Phosphorylation and Inactivation of Yeast 6-Phosphofructo-2-kinase Contribute to the Regulation of Glycolysis under Hypotonic Stress†

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ABSTRACT: Phosphorylation of yeast 6-phosphofructo-2-kinase and its role for the regulation of glycolysis under hypoosmotic conditions were investigated. 6-Phosphofructo-2-kinase was found to be phosphorylated in vitro by protein kinase C at serine 652 and thereby inactivated. Protein phosphatase 2A reversed the phosphorylative inhibition of the enzyme. When yeast cells were shifted to hypotonic media, 6-phosphofructo-2-kinase was found to be phosphorylated and inactivated. Under in vivo conditions, two phosphate residues were incorporated into the enzyme. One of them is bound to serine 652, indicating that this modification was probably caused by yeast protein kinase C1. The second phosphate is bound to Ser8 within the N-terminal peptide T_{1-41} which contains several serine residues but no protein kinase C recognition sequence. Site-directed mutagenesis confirmed that the phosphorylation of serine 652 but not the N-terminal modification is responsible for the in vivo inactivation of 6-phosphofructo-2-kinase. The obtained results suggest that the phosphorylation of 6-phosphofructo-2-kinase mediates a response of the cells to an activation of the hypoosmolarity MAP kinase pathway. Via a suppression of glycolysis, the inactivation of 6-phosphofructo-2-kinase is expected to be responsible for the observed accumulation of glucose 6-phosphate, an essential precursor of the cell wall glucans, and the decrease of glycerol, an important osmolyte.

In their natural environment, yeast cells are exposed to changes in extracellular osmolarity which affect the cellular hydration state and directly influence cell metabolism (*1, 2*). Exposure of yeast cells to hypotonic media induces a specific cellular response that includes changes in the activity of solute transporters and enzymes involved in solute accumulation (*3*), in the expression of genes encoding enzymes required for solute synthesis (*4*), in stress resistance (*5*), and in cell wall structure (*6*).

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity. The cell must remodel this rigid structure during vegetative growth and during pheromone-induced morphogenesis (*1*). Cell wall remodeling is monitored and regulated by the cell integrity signaling pathway controlled by protein kinase C (PKC1),[1] a serine/threonine-specific homologue of mammalian PKC (*7*). Hypotonic shock induces a rapid but transient activation of the hypoosmolarity MAP kinase pathway (*3*).

Yeast 6-phosphofructo-2-kinase (PFK2) catalyzes the synthesis of fructose 2,6-bisphosphate, which is the most powerful activator of glycolysis (*8*). In vitro phosphorylation of yeast PFK2 by PKA at Ser644 of the consensus phosphorylation site (RRYS) located within the C-terminal domain of the protein activates the enzyme (*9*). This is analogous to what occurs with the mammalian heart and placental PFK2s, and opposite to the liver isozyme which is inhibited by PKA. In contrast to the monofunctional yeast PFK2, the homologous mammalian enzymes are bifunctional, carrying the fructose-2,6-bisphosphatase activity on the same protein (*8*). The relative activities of the kinase and phosphatase depend on the type of isozyme and can be modulated for a given isozyme by phosphorylation and dephosphorylation of the protein. The rat liver PFK2/FBPas2 is phosphorylated at Ser32 within the N-terminal domain by cAMP-dependent protein kinase (PKA) (*10*). The bovine heart PFK2/FBPas2 is phosphorylated at Ser466 and Thr475 within the C-terminal domain by PKA and protein kinase C (PKC), respectively (*11*). Also the human placental isozyme is phosphorylated by PKA and PKC, and the two kinases share the same phosphorylation site (Ser460) (*12*).

In this report, we show that yeast PFK2 can be phosphorylated by PKC in vitro and that this modification causes an inactivation of the enzyme. The amino acid sequence of the peptide fragment carrying the phosphorylated residue shows that the phosphorylation sites for both PKC (Ser652) and PKA (Ser644) are in close proximity. We also report that the PFK2 is inactivated in vivo by hypotonic shock and this

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[1] Abbreviations: ACN, acetonitrile; 4HCCA, α-cyano-4-hydroxycinnamic acid; DTT, dithiothreitol; FBPase2, fructose-2,6-bisphosphatase; G-6-P, glucose 6-phosphate; MALDI-TOF MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; ME, mercaptoethanol; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PFK2, 6-phosphofructo-2-kinase; PKA, protein kinase A; PKC, protein kinase C; PP2A, phosphoserine/phosphothreonine-specific protein phosphatase 2A; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; T_{1-41}, tryptic peptide from amino acid 1 to amino acid 41.

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inactivation is the result of phosphorylation of the same amino acid residue which is phosphorylated in vitro by PKC. Under in vivo conditions, we found an additional phosphorylation site of PFK2 for a protein kinase different than PKC1.

EXPERIMENTAL PROCEDURES

Materials. Yeast nitrogen base and casamino acids were from Difco; adenine and tryptophan were from Merck. Restriction endonucleases were purchased from MBI Fermentas. T4 DNA ligase was obtained from Promega. The HiTrap affinity column and Sephacryl F3GA-S300 were from Amersham Pharmacia Biotech. The matrix used in all MALDI-TOF MS experiments was α-cyano-4-hydroxycinnamic acid obtained from Fluka. For mass spectrometric analysis, HPLC-grade water (Sigma), acetonitrile (Amersham Pharmacia Biotech), and trifluoroacetic acid (Baker) were used. The expression vector pMK11PFK2 was a gift from Dr. M. Kretschmer, Boston. PKC and the phosphoserine/phosphothreonine-specific protein phosphatase 2A were purchased from Promega.

Yeast Expression Vector. The plasmid pMK11PFK2 containing the open reading frame of yeast PFK2 fused to the Gal1 promoter was modified with a His6-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 PFKhis.

Culture Media. The *Saccharomyces cerevisiae* strain DFY658 (*pfk26::LEU2, fbp26::HIS3, leu2, his3, ura3*) (*13*) was used as host for pMK11PFK2His. The yeast cells were grown at 30 °C in minimal medium (YNB-P) (0.67% yeast nitrogen base without amino acids, 0.2% casamino acids, 50 μg/mL tryptophan and adenine, 2% galactose) containing no uracil. The low-phosphate culture medium (YNB-LP) was prepared according to Rubin (*14*). The medium was supplied with the same additives as YNB-P. Fresh culture media were inoculated from overnight cultures and incubated at 30 °C for the indicated times. For inhibition of the PKC1-signaling pathway, sterile staurosporine was added to YNB-P to a final concentration of 1 μg/mL.

Assay of Enzyme Activity. PFK2his activity was measured at 30 °C in 50 mM Tris-HCl, pH 7.2, 6 mM ATP, 2 mM fructose 6-phosphate, 6.5 mM glucose 6-phosphate, 20 mM magnesium chloride, 2 mM potassium phosphate, and 5 mM ME. Samples were withdrawn at 0, 2, and 5 min and assayed for fructose 2,6-bisphosphate as described (*15*). Protein concentration was determined by the method of Bradford using bovine serum albumin as standard protein (*16*).

