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# Evidence for phosphorylation of yeast phosphofructokinase

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Radioactively labelled material from yeast cells grown in the presence of [$^{32}$P]phosphate was specifically recognized by antibodies raised against yeast phosphofructokinase. Purified yeast phosphofructokinase was phosphorylated in a cyclic AMP-independent manner by a protein kinase enriched from yeast extracts. This phosphorylation occurred specifically on the $\beta$-subunit, and 0.56 mol of phosphate/mol of subunit was incorporated. The results indicate the phosphorylation of yeast phosphofructokinase both in vivo and in vitro. Phosphofructokinase phosphorylated in vitro was more stable against proteolytic degradation compared to the non-phosphorylated enzyme.

Phosphofructokinase; Yeast; Protein kinase; Protein phosphorylation

## 1. INTRODUCTION

Glycolysis and gluconeogenesis are the principal pathways of glucose metabolism in yeast cells with phosphofructokinase and fructose-1,6-bisphosphatase as the key enzymes. Addition of glucose as glycolytic carbon source to the growth medium results in catabolite inactivation of the gluconeogenic enzymes fructose-1,6-bisphosphatase, cytoplasmic malate dehydrogenase and phospho*enol*pyruvate carboxykinase [1]. In the case of fructose-1,6-bisphosphatase a biphasic mechanism of its glucose-induced inactivation has been demonstrated [2]. The proteolytic degradation is preceded by a rapid phosphorylation controlled by cyclic AMP. The rate of this reaction is modulated by the kinetic inhibitors of the enzyme fructose-2,6-bisphosphate and AMP [3].

Phosphate incorporation into phosphofructokinase, the other enzyme of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle, has been demonstrated in higher organisms [4]. We now provide evidence for the phosphorylation also of the yeast enzyme which is accompanied by a stabilization against proteolytic degradation.

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*Abbreviation:* PFK, phosphofructokinase

## 2. EXPERIMENTAL

### 2.1. *Materials*

*Saccharomyces cerevisiae* wild type strain DFY-1 (a lys1) was kindly provided by Dr Dan Fraenkel (Harvard Medical School, Boston). It was grown at 30°C on a rotary shaker in rich medium M 63 supplemented with Bacto tryptone (10 g/l) and Bacto yeast extract (4 g/l). Glucose (20 g/l) was the carbon source.

Phosphofructokinase from baker's yeast (VEB Backhefe, Leipzig) was purified according to [5]. Enzymes and substrates were from Boehringer. [$\gamma$-$^{32}$P]ATP was synthesized according to [6]. Protamine sulphate and buffer substances were from Sigma.

### 2.2. *Phosphorylation of yeast proteins in vivo*

1 ml of an exponentially growing culture of DFY-1 ($A_{580}$ = 8) was diluted with 8 ml of fresh medium containing 150 $\mu$Ci [$^{32}$P]phosphoric acid. The cells were then allowed to grow for 6 h. They were collected by centrifugation and washed 5 times, each with 1 ml of ice-cold 50 mM Tris-HCl, pH 7.6 (buffer A). The cells were disrupted by shaking with 1 g of glass beads (0.5 mm in diameter) in 0.5 ml of buffer A also containing 0.5 mM EDTA and 1 mM 2-mercaptoethanol. After centrifugation (50000 × *g*, 20 min), 0.2 ml of the supernatant was diluted with 4 ml of buffer A containing 20 g/l of bovine serum albumin and divided into 1-ml aliquots. These were used for binding studies to immobilized PFK antibodies.

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2.3. *Immobilization of antibodies on nitrocellulose filters*

Antibodies against highly purified yeast PFK were raised in rabbits, and immunoglobulins were enriched from the antisera [7]. Using an immunoblot technique [17] with the DFY-1 extract these antibodies recognize only the two phosphofructokinase subunits.

