The results of the in vitro experiments described above suggest the presence of more than one potential in vivo PKA phosphorylation site within

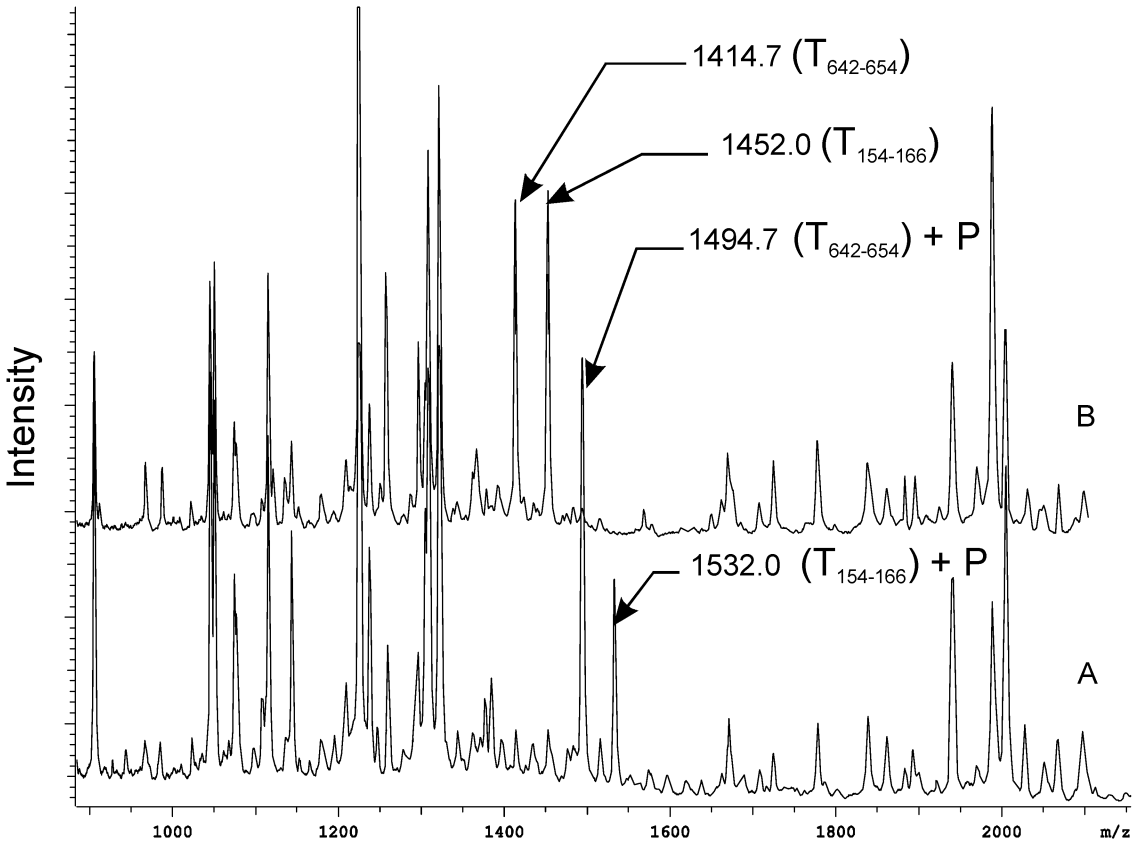

FIGURE 2: Comparison of MALDI-TOF mass spectra of trypsinized PFK2 after enzymatic phosphorylation and phosphorylation/dephosphorylation. Purified PFK2 was incubated with [γ-^{32}P]ATP and the catalytic subunit of PKA for 30 min. After the phosphorylation and SDS−PAGE analysis the samples were subjected to in-gel digestion with trypsin followed by MALDI-TOF MS analysis. (A) Tryptic digest of in vitro PKA phosphorylated PFK2. (B) Tryptic digest of phosphorylated PFK2 after dephosphorylation with PP-2A.

