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CYCLIC AMP AND FRUCTOSE-2,6-BISPHOSPHATE STIMULATED IN VITRO PHOSPHORYLATION OF YEAST FRUCTOSE-1,6-BISPHOSPHATASE

Gabriele Pohlig[1], Ruth Wingender-Drissen[2], Takashi Noda[1] and Helmut Holzer[1,3]

[1]Biochemisches Institut der Universität, D-7800 Freiburg im Breisgau (West Germany)

[2]Botanisches Institut der Universität Bonn, D-5300 Bonn 1 (West Germany)

[3]GSF-Abteilung für Enzymchemie, D-8042 Neuherberg bei München (West Germany)

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Summary: Phosphorylation of purified yeast fructose-1,6-bisphosphatase was studied using purified preparations from yeast of two different cyclic AMP-independent protein kinases and a cyclic AMP-dependent protein kinase. Incorporation of ^{32}P into fructose-1,6-bisphosphatase could be demonstrated only with the cyclic AMP-dependent protein kinase. Phosphorylation of fructose-1,6-bisphosphatase was stimulated by 3 µM fructose-2,6-bisphosphate and inhibited by 1 mM 5'-AMP.

Introduction: The addition of glucose to glucose-derepressed yeast cells causes a disappearance of 60% of Fru-P_2ase activity within 3 to 5 min (1). During this rapid inactivation, phosphorylation of serine residues of the enzyme was demonstrated in experiments with intact yeast cells (2,3). It was further shown that an increase in the intracellular concentration of cyclic AMP preceeds the phosphorylation (4). The inactivation of Fru-P_2ase was also observed in a cell free extract after the addition of ATP, Mg^{2+} and cyclic AMP (4,5). These findings strongly suggest an involvement of a cyclic AMP-dependent protein

Abbreviations: Fru-P_2ase, fructose-1,6-bisphosphatase; Fru-2,6-P_2, fructose-2,6-bisphosphate; SDS, sodium dodecyl sulfate; MES, 2-(N-morpholino) ethanesulfonic acid; TCA, trichloroacetic acid; Tris, Tris(hydroxymethyl)aminomethane

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kinase in the catabolite inactivation of Fru-P_2ase. We now show that highly purified cyclic AMP-dependent protein kinase from baker's yeast catalyzes the phosphorylation of yeast Fru-P_2ase when incubated with (γ-^{32}P)ATP, and that the phosphorylation is stimulated by Fru-2,6-P_2.

Materials and Methods: Enzymes: Glycogen phosphorylase kinase (6), casein kinase (7) and cyclic AMP-dependent protein kinase (8) were isolated from commercial baker's yeast (Deutsche Hefe Werke, Hamburg, FRG). Fru-P_2ase was purified from commercial baker's yeast (Pleser, Darmstadt, FRG) according to a modification[1] of the procedure described by Funayama et al. (9).
Phosphorylation assay: The measurement of incorporated phosphate was basically done according to Corbin and Reimann (10). After incubation at 25°C for various times, aliquots of 10 µl were taken from the reaction mixture, applied to Whatman GF/C filters (ø 2.5 cm) and immediately immersed into 10% TCA to stop the phosphorylation reaction. Further washing of the papers was performed in 5% TCA, 2 times, 20 minutes each. After another wash in water and acetone, filters were dried and radioactivity was checked in a Packard scintillation counter. The reaction mixture (total volume 80 µl) contained 10 µg of cyclic AMP-dependent protein kinase, 40 mM Mes pH 6.0, 12 mM $MgCl_2$, 0.1 mM (γ-^{32}P)ATP (3.6 x 10^6 cpm/nmol) with addition of 13 µg of Fru-P_2ase, 6 µM cyclic AMP, 1 mM 5'-AMP, 3 µM Fru-2,6-P_2, respectively,
Gel electrophoresis and autoradiography: Reaction mixtures were incubated for 30 minutes, boiled in SDS-solution and subjected to SDS-polyacrylamide slab gel electrophoresis as described by King and Laemmli (11). Gels were stained for protein with Coomassie brilliant blue R-250, dried and applied to autoradiography.
Materials: Whatman GF/C filters were purchased from Whatman (Springfield Mill, UK). X-ray films (XAR-5) were a product of Kodak (Rochester, USA). All chemicals were of analytical purity and obtained from Sigma (München, FRG) or Boehringer (Mannheim, FRG).