Purification of Recombinant PFK2his. Yeast 6-phosphofructo-2-kinase was purified using the His-tag technology combined with affinity chromatography on Cibacron blue. Yeast cells were disrupted by shaking vigorously with glass beads using a vortex mixer. The crude extract was applied to a Sephacryl F3GA-S300 column preequilibrated with lysis buffer (100 mM NaH_2PO_4, pH 7.4, 600 mM NaCl, 10 mM imidazole). After being washed with lysis buffer, proteins were eluted with this buffer containing 2 M KCl. The fractions containing enzyme activity were pooled, desalted, and loaded on a HiTrap column equilibrated with the lysis buffer. After being washed with lysis buffer containing 20 mM imidazole, the bound enzyme was eluted by 120 mM imidazole in lysis buffer. The isolation of PFK2his from yeast strain DFY658/pMK11PFK2His leads to an enzyme preparation of more than 85% purity with a specific activity of 900 milliunits/mg.

In Vitro Phosphorylation of PFK2his by Protein Kinase C. Recombinant wild-type and mutant PFK2his were phosphorylated in vitro using rat brain PKC at 30 °C. The reaction mix (100 μL) contained 200 μg of PFK2his, 2 mM ATP, 20 mM HEPES, pH 7.4, 1 mM DTT, 0.34 mM EDTA, 0.34 mM EGTA, 44 mM $MgCl_2$, 1 mM $CaCl_2$, 200 μg/mL phosphatidylserine, and 0.2 unit/mL PKC. Phosphate incorporation and enzyme activity were determined after different incubation times. For ^{32}P incorporation, purified yeast PFK2his was incubated with 10 μCi of [γ-^{32}P]ATP. The reaction was terminated by diluting 10 μL aliquots with 5 μL of sample buffer [5% (w/v) SDS, 20% (v/v) glycerol, 0.02% (w/v) bromphenol blue, 100 mM DTT, and 65 mM Tris-HCl, pH 6.8] before boiling for 5 min and SDS−PAGE on 8% acrylamide minigels. To determine ^{32}P incorporation, gels were silver-stained and dried, and the radioactivity of the bands corresponding to PFK2his was determined by a PhosphorImager (Molecular Dynamics) using aliquots of a dilution series of the stock solution of ^{32}P-labeled inorganic phosphate for calibration.

In Vivo Phosphorylation of PFK2his after Hypotonic Shock. The induction of hypotonic shock to yeast cells cultured in YNB-P was performed according to Davenport et al. (*3*). After reaching log phase, the cells were centrifuged, and the pellet was resuspended either in YNB-P (control) or in hypotonic medium (20% YNB-P). After the indicated incubation times, yeast cells were quickly chilled and collected by centrifugation. Ice-cold buffer containing protease and phosphatase inhibitors (100 mM NaH_2PO_4, pH 7.4, 600 mM NaCl, 10 mM imidazole, 5 mM ME, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1 mM sodium vanadate, 1mM PMSF, 0.01 μg/μL leupeptin, 0.01 μg/μL pepstatin) was added to the cell pellet, and the cells were disrupted using glass bead agitation. The time-dependent change of the PFK2his activity under hypotonic shock was followed.

To determine ^{32}P incorporation during the hypotonic shock, yeast cells were grown on YNB-LP. After reaching log phase, the cells were gently centrifuged and resuspended in hypotonic medium (20% YNB-LP) with ^{32}P-labeled inorganic phosphate for different times. After rapid cooling, PFK2his was prepared and phosphate incorporation analyzed by phosphorimaging.

For the identification of the PFK2 in vivo phosphorylation sites by MALDI-TOF MS analysis, yeast cells were cultured to log phase in YNB-P and then transferred into 20% YNB-P. After 60 min of incubation under shaking, the cells were harvested, and the PFK2 was purified as described above. After SDS−PAGE and in-gel digestion, the peptide fragments were analyzed with MALDI-TOF MS.

Identification of Phosphorylated Peptide Fragments of PFK2his. After MALDI-TOF MS analysis of trypsin or Glu-C-digested PFK2his, the phosphorylated peptides were identified with the help of the Internet tool FindMod from

ExPASy (www.expasy.ch/sprot/findmod.html) for the matching of user-specified peptides to those generated theoretically.

In Vitro Dephosphorylation of PFK2his by Protein Phosphatase 2A. After in vitro phosphorylation of PFK2his, the reaction was stopped by the addition of staurosporine (*17*). After in vivo phosphorylation, the PFK2his was purified (see above). The phosphorylated enzyme was equilibrated with 50 mM Tris-HCl buffer, pH 7.4, 18 mM $MgCl_2$, 1 mM DTT, 0.01 mM EGTA, 5 mM ME, 0.1 mg/mL BSA. Treatment with PP2A (2.5 units/mL) was carried out at 37 °C according to Levin et al. (*18*).

For the peptide fragments resulting from in-gel digestion of phosphorylated protein, dephosphorylation was carried out after taking the dried tryptic digest in the dephosphorylation buffer. The reaction was stopped by addition of 2% TFA.

In-Gel Digestion and MALDI-TOF Analysis. Protein separation by SDS−PAGE, gel staining and destaining, and in-gel digestion by trypsin or Glu-C were carried out according to (*19*). The matrix solutions were saturated with 4HCCA and were prepared according to Asara and Allison (*20*). For augmentation of the detection efficiency of the phosphopeptide fragments, solid 4HCCA was added to 50% ACN in 20 mM diammonium citrate dissolved in HPLC water. The pellets obtained after protein digestion were dissolved in 0.1% TFA with 50% ACN to a final concentration of 50−80 ng/μL and desalted with ZipTip C18 (Millipore). The sample-matrix preparation was carried out as a two-layer sample preparation (*21*). A stainless-steel target was used.

MALDI-TOF mass spectrometry was performed on a Bruker BiflexIII mass spectrometer (Bruker Daltonik, Germany) used in the linear mode with internal calibration as described in (*19*).

Site-Directed Mutagenesis. Using pMK11PFK2His as template, codon 652 was changed from serine to alanine (Ser652/Ala) by oligonucleotide-directed mutagenesis (*22*). The following primers were used: first PCR: PKvI, AAC CGC TCC GCC AGC TGC AAG AAG CAG CTT (forward mutagenic primer with a double base mismatch to allow the Ser652 mutation to Ala and to introduce the restriction site for *Mbi*I without amino acid modification); PKrII, CTT GGT GTA GCC GCT GAC TTC (reverse hybrid primer); second PCR: PKvIII, TAA CGA GAA GTG GCG AGA GC (forward primer); PKrIV, AAG CTG CTT CTT GCA GCT GGC GGA GCG GTT (reverse primer, antisense mutagenic primer complementary to PKvI); third PCR: PKvIII, PKrII. The *Sph*I/*Kpn*I fragment in plasmid pMK11PFK2His was replaced by the mutated 791 bp fragment obtained by *Sph*I/*Kpn*I digestion of the third PCR product, resulting in a plasmid carrying the Ser652/Ala mutation which was transformed into the PFK2 mutant strain DFY658. The mutation was confirmed by DNA sequencing as well as in the protein after its purification and in gel-digestion with trypsin followed by MALDI-TOF MS analysis.