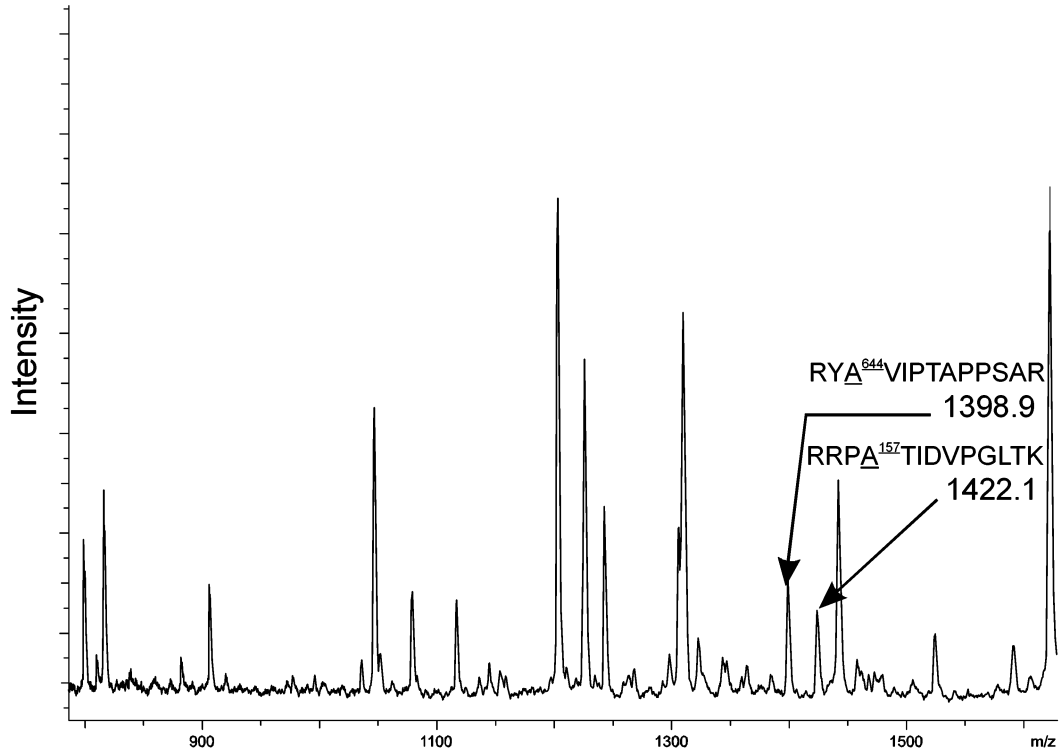

FIGURE 3: MALDI-TOF mass spectrum of the trypsin-digested in vitro phosphorylated Thr157,Ser644/Ala double mutant. The purified PFK2 double mutant was incubated with [γ-^{32}P]ATP and PKA catalytic subunit for 30 min. After the phosphorylation and the SDS−PAGE analysis the mutant protein was subjected to in-gel digestion with trypsin followed by MALDI-TOF MS analysis.

PFK2. To prove whether Ser644 is in fact the residue which is phosphorylated in vivo after glucose induction, whether Thr157 and/or Ser667 is also phosphorylated in vivo by PKA, and if there are other phosphorylation sites for PKA

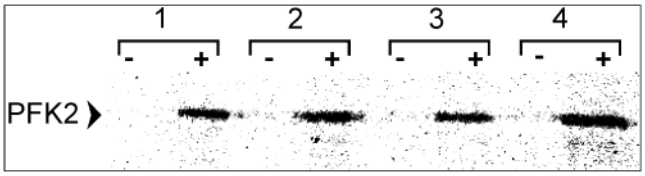

FIGURE 4: In vivo phosphorylation of wild-type PFK2 and mutants after glucose-induced activation of the Ras-cAMP pathway. The yeast cells (DFY658) were incubated for 30 min at 30 °C with [^{32}P]inorganic phosphate and 2% glucose. Lanes: 1, wild-type PFK2; 2, Thr157/Ala mutant; 3, Thr157,Ser644/Ala double mutant; 4, Thr157,Ser644,Ser667/Ala triple mutant. (−) Cells were incubated in the presence of [^{32}P]inorganic phosphate without glucose. (+) Cells were incubated in the presence of [^{32}P]inorganic phosphate and 2% glucose.

