Ser^{644} is important for catalytic activity but is not involved in cAMP-dependent phosphorylation of yeast 6-phosphofructo-2-kinase

Renate Keßler, Klaus Eschrich*

Institute of Biochemistry, University of Leipzig, School of Medicine, Liebigstraße 16, D-04103 Leipzig, Germany

Received 12 August 1996; revised version received 12 September 1996

Abstract To identify the target amino acid for the cAMP-dependent phosphorylation of yeast 6-phosphofructo-2-kinase Ser^{644} was mutated to Ala. The plasmid-encoded wild-type and mutant enzymes were overexpressed in *E. coli* TG2 cells and in the yeast strain DFY658. Like the wild-type enzyme, the $Ser^{644} \rightarrow$ Ala mutant was phosphorylated in vivo after addition of glucose to yeast cells and in vitro by the catalytic subunit of protein kinase A. The specific activity of the mutant enzyme was 6-fold lower than that of the wild-type yeast 6-phosphofructo-2-kinase, but both enzymes were activated in response to the addition of glucose to yeast cells.

Key words: Glycolysis; 6-Phosphofructo-2-kinase; *Saccharomyces cerevisiae*; Site-directed mutagenesis

1. Introduction

6-Phosphofructo-2-kinase (6PF-2-K, EC 2.7.1.105) from yeast (*Saccharomyces cerevisiae*) is an excellent substrate for cAMP-dependent protein kinase [1,2]. The activity of purified 6PF-2-K can be increased about 10-fold upon incubation with ATP and the catalytic subunit of cAMP-dependent protein kinase [1]. The in vivo phosphorylation induced by glucose causes a 7-fold activation of the enzyme [3]. In contrast to the yeast enzyme, 6PF-2-K of the bifunctional liver enzyme is inhibited by phosphorylation [4]. In rat liver 6PF-2-K Ser^{32} was identified as the primary residue phosphorylated by protein kinase A [5]. In yeast 6PF-2-K the consensus sequence Arg-Arg-Tyr-Ser in the C-terminal region was suggested to be the potential phosphorylation site for protein kinase A [6], in this respect resembling the enzyme from heart [7]. Here we show by in vivo and in vitro phosphorylation as well as by activity measurement that a conversion of Ser^{644} to Ala does affect the catalytic activity of the enzyme but is not involved in the cAMP-dependent phosphorylation of yeast 6PF-2-K.

2. Materials and methods

2.1. Strains and plasmids

E. coli TG2 cells were used for cloning and expression experiments. The yeast (*Saccharomyces cerevisiae*) strain DFY658 (*pfk26::LEU2, fbp26::His3, leu2, his3, ura3*) was described by Kretschmer et al. [8]. The plasmids pRK3 and pRK5 are expression vectors of yeast 6PF-2-K, described below. pRK6 carries the $Ser^{644} \rightarrow$ Ala mutant of the enzyme. The plasmid pMK11, containing the *PFK26* gene fused to the *Gal1* promoter, was kindly provided by Dr. M. Kretschmer (Auto-Immune Inc., Lexington, KY).

2.2. Expression of yeast 6PF-2-K in E. coli and preparation of cell-free extracts

The cDNA for 6PF-2-K isolated by Kretschmer and Fraenkel [6] was cloned into the *SalI*/*SacI* sites of pUC19 behind a Shine-Dalgarno sequence ligated into the *Hin*dIII/*Pst*I sites of the vector. Digestion with *Hin*cII/*Pst*I, treatment with Klenow fragment of DNA polymerase I and ligation yielded the plasmid pRK3. The PFK26 gene and the ribosome binding site were subcloned into the *Pst*I/*Hin*dIII sites of the expression vector pKK223-3 (Pharmacia Biotech) containing the strong *tac* promoter. The resulting plasmid was designated pRK5. *E. coli* TG2 cells were transformed with plasmids encoding the wild-type and the mutant enzyme, respectively. The preculture was grown overnight at 37°C in LB medium containing ampicillin (100 μg/ml). The overnight culture (5 ml) was added to 0.5 l LB medium and the cells were induced with 0.25 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 25°C for 20 h. The cultures were harvested by centrifugation, washed, and 1 g of the pellet was resuspended in 2 ml homogenization buffer (100 mM potassium phosphate, pH 7.2, 4 mM mercaptoethanol (ME), 1 mM phenylmethanesulfonyl fluoride, 1 μg leupeptin/ml). The cells were sonicated and centrifuged at $20\,000 \times g$ for 15 min.