Strips of nitrocellulose filter (1.5 × 3 cm, Schleicher & Schuell, 0.45 $\mu$m) were gently agitated first in buffer A for 30 min, then in buffer A containing 1 g/l of anti-yeast phosphofructokinase immunoglobulin (or $\gamma$-globulins of a non-immunized rabbit for control strips) for 10 h and finally with buffer A containing 20 g/l of bovine serum albumin for 1 h. Unbound material was removed by sequential washing in buffer A, buffer A containing 0.2% Nonidet P-40, and twice in buffer A.

2.4. *DEAE-cellulose chromatography of yeast protein kinases*

50 g of fresh baker's yeast were mixed with 500 g of crushed dry ice and homogenized in a blender. After thawing, the paste was mixed with 50 ml of buffer A, also containing 1 mM EDTA and 5 mM 2-mercaptoethanol (buffer B), and centrifuged as above. The pellet obtained was redissolved in buffer B and dialyzed against this buffer. The dialysate was applied to a 5 × 30 cm DEAE-cellulose (Whatman DE 52) column equilibrated with buffer B. After washing the column with two column volumes, a linear NaCl gradient (0–0.5 M NaCl in buffer B, total volume 800 ml) was applied. The flow rate was 60 ml/h and 15 ml fractions were collected.

2.5. *Analyses*

Protein kinase activity was assayed in a mixture (0.3 ml) containing 20 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 5 mM $CaCl_2$, 0.3 M NaCl, $10^{-5}$ M cyclic AMP (when indicated), 1 mM [$\gamma$-$^{32}$P]ATP (40–100 cpm/pmol), 0.6 mg protamine or 0.15 mg yeast PFK, and enzyme protein. After incubation at 22°C, 50 $\mu$l aliquots were spotted on filter paper discs (Whatman 3MM). Before counting in a liquid scintillator counter the discs were washed as described in [8] or [9] (trichloroacetic acid-$H_3PO_4$ method) when either protamine or PFK, respectively, was the substrate.

To obtain phosphorylated PFK the protein from the incubation mixture was concentrated by ammonium sulphate precipitation (55% saturation) besides an aliquot used for the estimation of the amount of phosphorylation. The precipitate was collected by centrifugation at 25000 × $g$ and desalted by Sephadex G-25 filtration. Protein was determined according to [10].

SDS-polyacrylamide gel electrophoresis was carried out using a modification [11] of the Neville procedure [12].

## 3. RESULTS

### 3.1. *Demonstration of in vivo phosphorylation*

Incubation of yeast extract, labelled in vivo with [$^{32}$P]phosphate, with antibodies to yeast PFK immobilized on nitrocellulose strips resulted in a substantial adsorption of radiolabelled material to the strips (table 1). This adsorption was approx. 3 times higher compared to strips coated with unspecific immunoglobulins, and it also decreased when phosphofructokinase was added to the incubations. Furthermore, a nitrocellulose coated control (not shown) with purified phosphofructokinase showed no retention of radioactivity on the filters. These results suggest that yeast PFK can become phosphorylated in vivo.

Table 1

Radioactivity retained on nitrocellulose strips coated with antibodies to yeast phosphofructokinase

| Coating | $\mu$g phosphofructokinase added | Radioactivity (cpm) |
|---|---|---|
| Control immunoglobulins | – | 6212 ± 1279 |
| Anti-yeast phosphofructokinase | – | 19025 ± 3479 |
| Anti-yeast phosphofructokinase | 10 | 9784 ± 1712 |
| Anti-yeast phosphofructokinase | 50 | 8636 ± 1890 |

Aliquots of cell extract from yeast grown in the presence of [$^{32}$P]phosphate were incubated for 3 h without or with the indicated amounts of phosphofructokinase (final volume 1.1 ml). The strips were washed with 5 ml each of buffer A, buffer A containing 0.2% Nonidet P-40, and buffer A (each step for 30 min) before liquid scintillation counting. Non-specific adsorption was monitored using strips coated with $\gamma$-globulins of a non-immunized rabbit. Results are from 5 measurements ± SD