Table 1: Effect of the in Vivo Phosphorylation on the Specific Activity of the Yeast PFK2 and PFK2 Mutants

	PFK2 (milliunits/mg of protein)		activation
	−glucose	+2% glucose	(*x*-fold)
wild-type PFK2	20.7	140.6	6.7
Ser644/Ala mutant	3.0	19.5	6.5
Thr157/Ala mutant	19.5	122.8	6.3
Thr157,Ser644/Ala mutant	2.8	15.2	5.4
wild-type PFK2 from RS13-58A (with reduced PKA activity)	18.6	62.3	3.3

or for other protein kinases in PFK2, activity and phosphorylation of recombinant wild-type PFK2 and PFK2 mutants were analyzed after activation of the Ras-cAMP pathway by glucose addition to yeast cells. To study the phosphorylation state of PFK2, yeast cells lacking endogenous PFK2 and expressing plasmid-encoded wild-type or mutant PFK2 were grown initially in medium depleted of inorganic phosphate and then incubated in medium containing [^{32}P]-inorganic phosphate and glucose. As shown in Figure 4, the in vivo labeling of the Thr157/Ala mutant does not differ significantly from that of the wild-type enzyme. Interestingly, the double mutant (Thr157,Ser644/Ala) and the triple mutant (Thr157,Ser644,Ser667/Ala) were also found phosphorylated in vivo (Figure 4). The effect of the in vivo phosphorylation on the specific activity of the different enzymes was investigated. When the cells are depleted of glucose, the specific activity of the wild-type PFK2 in cell-free yeast extracts was about 20 milliunits/mg of protein. The addition of glucose to the incubation medium caused a 7-fold increase in the specific activity of PFK2 (Table 1). Glucose addition also activated the Ser644/Ala, Thr157/Ala, and double mutant Thr157,Ser644/Ala but less strongly than the wild-type enzyme (Table 1). The obtained results do neither prove nor disprove an in vivo phosphorylation of the known in vitro PKA phosphorylation sites of PFK2. In addition, they suggest the existence of further, hitherto unknown in vivo phosphorylation sites of PFK2. In vivo phosphorylation of PFK2 was studied in yeast strains with mutations in the Ras-cAMP pathway. The strain T162-1A lacking two of the three catalytic subunits of the PKA and the strain RS13-58A attenuated in the remaining catalytic subunit were used. In both PKA-deficient yeast strains PFK2 was as intensively labeled as in DFY685, a wild-type yeast with functional PKA (Figure 5).

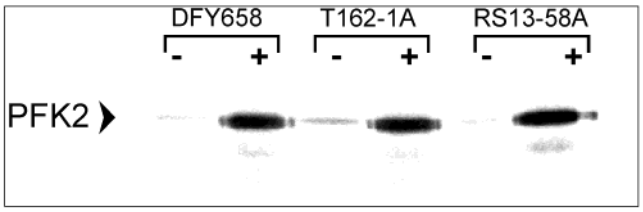

FIGURE 5: In vivo phosphorylation of the PFK2 expressed in different yeast strains. Yeast cells were incubated for 30 min at 30 °C with [^{32}P]inorganic phosphate and 2% glucose. Key: DFY658 (PKA wild-type strain); T162-1A (null mutant for PKA subunits TPK2 and TPK3); RS13-58A (null mutant for PKA subunits TPK2 and TPK3 and attenuated in TPK1). (−) Cells were incubated in the presence of [^{32}P]inorganic phosphate without glucose. (+) Cells were incubated in the presence of [^{32}P]inorganic phosphate and 2% glucose.

MALDI-TOF MS Identification of the PFK2 Sites Phosphorylated in Vivo after Glucose Induction. The identification of the in vivo phosphorylation site(s) of PFK2 was achieved by comparing the results of MALDI-TOF MS peptide mass fingerprinting of the tryptic digests of purified in vivo phosphorylated and dephosphorylated PFK2. The superposition of the mass spectra resulting from the two tryptic digests confirmed that the PFK2 was in vivo phosphorylated at Ser644. Figure 6 shows the peak *m*/*z* 1414.2 corresponding to the unphosphorylated peptide $T_{642-654}$ carrying Ser644 which could be identified in the tryptic digests from the phosphorylated and the dephosphorylated PFK2. Exclusively in the mass spectrum of the tryptic digest obtained from the phosphorylated enzyme an additional peak 80 Da larger (*m*/*z* 1494.2) than that of the unphosphorylated peptide $T_{642-654}$ was observed (Figure 6A). The absence of this peak in the spectrum of dephosphorylated PFK2 (Figure 6B) confirms that it results from the phosphorylation of the peptide fragment $T_{642-654}$. MALDI-TOF MS analysis of the tryptic digest of the in vivo phosphorylated Ser644/Ala mutant of PFK2 allowed the detection of the peak corresponding to the unmodified peptide carrying the Ser644/Ala mutation (*m*/*z* 1398.5; Figure 7). This strongly supports the notion that within this peptide only Ser644 is phosphorylated in vivo although the data are also compatible with the less probable assumption of a phosphorylation of Thr648 or Ser652 under the condition of the presence of Ser644 in its unphosphorylated state. Neither the peak *m*/*z* 1452.8 corresponding to the peptide fragment $T_{154-166}$ carrying Thr157 (Figure 7) nor the peak *m*/*z* 1863.7 (data not shown) corresponding to the peptide fragment $T_{661-670}$ carrying Ser667 was found modified after in vivo phosphorylation.