Determination of Glycerol and Glucose 6-Phosphate. Hypotonically stressed cells were prepared as described above. Samples were withdrawn by filtration, and the filters carrying the cells were quickly frozen in liquid nitrogen. After being thawed, the cells were disrupted in 0.5 M perchloric acid by shaking with an equal volume of glass beads (0.5 mm diameter) using a vortex mixer (5 × 30 s with a 1 min cooling period between each mixing). After centrifugation, the supernatant was neutralized with KOH. Glycerol determination was carried out in the supernatant by using glycerokinase and glycerophosphate dehydrogenase and absorbance measurement at 340 nm as described by Albertyn et al. (*4*). Glucose 6-phosphate was determined according to Lang and Michal (*23*) using glucose-6-phosphate dehydrogenase. Glycerol and glucose 6-phosphate concentrations were calculated per gram dry weight of cells.

MFKPVDFSETSPVPPDIDLAPTQSPHHVAPSQDSSYDLLSRSSDDKIDAEKGS**H**
DELSKHLPLFQKR**PL**SDTPISSNWNSPGITEENTPSDS**P**ENSATNLKSLHRLHIN
DETQLKNAKIP**T**NDTTDYMPPSDGANEV**T**RIDLKDIKSPTRHHKRRP**TT**IDVP
GLTKSKTSPDGLISKEDSGSKLVIVMVGLPA**TGK**SFI**TNK**LSRFLNYSLYYCKV
FNVGN**TRR**KFAKEHGLKDQDSKFFEPKNAD**STRL**RDKWAMDTLDELLDYLL
EGSGSVGIFDATN**TSR**ERRKNVLARIRKRSPHLKVLFLESVCSD**H**ALVQKNIR
LKLFGPDYKGKDPES**SLK**DFKSRLANYLKAYEPI**E**DDENLQYIKMIDVGKKV
IAYNIQGFLRSQTVYYLLNFNLADRQIWITRSGESEDNV**SGR**IGGNSHL**TPR**GL
RFAKSLPKFIARQREIFYQNLMQQKKNNENTDGNIYNDFFVWTS**MR**ARTIGT
AQYFNEDDYPIKQMKMLDELSAGDYDGMTYPEIKNNFPEEFEKRQKDKLRY
RYPGIGGESYMDVINRLRPVITE**L**ERIEDNVLII**THR**VVARALLGYFMNLSMGI
IANLDVPLHCVYCLEPKPYGITWSLWEYDEASDSFSKVPQTDLN**TTR**VKEVG
LVYNERRYSVIPTAPP**S**A**R**SSFASDFL**SRK**RSNPTSASSSQSELSEQPKNSVSAQ
TGSNNTTLIGSNFNIKNENGDSRIPLSAPLMATNTSNNILDGGGTSISIHRPRVV
PNQNNVNPLLANNNKAASNVPNVKKSAA**TPR**QIFEIDKVDEKLSMLKNKSFL
LHGK**D**YPNNA**D**NNDNEDIRA**K**TMNRSQSHV

FIGURE 1: Primary structure of yeast PFK2. The consensus sequences for putative protein kinase C phosphorylation sites are given in boldface type. Serine 652 which was changed to alanine by site-directed mutagenesis is underlined.

RESULTS

In Vitro Phosphorylation of PFK2his. An examination of the amino acid sequence of PFK2 with the help of NetPhos, a tool from ExPASy (www.expasy.ch), revealed 14 potential PKC consensus phosphorylation sites (Figure 1). To prove whether PFK2 is in fact a substrate for PKC, purified recombinant PFK2his was phosphorylated in vitro using ATP or [γ-^{32}P]ATP, respectively, and rat brain PKC. This treatment led to a time-dependent phosphorylation of PFK2his (Figure 2A), which paralleled the inhibition of the enzyme (Figure 2B). After 10 min of incubation, the phosphate incorporation showed a maximum of about 0.8 mol of phosphate incorporated per mole of subunit. At the maximum of phosphate incorporation, PFK2his is inhibited to more than 98%. During dephosphorylation of PFK2his by PP2A, the enzyme is reactivated (Figure 2B).

MALDI-TOF MS Analysis of PFK2his Phosphorylated in Vitro by PKC. To identify the PKC phosphorylation site(s) on PFK2his, tryptic peptides obtained by digestion of in vitro phosphorylated and dephosphorylated enzymes were compared. The tryptic map obtained after MALDI-TOF MS analysis covered about 85% of the PFK2his sequence (Table 1).

As shown in Figure 3 A, panel b, the mass-to-charge ratio (*m*/*z*) determined for the dephosphorylated PFK2his fragment $T_{642-654}$ (*m*/*z* 1414.2 Da) agrees sufficiently with the theoretical molecular mass predicted by the amino acid sequence.

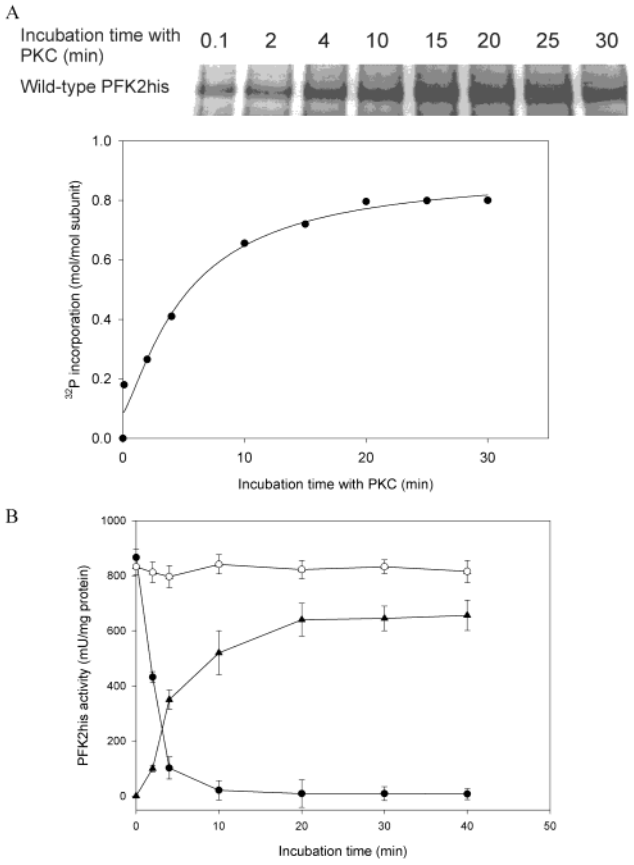

FIGURE 2: Time-dependent phosphorylation and inhibition of PFK2his by PKC. (A) Purified PFK2his was incubated with [γ-^{32}P]-ATP and PKC (0.2 unit/mL) in a final volume of 100 μL. At the indicated times, aliquots (10 μL) were removed for SDS−PAGE and quantified by phosphorimaging. (B) Purified PFK2his was incubated with 2 mM ATP with (●) or without (○) PKC (0.2 unit/mL) in a final volume of 100 μL. At the indicated times, aliquots of 10 μL were removed for PFK2his activity assay. (▲) PFK2his phosphorylated in vitro by PKC was incubated for different times with PP2A, and the enzyme activity was monitored.