2.3. Site-directed mutagenesis

For the introduction of the $Ser^{644} \rightarrow$ Ala mutation the method of PCR-enabled site-directed mutagenesis described by Nelson and Long [9] was used. The plasmid pRK3 was used for mutant construction. The selective amplification of the mutated sequence required four synthetic oligonucleotide primers (Table 1). The primers were synthesized on a Gene Assembler Plus (Pharmacia LKB) by β-cyanoethyl phosphoramidite chemistry and purified by gel filtration on Sephadex G-25. PCR was carried out with an Omnigene thermal cycler (MWG-Biotech) using Ultma polymerase (Perkin Elmer). The final 766 bp product was purified by a PCR purification kit (Qiagen), digested with *Sfu*I and *Kpn*I and the resulting 648 bp fragment was cloned into the parent plasmid replacing the corresponding wild-type fragment. The mutant plasmids were identified by *Xmn*I digestion and confirmed by DNA sequencing [10]. The mutant fragment was subcloned into the *Nco*I/*Kpn*I sites of pRK5 and the *Sph*I/*Kpn*I sites of pMK11, respectively.

2.4. Assay of enzyme activity

6PF-2-K activity was measured at 25°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 [3]. Protein concentration was determined by the method of Bradford [11].

2.5. Immunoblotting

For Western blotting standard protocols were used [12]. The blots were probed with antiserum raised against yeast 6PF-2-K in a rabbit. The amount of 6PF-2-K protein was estimated by densitometry using a video gel documentation and analysis system (E.A.S.Y., Herolab). Purified 6PF-2-K was used for calibration.

2.6. In vitro phosphorylation of 6PF-2-K

6PF-2-K eluted from protein G Sepharose was incubated for 30 min at 30°C in 0.1 M Tris-glycine, pH 7.5, 10 mM magnesium chloride, 1 μCi [γ-^{32}P]ATP with and without 3 μg/ml catalytic subunit of protein kinase A and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were analyzed by Phosphor Imager (Molecular Dynamics) scanning.

*Corresponding author. Fax: (49) (341) 9722119.
E-mail: eschrich@rz.uni-leipzig.de

PII S0014-5793(96)01045-9

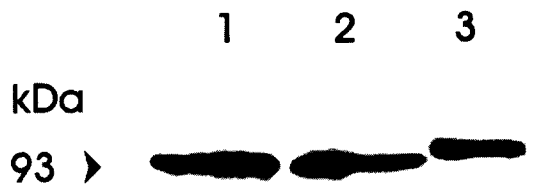

Fig. 1. Immunoblot of wild-type and mutant yeast 6PF-2-K expressed in *E. coli*. Cell extracts of TG2 cells carrying plasmid-encoded wild-type 6PF-2-K (lane 1) or $Ser^{644} \rightarrow Ala$ mutant 6PF-2-K (lane 2). Lane 3: 25 ng 6PF-2-K purified from yeast. 30 μg protein was loaded onto lanes 1 and 2. The position of phosphorylase b is indicated by the arrow on the left.

2.7. In vivo phosphorylation of 6PF-2-K

^{32}P-labeling of 6PF-2-K was performed in the yeast strain DFY658 transformed with pMK11 and pMK11/Ala^{644}, respectively. Cells were grown in low-phosphate YNB-A medium [13] supplemented with 0.2% casamino acids, 1% glucose, and 1% galactose to an A_{600} of 2. The in vivo labeling was performed according to Vojtek and Fraenkel [14].