The superposition of the mass spectra obtained from the tryptic digests of in vivo phosphorylated and dephosphorylated PFK2 revealed that the enzyme was phosphorylated not only at Ser644 but also on additional sites. The phosphorylation occurred close to the N-terminus of the protein. Up to five phosphate residues were incorporated into the peptide fragment $T_{67-101.}$ All possible phosphorylation states (one, two, three, four, five times) coexist (Figure 8A), and all phosphates were completely removed by treatment with PP-2A (Figure 8B). To investigate the functional significance of T_{67-101} and its phosphorylation, a PFK2 mutant lacking this peptide was constructed, expressed in, and purified from yeast. The in-gel digestion of the purified mutant protein followed by MALDI-TOF MS fingerprinting confirmed its

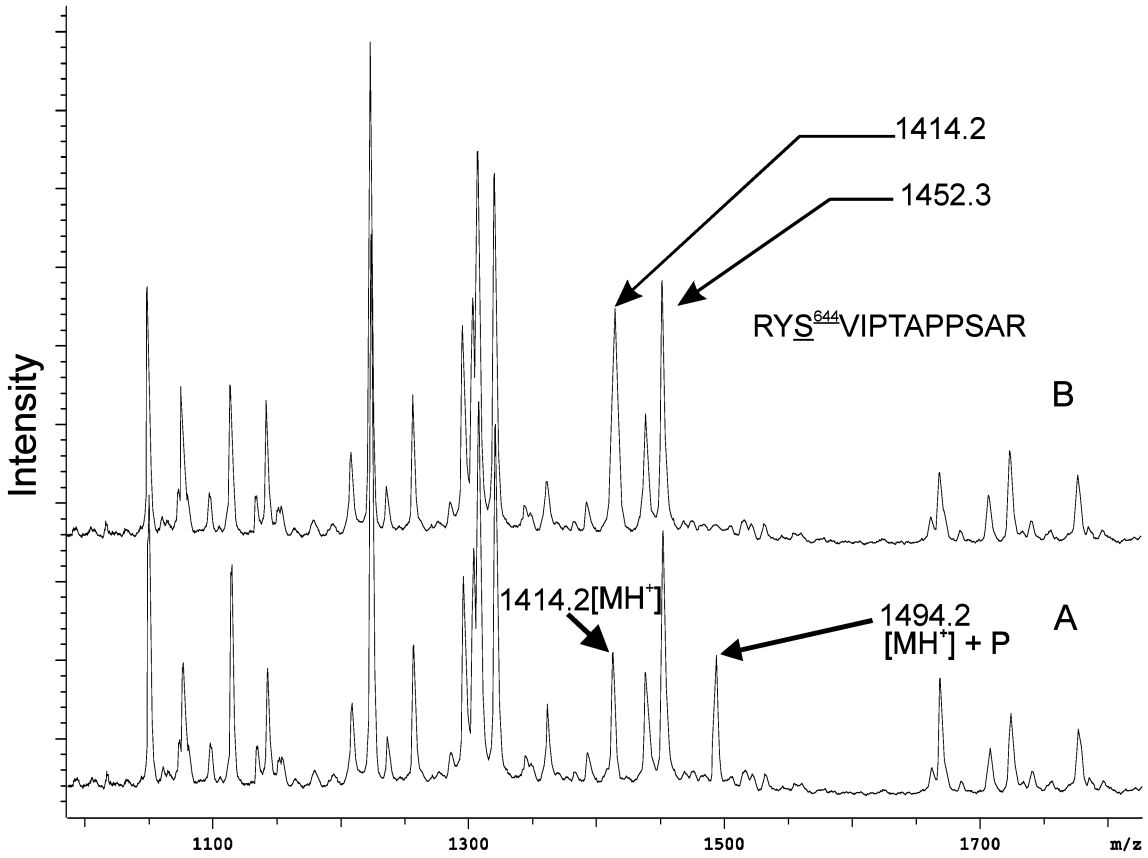

FIGURE 6: Identification of the C-terminal in vivo phosphorylation site of PFK2 by MALDI-TOF MS. (A) Tryptic digest of PFK2 phosphorylated in vivo after glucose induction of the yeast cells. The peak with *m*/*z* 1494.2 represents the phosphorylated peptide $T_{642-654}$. (B) MALDI-TOF mass spectrum of the tryptic digest of in vivo phosphorylated and in vitro dephosphorylated PFK2.