Table 1: Summary of MALDI-TOF MS Results Obtained after In-Gel Digestion of PFK2his with Trypsin[a]

PFK2his fragments	detected mass (Da)	theoretical mass (Da)	PFK2his fragments	detected mass (Da)	theoretical mass (Da)
T_{1-41}	5372.9	5372.8	$T_{431-434}$	444.2	444.3
T_{42-59}	1970.7	1970.9	$T_{439-451}$	1725.5	1725.8
T_{60-66}	882.3	882.5	$T_{441-451}$	1441.8	1441.7
T_{67-101}	3755.5	3755.7	$T_{452-452}$	147.2	147.1
$T_{102-105}$	ND[b]	512.5	$T_{453-472}$	2437.3	2437.1
$T_{106-115}$	1210.6	1210.6	$T_{475-490}$	1874.5	1874.8
$T_{116-139}$	2607.3	2607.2	$T_{494-512}$	2147.9	2147.9
$T_{119-139}$	2294.0	2294.0	$T_{513-521}$	1153.7	1153.5
$T_{140-143}$	488.3	488.3	$T_{513-522}$	1309.7	1309.6
$T_{144-146}$	375.2	375.2	$T_{523-530}$	ND	1107.3
$T_{147-153}$	863.1	862.9	$T_{531-545}$	1670.8	1670.7
$T_{154-166}$	1453.6	1453.8	$T_{546-555}$	1225.9	1225.7
$T_{155-166}$	1297.5	1297.7	$T_{556-566}$	1322.8	1322.7
$T_{155-177}$	2411.8	2411.4	$T_{546-566}$	2527.5	2527.3
$T_{178-202}$	2591.4	2591.4	$T_{567-620}$	6129.5	6129.1
$T_{184-202}$	1988.3	1988.2	$T_{621-630}$	1144.4	1144.5
$T_{206-215}$	1313.5	1313.6	$T_{631-642}$	1461.8	1461.8
$T_{206-223}$	2272.5	2272.1	$T_{633-641}$	1078.7	1078.5
$T_{229-249}$	2450.8	2450.6	$T_{642-654}$	1414.4	1414.6
$T_{239-249}$	1311.5	1311.6	**$T_{642-654}$**	**1494.4**	**1494.6**
$T_{250-283}$	3801.8	3801.8	$T_{643-654}$	1258.7	1258.6
$T_{254-283}$	3290.0	3289.6	$T_{655-664}$	1116.5	1116.5
$T_{284-294}$	1142.3	1142.3	$T_{667-684}$	1863.7	1863.8
$T_{288-301}$	1689.2	1689.1	$T_{685-713}$	3039.6	3039.4
$T_{302-322}$	2413.5	2413.8	$T_{706-743}$	3875.5	3875.9
$T_{318-320}$	402.4	402.3	$T_{714-743}$	3103.6	3103.6
$T_{323-329}$	839.2	839.4	$T_{744-760}$	1860.6	1860.8
$T_{330-331}$	204.1	204.1	$T_{761-769}$	ND	899.494
$T_{348-355}$	965.3	965.1	$T_{770-776}$	ND	730.421
$T_{350-364}$	1839.6	1839.8	$T_{777-783}$	ND	892.477
$T_{350-370}$	2483.2	2483.2	$T_{784-800}$	1304.5	1304.7
$T_{371-371}$	147.2	147.1	$T_{795-801}$	801.4	801.5
$T_{372-382}$	ND	1293.7	$T_{788-800}$	1617.2	1617.0
$T_{383-397}$	ND	1816.9	$T_{802-816}$	1778.5	1778.7
$T_{398-403}$	816.5	816.4	$T_{795-816}$	2561.1	2561.2
$T_{404-414}$	1136.4	1136.4	$T_{802-822}$	2480.1	2480.0
$T_{415-424}$	1051.5	1051.5	$T_{823-827}$	ND	557.268
$T_{428-430}$	365.3	365.2			

[a] For comparison, theoretical fragment masses are given. The only new fragment occurring after in vitro phosphorylation of PFK2his by PKC is given in boldface type. [b] ND: not detected.

As illustrated in Figure 3A, panel a, the mass spectrum of the tryptic digest of phosphorylated PFK2his shows that the peak corresponding to the $T_{642-654}$ fragment is about 80 Da larger (*m*/*z* 1494.4 Da). One phosphate group increases the mass of the peptide by 80 Da. Within the PKC consensus sequence S^{652}AR (see Figure 1), the serine represents the potential PKC phosphorylation site.

Site-Directed Mutation of Ser652. To prove that Ser652 is phosphorylated by PKC, this amino acid was substituted for alanine by site-directed mutagenesis. The amino acid exchange was confirmed by DNA sequencing as well as by MALDI-TOF MS when comparing the tryptic peptides of the wild-type PFK2his and the Ser652/Ala mutant. In the tryptic digest of the Ser652/Ala mutant, previously phosphorylated by PKC, the peak corresponding to fragment $T_{642-654}$ was found to be 96 Da smaller (*m*/*z* 1398.2, Figure 3B, panel b) than the corresponding peak of the phosphorylated wild-type enzyme (Figure 3B, panel a). This results from the mass difference between phosphorylated serine (*m*/*z* 167.1) and alanine (*m*/*z* 71.1). The activity of the Ser652/Ala mutant of PFK2his was controlled. There was no difference between the specific activities of purified wild-type and mutant PFK2his. After in vitro phosphorylation of the Ser652/Ala mutant by PKC, no inhibition of enzyme activity was observed (Figure 4). Phosphorylation experiments with the mutant enzyme using [γ-^{32}P]ATP showed no incorporation of radioactivity (data not shown).

Hypotonic Stress of Yeast Cells Induces in Vivo Phosphorylation and Inactivation of PFK2his. The in vitro phosphorylation results of PFK2his suggested a corresponding in vivo posttranslational modification of the enzyme. As PKC1 (the yeast homologue of PKC) is activated by hypotonic shock (*3*), we investigated the effect of PKC1 on PFK2his in vivo by exposing the yeast cells to hypotonic stress and studying the effect of this on the activity of PFK2his. We found that this modification of environmental conditions has the same effect on PFK2his as the in vitro phosphorylation by PKC. In the presence of ^{32}P-labeled inorganic phosphate in the culture medium, PFK2his is extensively phosphorylated (Figure 5 A). This in vivo phosphorylation leads to an inhibition of the enzyme (Figure 5B). Yeast cells grown in the presence of 1 μg/mL staurosporine showed no inactivation of PFK2 after hypoosmotic shock (data not shown). When yeast cells expressing the Ser652/Ala mutant of PFK2his were exposed to hypotonic stress, no significant inhibition of PFK2his activity was observed.