### 3.2. *Isolation of phosphofructokinase kinase activity*

When extracts were chromatographed on DEAE-cellulose two fractions of protein kinase activity capable of phosphorylating yeast phosphofructokinase were resolved (fig.1). One fraction eluted early in the salt gradient (at 0.05 M NaCl) slightly ahead of cyclic AMP-dependent protein kinase, whereas the second major fraction eluted later (at 0.25 M NaCl), well behind a cyclic AMP-independent protamine kinase activity. Quantitatively this second fraction of PFK kinase was rather constant in different yeast preparations, while the first one varied and was even absent in some preparations.

### 3.3. *Phosphorylation of yeast phosphofructokinase in vitro*

The major PFK kinase fraction isolated by

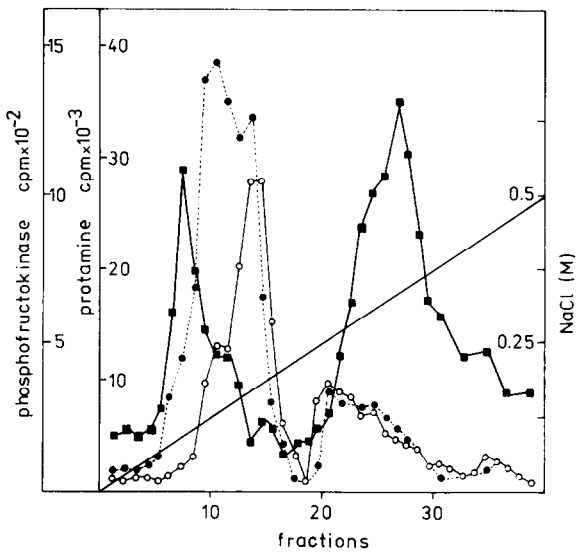


Fig.1. DEAE-cellulose chromatography of protein kinase activities of yeast extract. Substrates were phosphofructokinase (■) and protamine in the absence (○) or presence (●) of cyclic AMP. The profile of phosphofructokinase activity was identical with and without cyclic AMP.

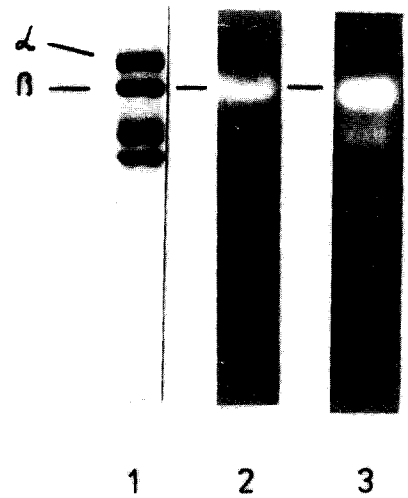


Fig.2. SDS-polyacrylamide gel electrophoresis of yeast phosphofructokinase phosphorylated by the isolated protein kinase. Lanes: 1, protein stain (20 μg protein applied); 2,3, autoradiographs from the same run, exposure times 16 and 52 h, respectively. Note the fast-moving band in addition to phosphofructokinase subunits in lane 1 indicating proteolysis during in vitro phosphorylation.

DEAE-cellulose chromatography was concentrated 5-fold by ultrafiltration and used for further analyses. The rate of phosphorylation of yeast PFK by this preparation was not affected significantly by 10 μM cyclic AMP, 5 μM cyclic GMP, 2 mM AMP or 20 μM fructose-2,6-bisphosphate. The maximum amount of phosphate incorporated after prolonged incubation was 0.28 mol/mol of phosphofructokinase subunit ($M_r$ 100000 was used for the calculation).

The PFK subunit specificity of the isolated protein kinase was examined by SDS-polyacrylamide gel electrophoresis and autoradiography after phosphorylation with [$\gamma$-$^{32}$P]ATP (fig.2). Only the faster moving $\beta$-subunit became phosphorylated to a significant degree.