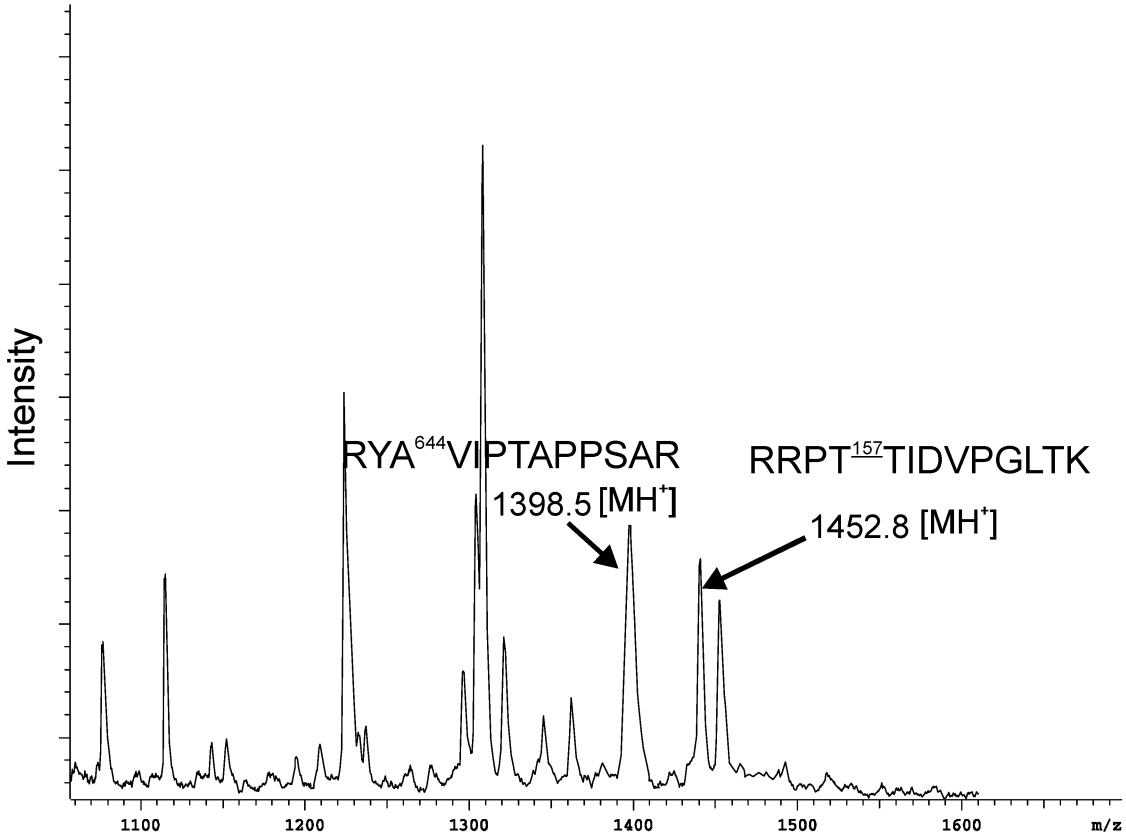

FIGURE 7: MALDI-TOF mass spectrum of the tryptic digest of the in vivo phosphorylated Ser644/Ala mutant. The peptide fragment $T_{642-654}$ carrying the Ser644/Ala mutation was not phosphorylated. The peptide fragment carrying the Thr157 was also not phosphorylated.