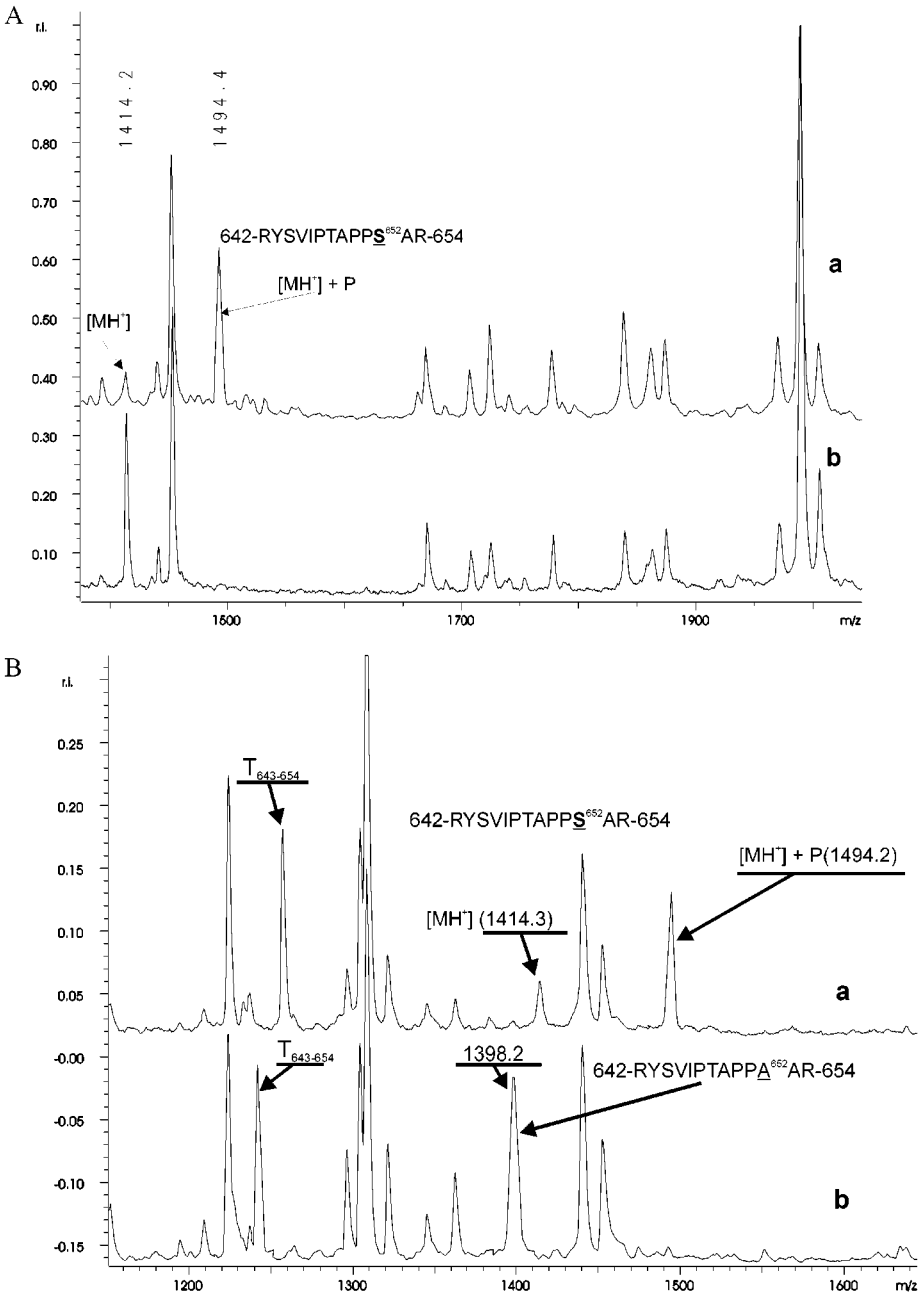

FIGURE 3: MALDI-TOF mass spectra of in vitro PKC-phosphorylated wild-type PFK2his and Ser652/Ala mutant. (A) Panel a: tryptic digest of in vitro PKC-phosphorylated PFK2his; panel b: tryptic digest of the dephosphorylated protein. (B) Panel a, tryptic digest of PKC-phosphorylated wild-type PFK2his; panel b, tryptic digest of PKC-phosphorylated Ser652/Ala mutant.

MALDI-TOF MS analysis was used to identify the site at which PFK2his is phosphorylated under hypotonic conditions. Purified PFK2his isolated from yeast cells after hypotonic shock was dephosphorylated with PP2A before the in-gel digestion to assess the relative level of in vivo protein phosphorylation. A comparison of the MALDI-TOF mass spectra of the tryptic digests of PFK2his proteins from the two experiments (in vivo phosphorylation and in vitro dephosphorylation) confirmed that PFK2his was phosphorylated in vivo. The peak (*m*/*z* 1494.4) representing the phosphorylated peptide $T_{642-654}$ is present in the spectrum obtained from the phosphorylated protein (Figure 6 A, panel a) but absent in the spectrum of the dephosphorylated protein (Figure 6A, panel b). The results show that the modification affects the same fragment, $T_{642-654}$, which was phosphorylated in vitro by PKC (Figure 3A, panel a).

In addition, the MALDI-TOF MS analysis of the in vivo phosphorylated PFK2his showed another modified peak (*m*/*z* 5452.7) corresponding to a single phosphorylation of the tryptic fragment T_{1-41} (Figure 6B, panel a). This peak vanishes after dephosphorylation of the tryptic fragments of PFK2his by PP2A (Figure 6B, panel b). MALDI-TOF MS analysis of the in vivo phosphorylated and Glu-C-digested PFK2his reveals Ser8 as the second phosphorylated residue (Figure 7).

PFK2his Inactivation under Hypotonic Stress Increases Glucose 6-Phosphate and Decreases Glycerol. G-6-P was measured in the DFY658 strain expressing the wild-type PFK2his, the Ser652/Ala mutant, or no PFK2. Under isoosmotic conditions, the G-6-P concentration was 2-fold higher in the cells without PFK2his than in those expressing the enzyme (Figure 8A). After application of hypoosmotic

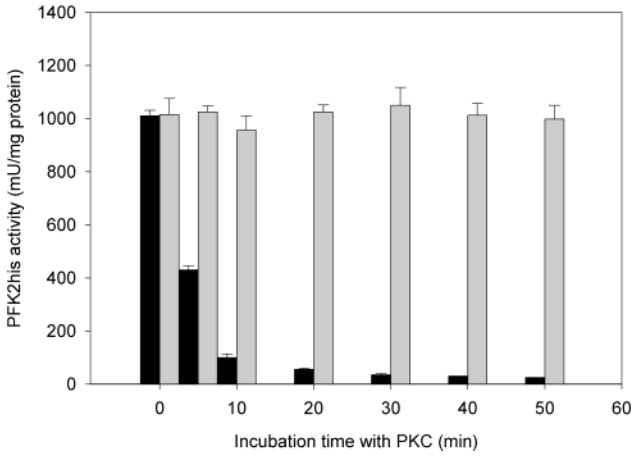

FIGURE 4: Time-dependent activity changes of wild-type PFK2his and Ser652/Ala mutant after PKC phosphorylation. Black bars, wild-type protein; gray bars, Ser652/Ala mutant.

stress to the yeast strain with wild-type PFK2his, G-6-P accumulates during the first 20 min to reach a 3-fold higher concentration than that observed under isoomotic conditions. After this, the G-6-P concentration dropped to lower values than under isoosmosis (Figure 8A). In contrast, in cells expressing either the mutant PFK2his or no PFK2 the G-6-P concentration decreased rapidly after induction by hypotonic stress.