Results: A purified preparation of cyclic AMP-dependent protein kinase from baker's yeast (8) was incubated with purified Fru-P_2ase in the presence of (γ-^{32}P)ATP and Mg^{2+}. The radioactivity of trichloroacetic acid-insoluble ^{32}P was measured. Incubation of the protein kinase alone leads to some incorporation of ^{32}P (Table I), probably due to

[1] T. Noda, H. Hoffschulte and H. Holzer: manuscript in preparation

Table 1 Incorporation of ^{32}P from (γ-^{32}P)ATP into trichloroacetic acid-insoluble material

Incubation mixture	Incorporation (cpm)
Kinase alone	520
+ cAMP	613
+ Fru-P_2ase	470
+ Fru-P_2ase + cAMP	2100
+ Fru-P_2ase + cAMP + AMP	310
+ Fru-P_2ase + cAMP + Fru-2,6-P_2	3600
+ Fru-P_2ase + cAMP + Fru-2,6-P_2 + AMP	720

Assays of incorporated ^{32}P were performed as described in Materials and Methods. For concentrations of the additions indicated in the table see Materials and Methods. Values represent net incorporation after 6 min of incubation. Zero time values of 600 - 1000 cpm were subtracted from the observed incorporations.

autophosphorylation of the kinase. The addition of Fru-P_2ase does not increase the level of ^{32}P-incorporation, however, further addition of 6 µM cyclic AMP doubles the incorporation of ^{32}P. When 3 µM Fru-2,6-P_2 is added together with cyclic AMP, another significant increase of ^{32}P-incorporation is observed. The addition of 1 mM 5'-AMP completely prevents the increase of ^{32}P-incorporation by cyclic AMP and Fru-2,6-P_2. The inhibitory effect of 5'-AMP does not appear to be the result of competition with cyclic AMP because a 30-fold increase of cyclic AMP concentration in the reaction mixture failed to reverse the inhibitory action of 5'-AMP (data not shown). The levels of ^{32}P-incorporation in Table 1 include a significant background of autophosphorylation. Thus, as will be shown later in Fig. 2, the added Fru-P_2ase is in fact phosphorylated although the total incorporation does not increase with the addition of the enzyme itself.

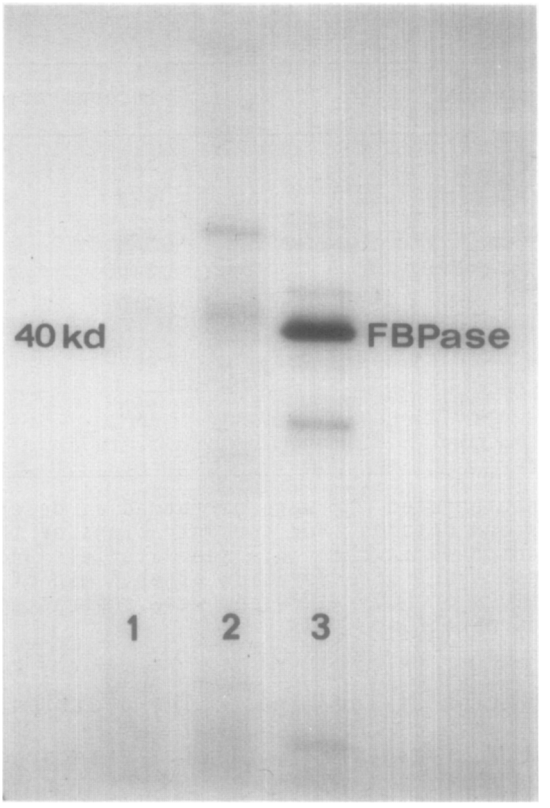

Fig. 1 Phosphorylation of Fru-P_2ase by yeast protein kinases.
Incubation of Fru-P_2ase was performed for 30 min with casein kinase (lane 1), phosphorylase kinase (lane 2) and cyclic AMP-dependent protein kinase (lane 3), all purified from baker's yeast. Reaction mixtures (total volume 120 µl) contained 40 mM Tris-Cl pH 7.5, 25 µg Fru-P_2ase, 0.1 mM (γ-^{32}P)ATP (3.6 x 10^6 cpm/nmol) in addition to 5 mM $MgCl_2$ and 1 µM cyclic AMP for cyclic AMP-dependent protein kinase, 5 mM $MgCl_2$ for casein kinase, and 20 mM $MgCl_2$ for phosphorylase kinase. The protein kinases were added in 100-fold concentration as compared to the concentration in the respective standard assays (6,7,8). Electrophoresis and autoradiography were carried out as described in Materials and Methods.

We next examined if Fru-P_2ase could also be phosphorylated by incubation with cyclic AMP-independent yeast glycogen phosphorylase kinase (6) or yeast casein kinase (7). No phosphorylation could be demonstrated by either of these cyclic AMP-independent protein kinases (cf. lanes 1 and 2 in Fig. 1) even though we used 100 times the amount of enzymes that is sufficient to phosphorylate their respective substrates.