2.8. Immunoprecipitation of 6PF-2-K

The procedure of Mönning et al. [15] was followed with some modifications. Cell-free extracts (1.5 ml) of *E. coli* or yeast cells, respectively, were diluted with a twofold volume of PBS and incubated for 2 h at 4°C with 50 μl antiserum against yeast 6PF-2-K raised in a rabbit. After addition of 75 mg protein G Sepharose (Pharmacia Biotech) the mixture was incubated 60 min at 4°C. Unbound proteins were removed from the Sepharose beads by washing with 20 mM Tris-HCl, pH 8.0, 150 mM sodium chloride. The protein G Sepharose was resuspended in 100 μl of 1×SDS loading buffer [16], heated for 10 min at 95°C and applied to SDS-PAGE. For in vitro labeling experiments 6PF-2-K was eluted from protein G Sepharose by 0.1 M glycine, pH 2.7, and neutralized with 1 M Tris-HCl, pH 9.0.

3. Results

3.1. Expression of wild-type and mutant enzymes in E. coli

The best results for expression of yeast 6PF-2-K in *E. coli* were obtained with TG2 cells containing the plasmid pRK5. After induction by IPTG at 25°C specific activities of 10 mU/mg protein were measured in cell-free extracts. This value is 4-times higher than that obtained at 37°C. In the soluble fraction of *E. coli* extracts one protein band was detected by immunoblotting with antiserum against purified yeast 6PF-2-K (Fig. 1).

Taking the intensity of this band as a basis, the specific activity of 6PF-2-K was 7.5 U/mg. This value corresponds well with the specific activity of the purified enzyme (8.8 U/mg) [8].

The 6PF-2-K expressed in *E. coli* migrates somewhat faster than the 96 kDa band of the enzyme isolated from yeast. Since there are no further bands visible this difference in the apparent molecular weight may result from a missing post-translational modification (e.g. glycosylation) of the recombinant 6PF-2-K in *E. coli* rather than from a proteolytic degradation.

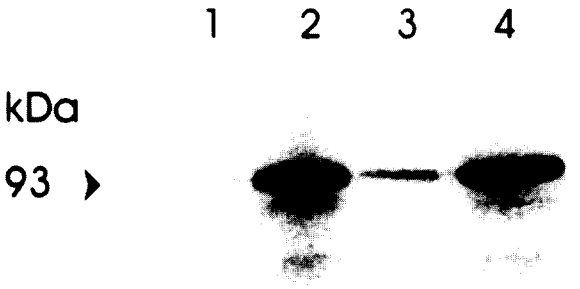

Fig. 2. In vivo labeling of yeast 6PF-2-K. Yeast cells grown in low-phosphate YNB-A medium with 1% glucose and 1% galactose to low absorbance were incubated in medium containing $^{32}P_i$ (lanes 1 and 3) or $^{32}P_i$ and 2% glucose (lanes 2 and 4). Immunoprecipitates of cell extracts (1.5 ml) were subjected to SDS-PAGE and autoradiography. Lanes 1 and 2: wild-type 6PF-2-K; lanes 3 and 4: $Ser^{644} \rightarrow Ala$ mutant. The position of phosphorylase b is indicated by the arrow on the left.

The $Ser^{644} \rightarrow Ala$ mutant was also expressed in *E. coli*. As shown by immunoblotting the mutant protein was visible at the same position as the wild-type 6PF-2-K (see Fig. 1). A comparison of the intensity of the wild-type and mutant 6PF-2-K protein bands (lanes 1 and 2 in Fig. 1) shows no significant difference. The specific activity of the mutant enzyme based on the amount of 6PF-2-K protein (1.2 U/mg) was 6.2-fold lower than that of the wild-type enzyme.