In the yeast cells expressing either the wild-type or the mutant PFK2his, the glycerol concentration under isoosmotic conditions is higher than in the cells without PFK2. This could be caused by overexpression of the enzyme, leading to both activation of glycolysis and glycerol overproduction. In all cases, after hypotonic shock glycerol decreased rapidly to reach a lower level than under isoosmotic conditions (Figure 8B).

DISCUSSION

In Vitro Phosphorylation/Dephosphorylation of Yeast PFK2his. While mammalian PFK2/FBPase2 isoforms are known to be phosphorylated by protein kinases A and C, yeast PFK2 was only known to be phosphorylated in vitro by PKA (*9*). Whether yeast PFK2 is also a substrate for PKC and, if so, what effect phosphorylation by PKC would have on enzyme activity were unknown.

The time-dependent phosphate incorporation into yeast PFK2his demonstrates that this enzyme can be phosphorylated by PKC in vitro and is a good substrate for this protein kinase (Figure 2A). However, in contrast to the phosphorylation by PKA, the action of PKC results in a strong inhibition of PFK2his. A substantial but incomplete reactivation of PKC phosphorylated PFK2his was achieved by mammalian PP2A (Figure 2B). The catalytic subunit of the mammalian PP2A is 80% identical to the yeast protein phosphatases encoded by yeast genes *PPH21* and *PPH22* (*24*).

Identification of the PKC Phosphorylation Site of Yeast PFK2. MALDI-TOF MS combined with tryptic digest mapping was used to identify the amino acid residue of PFK2his which is modified by PKC. In general, the detection of phosphorylation sites by MALDI-TOF MS is impeded by the low yield of phosphopeptides in the MALDI process when compared to that of their nonphosphorylated counterparts (*25*). A combination of two optimization steps allowed

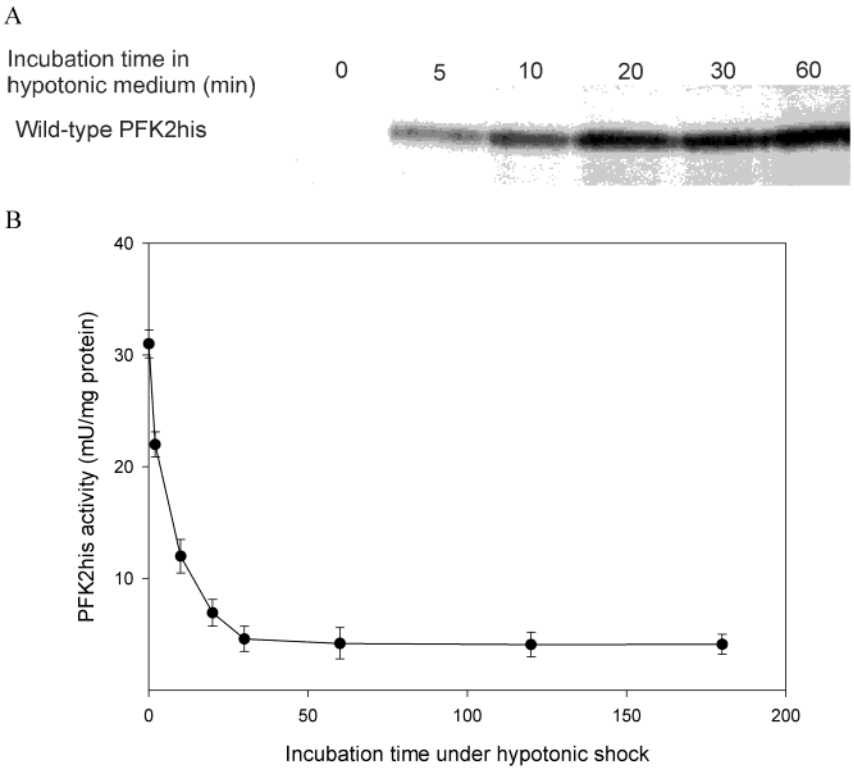

FIGURE 5: In vivo phosphorylation and inactivation of PFK2his after transferring yeast cells to hypotonic medium. (A) Time-dependent changes of the phosphorylation state of PFK2his after hypotonic shock. The cells were incubated for the indicated times with ^{32}P-labeled inorganic phosphate in hypotonic YNB-LP. PFK12his was purified on a HiTrap column and applied to SDS−PAGE. The radioactivity was detected by phosphorimaging. (B) Time-dependent inactivation of PFK2his after hypotonic shock. Enzyme activity was determined in cell-free extracts.