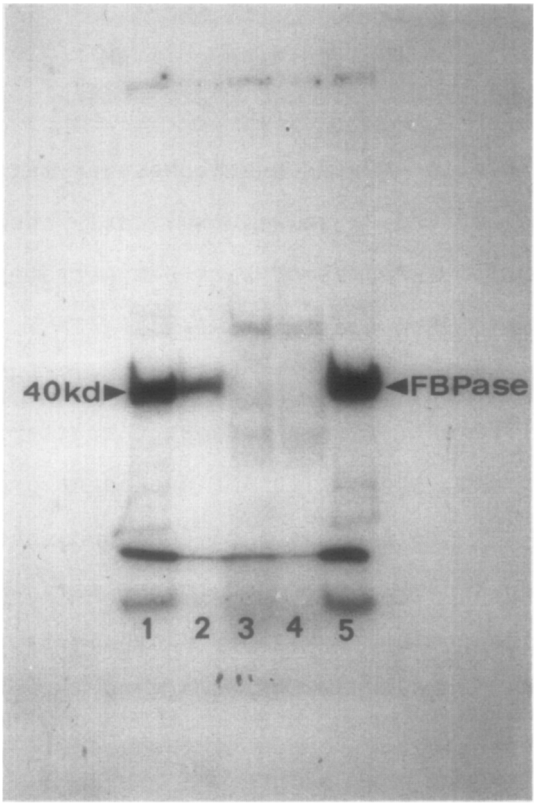

Fig. 2 ^{32}P-incorporation into Fru-P_2ase by cyclic AMP-dependent protein kinase from yeast. Incubation mixtures with cyclic AMP-dependent protein kinase, (γ-^{32}P)ATP and $MgCl_2$ were performed as described in Materials and Methods. Lane 3 and 4 show incubations of the kinase alone in the presence (lane 3) and absence (lane 4) of cyclic AMP. Lane 1 and 2 show incubations with added Fru-P_2ase in the presence (lane 1) and absence (lane 2) of cyclic AMP. Lane 5 shows the incubation of Fru-P_2ase in the presence of cyclic AMP and Fru-2,6-P_2. Electrophoresis and autoradiography were carried out as described in Materials and Methods

Aliquots of the incubation mixtures with cyclic AMP-dependent protein kinase (cf. Table 1), were submitted to SDS-slab gel electrophoresis and autoradiography. As shown in Fig. 2 incubation of the kinase alone gave several faint bands of ^{32}P-incorporation (lanes 3 and 4 in Fig. 2) whereas the incubation with Fru-P_2ase gave rise to an appearance of a distinct band at 40 kd (lane 2 in Fig. 2). The labelling was significantly increased

by the addition of 6 μM cylic AMP (lane 1 in Fig. 2) and further increased by the addition of 3 μM Fru-2,6-P_2 (lane 5 in Fig. 2).

Discussion: Cyclic AMP-dependent phosphorylation of serine residues of Fru-P_2ase was previously shown in intact yeast cells (2,3). The present work demonstrates that the phosphorylation of Fru-P_2ase with (γ-^{32}P)ATP is catalyzed by cyclic AMP-dependent protein kinase purified from yeast. Among three different protein kinases characterized in yeast (6,7,8), only the cyclic AMP-dependent protein kinase, which was isolated as a glycogen phosphorylase phosphorylating kinase, was found to be active against Fru-P_2ase. Evidence for regulatory phosphorylation of other yeast enzymes has been presented for glycogen phosphorylase (12,13), glycogen synthetase (14), NAD-dependent glutamate dehydrogenase (15) and trehalase (16). We found that Fru-2,6-P_2 stimulates phosphorylation of Fru-P_2ase by cyclic AMP-dependent protein kinase. The concentrations of Fru-2,6-P_2 (3 μM) and cyclic AMP (6 μM) used in the present experiments correspond to the concentrations of these effectors found in yeast cells immediately after the addition of glucose (17,4). A similar effect of Fru-2,6-P_2 on phosphorylation of yeast Fru-P_2ase catalyzed by a commercially available protein kinase from bovine heart muscle was reported by M.J. Mazón, J.M. Gancedo and C. Gancedo (7th International Symposium on Metabolic Interconversion of Enzymes, May 16-18, 1983, Avila, Spain).

Fru-P_2ase from S. cerevisiae exhibits a molecular weight of 115.000 consisting of two 56 kd subunits (9). Similar results have been obtained with purified Fru-P_2ase

from baker's yeast in this laboratory[1]. When highly purified Fru-P_2ase or immunoprecipitates of Fru-P_2ase are submitted to SDS-polyacrylamide gel electrophoresis, the bulk of the protein appears at 40 kd[1]. Most probably SDS-activated limited proteolysis by traces of proteinases firmly bound to Fru-P_2ase degrades the native 56 kd subunits to 40 kd material[1]. This explains why the labelling with ^{32}P shown in Figs. 1 and 2 is observed at 40 kd.

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