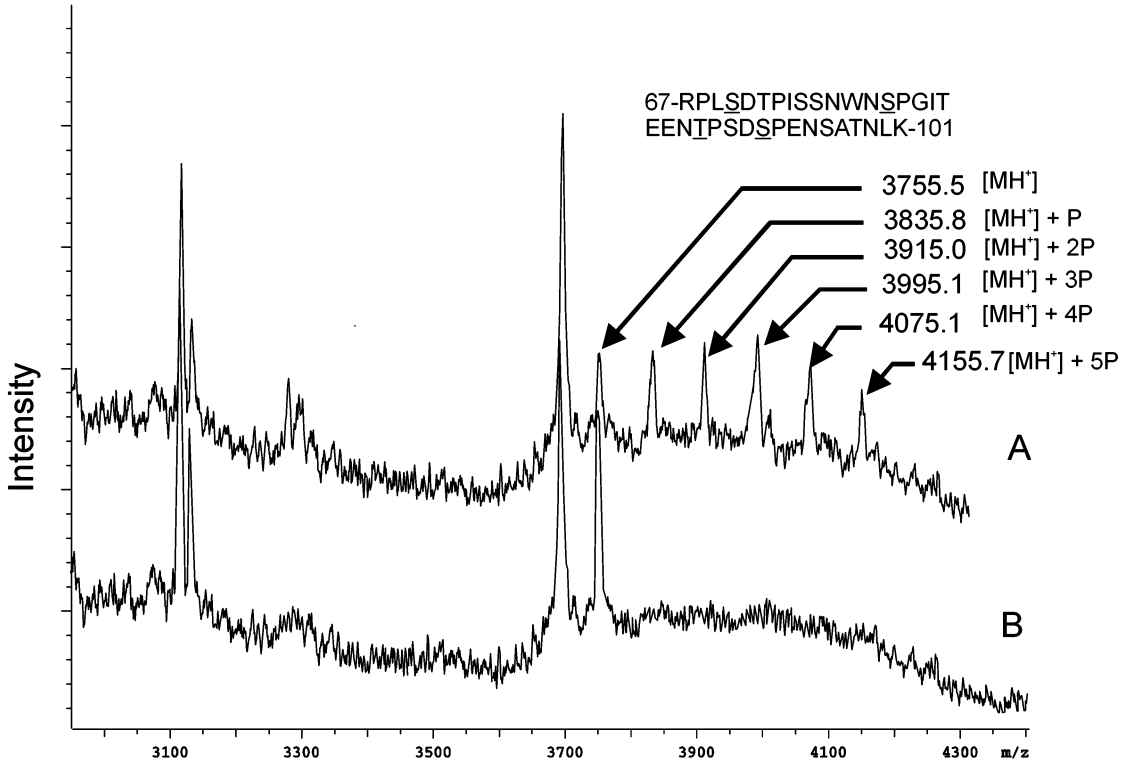

FIGURE 8: Identification of the N-terminal in vivo phosphorylation of the PFK2 by MALDI-TOF MS. (A) Tryptic digest of PFK2 phosphorylated in vivo after incubation of the yeast cells with 2% glucose. The peak with *m/z* 3755.5 represents the unmodified peptide T_{67-101}. The peptide fragment is up to 5-fold phosphorylated. (B) MALDI-TOF mass spectrum of the tryptic digest of in vivo phosphorylated and in vitro dephosphorylated PFK2. All phosphate residues were removed.

identity. Activity tests in cell-free extracts showed that this mutant was inactive. Also, after glucose induction of yeast expressing this mutant no activity could be detected in supernatants after cell disruption.

Wild-type PFK2 expressed in RS13-58A was found to be four times phosphorylated at the N-terminal peptide T_{67-101} but not on Ser644 (data not shown).

DISCUSSION

Purification of Wild-Type Yeast PFK2 and PFK2 Mutants. For the in vitro phosphorylation studies the enzymes were purified from cells growing on YNB-LP instead of YNB-P. Although the expression level of PFK2 is lower in YNB-LP than in YNB-P, the following two advantages make it profitable: the proteolytic degradation of PFK2 within the cells and during purification from cell-free extracts occurs much slower compared to YNB-P (*28*); in addition, the probability of an in vivo phosphorylation of PFK2 is very low, which simplifies the interpretation of the results of in vitro phosphorylations.