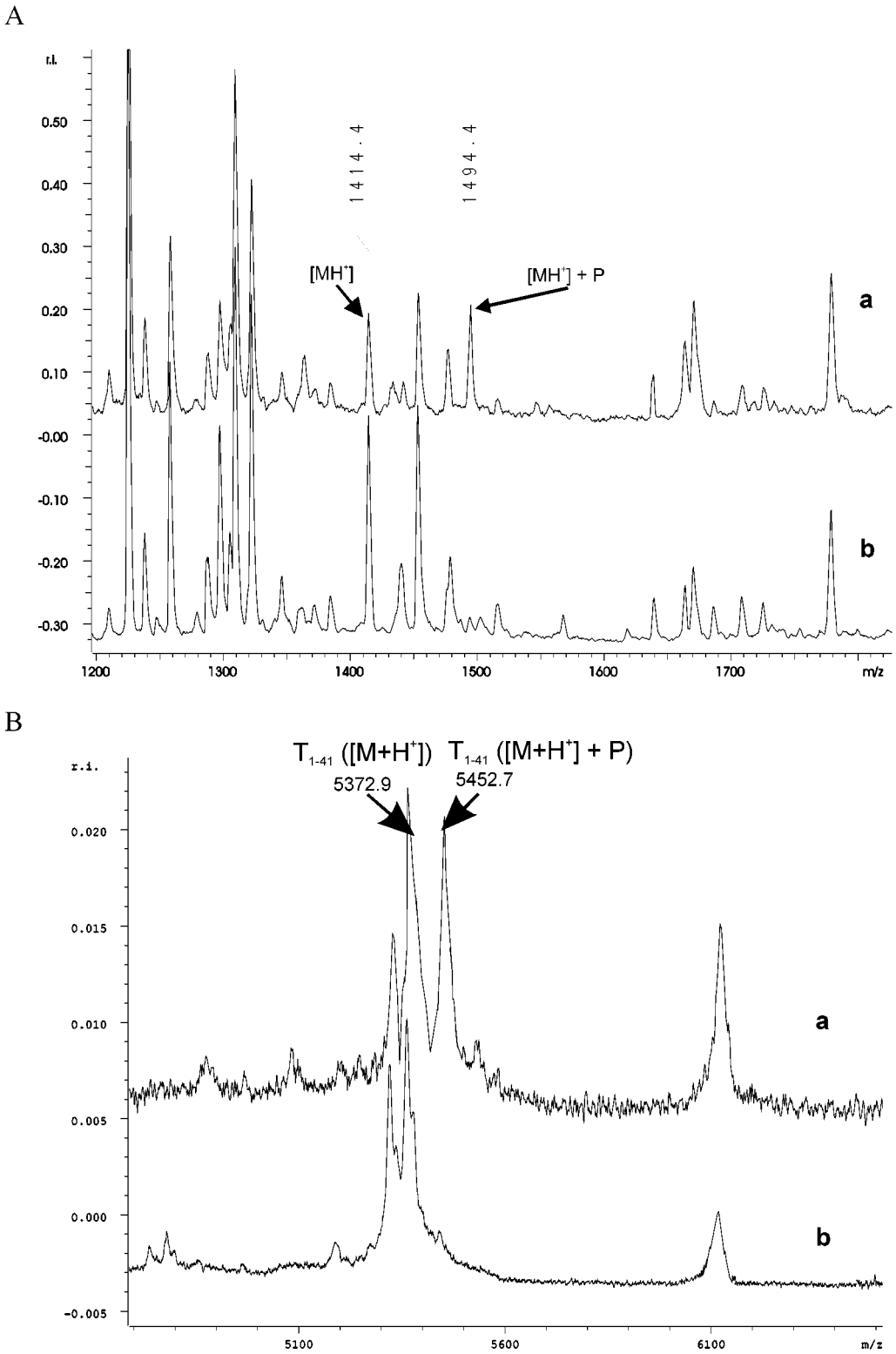

FIGURE 6: Identification of the in vivo phosphorylation sites of PFK2his by MALDI-TOF MS. Panels a: tryptic digest of PFK2his phosphorylated in vivo after incubation of the yeast cells under hypotonic conditions; panels b: tryptic digest of in vivo phosphorylated and in vitro dephosphorylated PFK2his after hypoosmotic stress. (A) Panel a: the peak with *m*/*z* 1494.4 represents the phosphorylated peptide $T_{642-654}$; (B) panel a: the peak with *m*/*z* 5452.7 represents the phosphorylated peptide T_{1-41}.

us to overcome this obstacle. The two-layer sample preparation method was applied for the sample matrix preparation (*21*) to allow better ionization of the peptide fragments; diammonium citrate was added to the matrix−analyte solution to enhance the signal for phosphopeptides (*20*). The combination of MALDI-TOF MS analysis with radioactive labeling and site-directed mutagenesis provided unambiguous evidence that PFK2his is phosphorylated in vitro at Ser652 by PKC. This modification results in the inactivation of PFK2his. Within the peptide $T_{642-652}$, the amino acid sequence Ser-Ala-Arg corresponds to a PKC recognition consensus sequence with Ser652 as the potential phosphorylation site. The incubation of the Ser652/Ala mutant protein with PKC and ATP resulted in no change of enzyme activity.

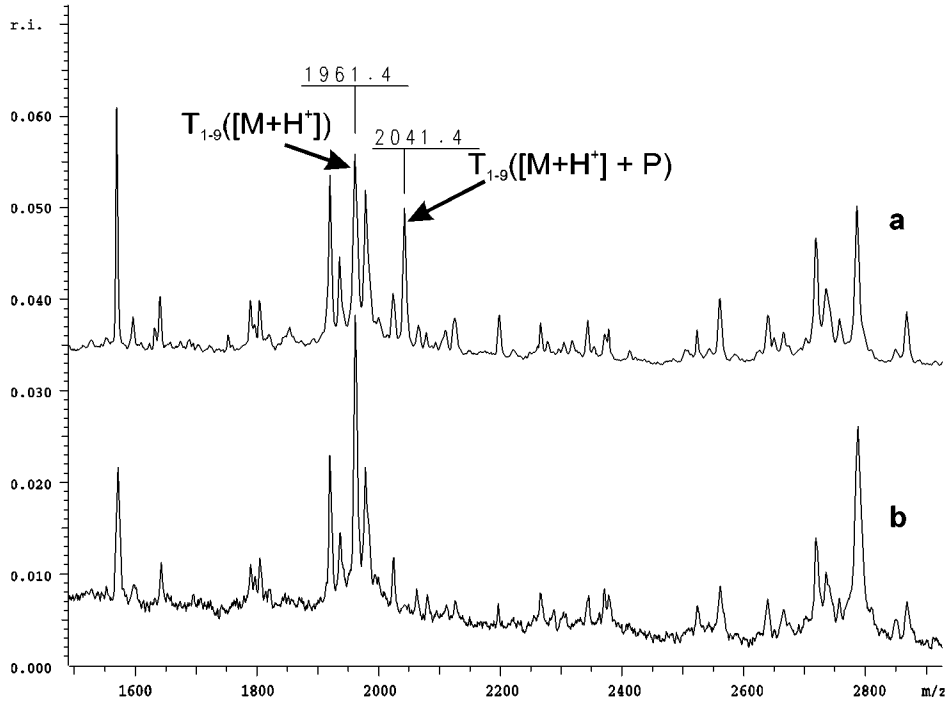

FIGURE 7: Identification of the second in vivo phosphorylation site of PFK2his by MALDI-TOF MS. Panel a: PFK2his digested with Glu-C after incubation of the yeast cells under hypotonic conditions. The peak with *m*/*z* 2041.4 represents the phosphorylated peptide T_{1-9} (*m*/*z* 1961.4); Ser8 is the only residue that can be phosphorylated within this peptide. Panel b: spectrum of in vivo phosphorylated and in vitro dephosphorylated PFK2his after hypoosmotic stress and treatment with Glu-C.

MALDI-TOF MS analysis confirmed that the mutant was not phosphorylated by PKC. In addition to Ser652, the peptide $T_{642-652}$ contains two other potentially phosphorylable residues, Ser644 and Thr648. A Ser644/Ala mutant could be phosphorylated by PKC (data not shown), excluding this amino acid as a target for PKC phosphorylation. Thr648 is not part of a PKC consensus sequence (see Figure 1).