3.2. In vivo phosphorylation of 6PF-2-K

S. cerevisiae DFY658, a strain with 6PF-2-K activity of 0.01 mU/mg protein and no phosphoprotein-forming fructose-2,6-bisphosphatase, was chosen for in vivo labeling experiments. Yeast cells expressing plasmid-encoded wild-type and mutant 6PF-2-K were grown initially in medium depleted of inorganic phosphate and then incubated in medium containing ^{32}P and glucose. As shown in Fig. 2, the in vivo labeling of the $Ser^{644} \rightarrow Ala$ mutant was not significantly different from that of the wild-type enzyme. In the presence of glucose the ^{32}P-labeling of both enzymes was strongly increased (lanes 2 and 4) in contrast to the reduced labeling in absence of glucose (lanes 1 and 3). Additionally, the effect of in vivo phosphorylation on the specific activities of the wild-type and the mutant enzymes was determined. In the stationary growth phase when the cells are depleted of glucose the specific activity of the wild-type 6PF-2-K was about 10 mU/mg protein measured in cell-free yeast extracts. The addition of glucose to the incubation medium caused a 7-fold increase in the activity of 6PF-2-K (Table 2). Glucose induced also a 7-fold activation of the $Ser^{644} \rightarrow Ala$ mutant 6PF-2-K, however,

Table 1
Oligonucleotide primers used for PCR mutagenesis

Primer	Sense	Sequence
		↓
A	forward	5′ TGAAAGAAGATATGCTGTTATACCAAC 3′
B	reverse	5′ CGTCACTTGAGTTACAGCATTTGGAACAACCCTGGG 3′
C	forward	5′ AAGTGCAGGTGATTATGATGG 3′
D	reverse	5′ CGTCACTTGAGTTACAGC 3′

The arrow indicates the position of the base exchange (T→G) introduced into the *PFK26* gene. The underlined bases comprise the *Xmn*I recognition sequence which is destroyed by the mutation.

Table 2
Effect of glucose on the activity of yeast 6PF-2-K

	6PF-2-K [mU/mg protein] (−glucose)	6PF-2-K [mU/mg protein] (+2% glucose)	Activation factor
Wild-type 6PF-2-K (pMK11)	10.7	70.6	6.6
Mutant 6PF-2-K ($Ser^{644} \rightarrow Ala$)	1.6	10.4	6.5

Yeast cells transformed with pMK11 or pMK11/$Ser^{644} \rightarrow$ Ala were grown in YNB-A medium supplemented with 0.2% casamino acids, 1% glucose and 1% galactose to $A_{600} = 6$ and then transferred to fresh medium. The cell suspensions were incubated at 30°C for 30 min with or without 2% glucose. Cell extracts were prepared with glass beads as described by Vojtek and Fraenkel [14] and assayed for 6PF-2-K activity.

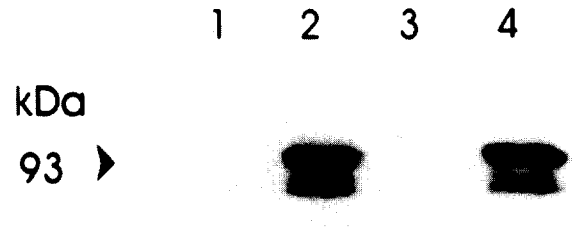

Fig. 3. In vitro phosphorylation of yeast 6PF-2-K. The wild-type and mutant enzymes were partially purified from *E. coli* extracts by immunoprecipitation. 1 μg 6PF-2-K was incubated with [γ-^{32}P]ATP and catalytic subunit of bovine heart cAMP protein kinase (see Section 2). Lanes 1 and 2: recombinant wild-type 6PF-2-K; lanes 3 and 4: $Ser^{644} \rightarrow$ Ala mutant of 6PF-2-K. In lanes 1 and 3 the protein kinase was omitted. The position of phosphorylase b is indicated by the arrow on the left.

the specific activities of the mutant enzyme in the presence and in the absence of glucose were substantially lower than those of the wild-type enzyme.

3.3. *In vitro phosphorylation of 6PF-2-K*

In order to verify the results obtained under in vivo conditions it was tested whether or not the $Ser^{644} \rightarrow$ Ala mutant could be phosphorylated in vitro. For this experiment both wild-type and mutant enzyme were partially purified from *E. coli* extracts. As shown in Fig. 3, both enzymes were phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase (lanes 2 and 4).