Advantages of the Chosen Method for the Identification of Phosphorylated Sites. Reversible protein phosphorylation is known to control a wide range of biological functions and activities (*10*). Thus, the determination of the phosphorylation site(s) of a given protein is an essential step in the analysis of the control mechanisms of many biological systems. Nevertheless, the direct determination of individual phosphorylation sites occurring within phosphoproteins in vivo has been difficult to date. The combination of the MALDI-TOF MS, radioactive labeling, and site-directed mutations for the identification of the protein phosphorylation sites as practiced in this paper proved to be successful and reliable. It should be assumed that postsource decay (PSD) analysis would allow a straightforward identification of the phosphorylated residues without the need to study mutant proteins. In fact, this method is applicable in the case of phosphorylated tyrosines (*34*). However, phosphoserine or phosphothreonine is more labile, and the phosphate group is rapidly lost during the MALDI process, resulting in the formation of a dehydroalanyl residue instead of a phosphoserine (*35*).

In Vitro Phosphorylation of PFK2 by PKA. The consensus sequences of cAMP-dependent protein kinase are characterized by the presence of basic amino acids, particularly arginine, N-terminally to the phosphoacceptor serine or threonine (*36*). In yeast PFK2 potential PKA phosphorylation sites are Thr157 in the N-terminal part and Ser644 and Ser667 in the C-terminal part of the protein. We have demonstrated in this study that in addition to Ser644, which was shown earlier to be phosphorylated in vitro (*27*), also Thr157 is a good substrate of PKA under in vitro conditions. In contrast, the third potential PKA phosphorylation site Ser667 could not be phosphorylated in vitro. Phosphorylation studies using the single mutant Thr157/Ala or Ser644/Ala confirmed that only the Ser644 phosphorylation was responsible for the increase of the PFK2 activity whereas the Thr157 phosphorylation had no effect on the enzyme activity (data not shown).

The identification of serine or threonine phosphorylations by MALDI-TOF MS is often compromised by the low efficiency of detection of the phosphorylated peptides (*35*). Both peptide fragments $T_{154-166}$ and $T_{642-654}$ of PFK2 which carry the in vitro phosphorylation sites for PKA contain an arginine at their N-termini, respectively. This turned out to

be an advantage for their MALDI-TOF MS identification in the phosphorylated state since arginine favors the ionization of the fragments and by this counterbalances the negative effect of the incorporated phosphate on protonization.

Glucose-Induced in Vivo Phosphorylation of PFK2. It has been reported that yeast PFK2 is activated after the addition of fermentable sugars to the cells via the RAS-cAMP pathway (*34*). The serine residue at position 644 of PFK2 has been presumed to be phosphorylated by PKA after glucose addition, with this modification being responsible for the observed activation of the enzyme (*19*, *21*, *37*). By a combination of site-directed mutagenesis, MALDI-TOF MS, and the use of yeast strains deficient in PKA, we could prove that the Ser644 is in fact phosphorylated in vivo after glucose induction. MALDI-TOF MS analysis of the in vivo phosphorylated and dephosphorylated protein showed that, in contrast to what occurs in vitro, Thr157 is not accepted as the substrate of yeast PKA. Our earlier work (*27*) and that of others (*37*) suggested that, in addition to Ser644, other sites of PFK2 might be phosphorylated in vivo and their modification might contribute to the activation of the enzyme after glucose addition. The results of the present paper strongly implicate protein kinases other than PKA to be responsible for the additional phosphorylation and, hence, to contribute to the regulation of yeast glycolysis after activation of the RAS-cAMP pathway. Both the triple mutant (Thr157,Ser644,Ser667/Ala) and the wild-type PFK2 expressed in PKA-deficient yeast strains were phosphorylated in vivo. This finding suggests that PKA is not the only protein kinase which phosphorylates PFK2 after glucose induction. The mass spectral analysis reinforces these results. The N-terminal peptide T_{67-101} was 5-fold phosphorylated in vivo. An examination of the primary structure of PFK2 shows that there are several potential phosphorylation sites with different consensus sequences within the peptide T_{67-101}. Ser70 is a potential site for both calmodulin-dependent protein kinase and PKA, Thr88 for casein kinase II, Ser92 for casein kinase I, and Ser80 and Ser96 for hitherto unknown protein kinases. The in vivo activation of the Ser644/Ala mutant showed that the phosphorylation near the N-terminus of PFK2 plays an important role in controlling the enzyme activity after glucose induction (Table 1). The fact that PFK2 lacking the peptide T_{67-101} is inactive further illustrates the importance of this peptide not only for the regulation after glucose induction but also for the structural integrity and activity of the protein. Yeast strain RS13-58A mutant in PKA shows only a 4-fold phosphorylation of the N-terminal T_{67-101}. This points to the fact that Ser70 is an additional in vivo phosphorylation site for PKA.

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