In placental PFK2/FBPase2, PKA and PKC share the same phosphorylation site (Ser460), which corresponds to the PKA phosphorylation site in yeast PFK2 (Ser644). The placental isozyme shows a very small activation after phosphorylation with PKA (1.8-fold) and no activation by PKC (1.1-fold; *12*). For the heart isozyme, the phosphorylation sites of the two kinases are located close to each other (*11*). Treatment of heart PFK2/FBPase2 by PKA leads to a small activation while phosphorylation by PKC has no effect on activity of the isozyme (*26*, *27*). The liver isozyme is also a substrate of PKA, but its phosphorylation results in inactivation (*28*). The PKC phosphorylation site (Ser652) in yeast PFK2 is in close proximity to the PKA site (Ser644) within the C-terminal region of the enzyme (*9*), resembling the situation in heart PFK2/FBPase2 (*11*). In contrast, placenta PFK2/FBPase2 contains only one phosphorylable residue in the corresponding region. This might be the reason PKA and PKC phosphorylate the same serine residue in the placental enzyme.

Among the 14 potential PKC phosphorylation sites in the primary structure of yeast PFK2 that were predicted by computer-based analysis, only Ser652 could be phosphorylated under in vitro conditions. Among the predicted phosphorylation sites, there are several residues which are not embedded in an optimal sequence to be recognized by protein kinases (*25*). In addition, most of the phosphorylation sites recognized by the software are in the middle of the primary structure of PFK2his (see Figure 1). This part of the protein constitutes the core of its tertiary structure and is most probably inaccessible to modifying enzymes. This assumption is in agreement with the observation that phosphorylation sites are found preferentially within either the N- or the C-terminal regions of the target proteins (*29*).

In Vivo Phosphorylation of PFK2his. Yeast PKC1 is activated by hypotonic stress conditions. As a consequence, this leads to activation of a cascade of phosphorylation steps organizing the adaptation of the yeast cells to this environmental change (*3*). It was unknown whether PFK2 is included in the response of yeast toward hypotonic stress, whether the enzyme is thereby phosphorylated, and, if so, by which protein kinase and with which effect on its activity. When cells expressing recombinant wild-type PFK2his were exposed to hypotonic stress, a strong decrease of PFK2his activity was observed (Figure 5B). MALDI-TOF mass spectrometric peptide mapping of PFK2his phosphorylated under hypotonic stress and dephosphorylated in vitro with PP2A revealed a 2-fold phosphorylation of the enzyme within the stressed cells. The first modification affects the same tryptic fragment ($T_{642-654}$) as in vitro with PKC. Cells grown on YNB-P in the presence of the PKC1 inhibitor staurosporine (*30*) showed no PFK2 inactivation after hypotonic stress. This supports the assumption that PFK2his was in vivo phosphorylated by PKC1 or by another protein kinase member of the MAPK pathway. The fact that Ser652 is N-adjacent to two proline residues indicates a typical consensus sequence also for MAP kinases (*31*). The second in vivo phosphorylation occurs within peptide T_{1-41}. Among the two potential phosphorylation sites Ser8 and Ser34, the first one could be experimentally verified (Figure 7) since fragment T_{1-9} contains no other serine or threonine residues. The fact that the Ser652/Ala PFK2his mutant was not

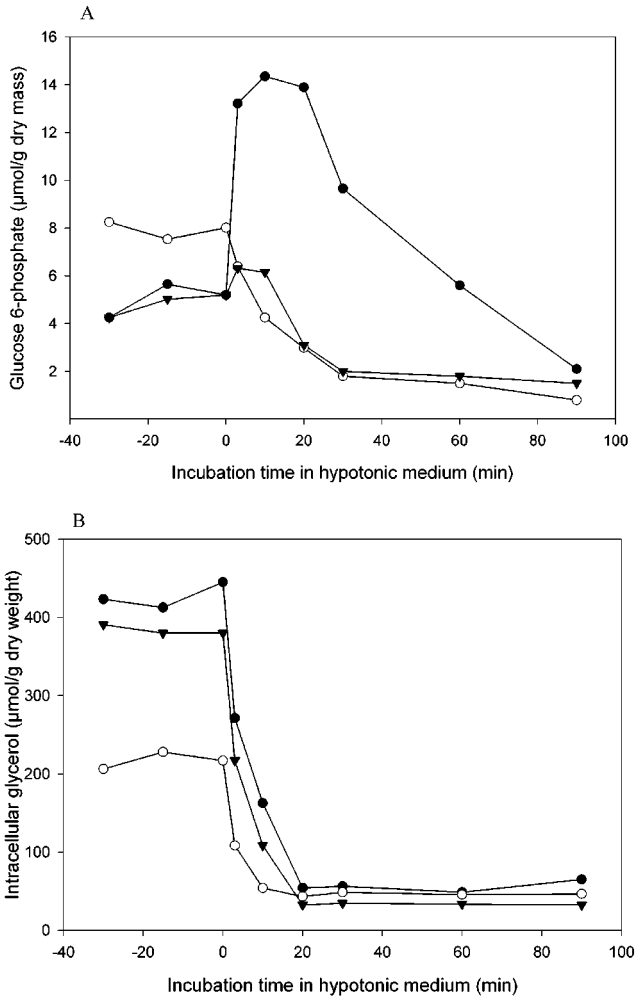

FIGURE 8: Analysis of yeast metabolites under hypotonic stress. (A) Time-dependent changes of glucose 6-phosphate concentration in DFY658 cells expressing wild-type PFK2his (●), Ser652/Ala mutant (▼), or no PFK2his (○) after transferring the yeast cells to hypotonic medium. (B) Glycerol concentration changes under the same experimental conditions. Data points represent the means of three independent experiments.

inhibited after hypotonic stress proves that only Ser652 is involved in the inactivation of PFK2his during a decrease in environmental osmolarity.

Role of PFK2 Inactivation in the Regulation of Glycolysis under Hypotonic Stress. Hypotonic stress activates in yeast cells the hypoosmolarity MAP kinase pathway by activating PKC1 (*32*). The observed in vivo phosphorylation and inhibition of PFK2 in yeast cells under hypoosmotic stress, which is probably catalyzed by PKC1, may represent one of the cellular responses to counteract the change of the environment. It is expected to reduce the rate of glycolysis, leading to an accumulation of G-6-P. Similar reactions of yeast cells were found after mutations in the 6-phosphofructo-1-kinase (*33*) or PFK2 genes (*34*). The increased hexose monophosphates can be metabolized by the pentose phosphate pathway, which provides the precursors of the synthesis of glucans, the main constituents of the yeast cell wall (*35*). This would be in accordance with the fact that under hypoosmotic conditions an enhanced cell wall remodeling occurs (*36*, *37*). The suppression of glycolysis may contribute to the cell wall maintenance by reducing the synthesis of glycerol which acts as strong osmolyte. This assumption is in line with the observed decrease of the cellular glycerol content after hypotonic stress. The PFK2-mediated effect on glycerol synthesis would be synergistic to the known release of glycerol via the metabolite transporter Fps1p, a member of the MIP family (*38*), which is essential for the adaptation to hypoosmolarity.

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