### 3.4. *Effect of phosphorylation on phosphofructokinase activity*

No difference in the activities of phosphorylated (0.18 mol phosphate incorporated/mol of subunit) and non-phosphorylated PFK was detectable. Both forms are similarly activated by AMP and fructose-2,6-bisphosphate and inhibited by ATP (not shown). However, the data from table 2 indicate a stabilization of the phosphorylated enzyme against proteolytic attack by $\alpha$-chymotrypsin. Within 2 h at a PFK/protease ratio of 20:1 the activity of the non-phosphorylated PFK dropped continuously to about 25% of the initial and control value. In contrast, the phosphorylated form lost its activity only during the initial phase and remained constant after 20 min at about 80% of the control.

Table 2

Inactivation of phosphorylated and non-phosphorylated yeast phosphofructokinase by $\alpha$-chymotrypsin

| Time of incubation (min) | Phosphofructokinase activity (U/ml) | |
|---|---|---|
| | Phosphorylated | Non-phosphorylated |
| 0 | 74 | 78 |
| 20 | 68 | 42 |
| 60 | 62 | 28 |
| 120 | 63 (75) | 19 (72) |

Phosphorylated (phosphate incorporated: 0.18 mol/mol of PFK subunit) and non-phosphorylated PFK (1 mg/ml of buffer B each) were incubated with $\alpha$-chymotrypsin (50 μg/mg of PFK-protein) at 25°C. Aliquots were taken at the indicated times and assayed for phosphofructokinase activity. The values in parentheses are control activities of samples which were incubated without $\alpha$-chymotrypsin and assayed after 120 min

## 4. DISCUSSION

The present work demonstrates the phosphorylation of yeast phosphofructokinase both in vivo and in vitro. Thus, $^{32}$P-labelled material from extracts of yeast cells grown in the presence of [$^{32}$P]phosphate was recognized by antibodies raised against yeast PFK. The specificity of this reaction was further supported by the decrease in binding of radioactive material to the antibodies brought about by non-labelled PFK. Yeast PFK could also be phosphorylated in vitro by a protein fraction isolated from yeast extract. We have not yet characterized this PFK kinase activity in detail, but it could be resolved from cyclic AMP-dependent protein kinase, and cyclic AMP did not affect its activity when phosphofructokinase was used as substrate. Thus, the phosphorylation of phosphofructokinase seems to be regulated differently from that of fructose-1,6-bisphosphatase, the other enzyme of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle, whose phosphorylation is regulated by cyclic AMP both in vitro [3] and in vivo [13].

Interestingly, only the $\beta$-subunit of the two different subunits of PFK was phosphorylated by the isolated protein kinase. The maximum phosphorylation observed corresponded to 0.56 mol phosphate/mol of $\beta$-subunit. However, since purified yeast PFK already contains some bound phosphate (analysis according to [12], data not shown), the total extent of phosphorylation may be even higher. The substantial modification suggests a regulatory role of the phosphorylation, as has been demonstrated for other yeast enzymes [14,15]. The specific phosphorylation of the $\beta$-subunit which is considered to be the catalytic one [16,17] made the idea of a regulatory effect of the PFK phosphorylation even more attractive. However, we were unable to detect a significant change in catalytic or regulatory properties of yeast PFK as a consequence of the in vitro phosphorylation, so having the same situation as in the case of the mammalian enzyme. In contrast, PFK from *Ascaris suum* and *Fasciola hepatica* were activated by phosphorylation [18].

For fructose-1,6-bisphosphatase from yeast the phosphorylation initiated a proteolytic degradation [2,3,19]. In this respect the increased stability of phosphorylated yeast PFK during treatment with $\alpha$-chymotrypsin might be an indication for a regulatory mechanism to switch effectively from gluconeogenic to glycolytic growth triggered by simultaneous phosphorylation of fructose-1,6-bisphosphatase and phosphofructokinase.

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