4. Discussion

Since they are devoid of 6PF-2-K and fructose-2,6-bisphosphatase bacteria are expected to be a convenient host for expression of wild-type and mutant 6PF-2-Ks. After optimization of induction and growth conditions in cell-free *E. coli* extracts enzyme activities up to 100-times higher than in yeast could be measured for wild-type 6PF-2-K. The increased recovery of active yeast 6PF-2-K at lowered induction temperature may be due to the slower rate of protein synthesis and the proper folding of the enzyme [17]. Although 6PF-2-K can also be overexpressed in *S. cerevisiae* [8], there are some advantages when the recombinant protein is obtained from bacteria. Cell cultivation is easier and in crude extracts or partially purified preparations no 'background' of other yeast proteins is present.

The specific consensus sequences of cAMP-dependent protein kinase are characterized by the presence of basic amino acids, particularly arginine, N-terminal to the phosphoacceptor serine or threonine [18]. In yeast 6PF-2-K a putative protein kinase target might be Ser^{644} in the Arg-Arg-Tyr-Ser sequence close to the C-terminus of the protein. We have shown in this study that the Ser^{644} is apparently not involved in cAMP-dependent phosphorylation. The $Ser^{644} \rightarrow$ Ala mutant 6PF-2-K was phosphorylated in vitro by protein kinase A like the wild-type enzyme. In yeast cells an in vivo phosphorylation of the mutant enzyme induced by glucose could also be observed. The effect of glucose on the in vivo phosphorylation of 6PF-2-K is mediated by the successive activation of adenylate cyclase and of cAMP-dependent protein kinase [3]. The activation of the $Ser^{644} \rightarrow$ Ala mutant 6PF-2-K in response to the addition of glucose to yeast cells correlated with the results of in vivo labeling experiments. However, the mutation of Ser^{644} to alanine resulted in a 6-fold decrease in the specific activity of yeast 6PF-2-K, indicating that this residue is important for the catalytic activity. It still remains to be elucidated which amino acid is modified by phosphorylation. The most likely candidate is Thr^{157} in the N-terminal consensus sequence Arg-Arg-Pro-Thr. Also serines 656, 667 and 790 have to be considered. Experimental work is in progress to pinpoint the target of protein kinase A in 6PF-2-K.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, Project Es 87/2-1.

References

[1] François, J., van Schaftingen, E. and Hers, H.-G. (1988) Eur. J. Biochem. 171, 599–608.
[2] Kretschmer, M., Tempst, P. and Fraenkel, D.G. (1991) Eur. J. Biochem. 197, 367–372.
[3] François, J., van Schaftingen, E. and Hers, H.-G. (1984) Eur. J. Biochem. 145, 187–193.
[4] Van Schaftingen, E. and Hers, H.-G. (1982) Eur. J. Biochem. 124, 143–149.
[5] Kurland, I.J., El-Maghrabi, M.R., Correia, J.J. and Pilkis, S.J. (1992) J. Biol. Chem. 267, 4416–4423.
[6] Kretschmer, M. and Fraenkel, D.G. (1991) Biochemistry 30, 10663–10672.
[7] Sakata, J. and Uyeda, K. (1990) Proc. Natl. Acad. Sci. USA 87, 4951–4955.
[8] Kretschmer, M., Langer, C. and Prinz, W. (1993) Biochemistry 32, 11143–11148.
[9] Nelson, R.M. and Long, G.L. (1989) Anal. Biochem. 180, 147–151.
[10] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[11] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[12] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[13] Rubin, G. (1973) J. Biol. Chem. 247, 3860–3875.
[14] Vojtek, A.B. and Fraenkel, D.G. (1990) Eur. J. Biochem. 190, 371–375.
[15] Mönning, U., Sandbrink, R., Banati, R.B., Masters, C.L. and Beyreuther, K. (1994) FEBS Lett. 342, 267–272.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Lin, K., Kurland, I., Xu, L.Z., Lange, A.J., Pilkis, J., El-Maghrabi, M.R. and Pilkis, S.J. (1990) Protein Express. Purif. 1, 169–176.
[18] Kennely, P.J. and Krebs, E.G. (1991) J. Biol. Chem. 266, 15555–15558.