Phosphorylated Fructose-1,6-bisphosphatase Dephosphorylating Protein Phosphatase from Saccharomyces cerevisiae

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Phosphorylation of fructose-1,6-bisphosphatase with cyclic AMP-dependent protein kinase from yeast is accompanied by a 50% decrease in the catalytic activity (Pohlig, G. and Holzer, H. (1985) J. Biol. Chem. 260, 13818–13823). Using reactivation of phosphorylated fructose-1,6-bisphosphatase as assay, a protein phosphatase was about 2,000-fold purified to electrophoretic homogeneity from Saccharomyces cerevisiae. Upon incubation with phosphorylated fructose-1,6-bisphosphatase the purified protein phosphatase not only reverses the 50% inactivation caused by phosphorylation, but also the previously observed change in the pH optimum and in the ratio of activity with Mg²⁺ or Mn²⁺. The phosphatase is strongly inhibited by heparin and fluoride. L-Carnitine, orthophosphate, pyrophosphate, and succinate inhibit to 50% at concentrations from 1 to 10 mM. The molecular mass of the native phosphatase was found to be 180,000 Da. Sodium dodecyl sulfate-gel electrophoresis suggested four subunits with a molecular mass of 45,000 Da each. Half-maximal activity was observed with 5 mM Mg²⁺ or Mn²⁺, the pH optimum of activity was found at pH 7. Using polyclonal antibodies, disappearance of [³²P]labeled fructose-1,6-bisphosphatase and concomitant liberation of the expected amount of inorganic [³²P]phosphate was demonstrated.

After addition of fermentable sugars or of ionophores to starved yeast cells, serine residues of fructose-1,6-bisphosphatase are rapidly phosphorylated (1, 2). Fructose-1,6-bisphosphatase consists of four, probably identical subunits (3). Incorporation of about 0.7 mol of [³²P]phosphate from [γ-³²P]ATP/mol of subunit of fructose-1,6-bisphosphatase was observed (4). The phosphorylated enzyme is therefore designated phosphorylated fructose-1,6-bisphosphatase. Cyclic AMP-dependent, fructose-2,6-bisphosphate stimulated phosphorylation of fructose-1,6-bisphosphatase according to the equation: fructose-1,6-bisphosphatase + 4ATP → phosphorylated fructose-1,6-bisphosphatase + 4ADP has been demonstrated in vitro with purified enzymes from yeast (4). In intact yeast cells, liberation of [³²P]fructose-1,6-bisphosphatase dephosphorylating protein phosphatase was postulated (1, 2, 5). The observation that the phosphorylated fructose-1,6-bisphosphatase exhibits only about 50% of the catalytic activity of the nonphosphorylated enzyme (1, 2, 4, 6) inspired us to develop an assay for phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase based on the measurement of the increase in catalytic activity which was expected to accompany the reaction: phosphorylated fructose-1,6-bisphosphatase → fructose-1,6-bisphosphatase + 4 orthophosphate. In the present paper such an assay and its application for the elaboration of a purification procedure and for characterization of phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase from Saccharomyces cerevisiae are described.

Materials and Methods

Results

Using "assay 1" (cf. "Materials and Methods") phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase was purified from S. cerevisiae M1 in a six-step procedure as summarized in Table I. An about 2000-fold purification with a yield of 2% was obtained. SDS-polyacrylamide gel electrophoresis of the crude extract and of aliquots from the steps 4, 5, and 6 of the purification procedure is shown in Fig. 3. The electrophoretically homogenous enzyme resulting from step 6 was applied in its native form to gel filtration on Sephacryl S-300. With the assumption of the same relationship between Stokes radii and molecular weight for the standards and phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase, a molecular mass of 180,000 Da was calculated (Fig. 4). From SDS-polyacrylamide gel electrophoresis, a molecular mass of 45,000 Da was calculated. The native enzyme is therefore very probably a tetramer of four subunits with identical molecular mass.

When incubated at 50 °C in 10 min 50% of the activity of

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†The designation of phosphorylated fructose-1,6-bisphosphatase follows the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (J. Biol. Chem. (1977) 252, 5938–5941).
Yeast Fructose-1,6-bisphosphatase Dephosphorylating Phosphatase

Table I

Purification of phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase from S. cerevisiae strain M1

| Step                  | Volume (ml) | Total activity (units) | Specific activity (units/mg) | Purification fold | Yield (%) |
|-----------------------|-------------|------------------------|-----------------------------|-------------------|-----------|
| 1. Crude extract      | 120         | 1.25                   | 0.00019                     | 1                 | 100       |
| 2. High speed supernatant | 109       | 1.14                   | 0.00038                     | 2                 | 91        |
| 3. Ammonium sulfate   | 154         | 0.87                   | 0.00076                     | 4                 | 70        |
| 4. DEAE-Sepharose chromatography | 27      | 0.61                   | 0.0003                     | 16                | 49        |
| 5. Phenyl-Sepharose chromatography | 12      | 0.21                   | 0.019                       | 100               | 17        |
| 6. Heparin-Sepharose chromatography | 2.4   | 0.019                  | 0.43                        | 2260              | 2         |

Fig. 3. SDS-polyacrylamide gel electrophoresis of phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase. Samples obtained after different purification steps (see Table I) were solubilized and subjected to SDS-polyacrylamide gel electrophoresis (see "Materials and Methods"). Lane 1, marker proteins: phosphorylase b (Mr = 94,000), bovine serum albumin (Mr = 67,000), ovalbumin (Mr = 43,000), carbonic anhydrase (Mr = 30,000), trypsin inhibitor (Mr = 20,000). Lane 2, 50 µg of protein from crude extract. Lane 3, 50 µg of protein after DEAE-Sepharose chromatography (step 4). Lane 4, 10 µg of protein after phenyl-Sepharose chromatography (step 5). Lane 5, 10 µg of phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase after heparin-Sepharose chromatography (step 6).

The purified enzyme disappear. The presence of 25 mM MgCl₂ somewhat stabilizes the enzyme: 50% inactivation in 10 min is now observed at 55 °C.

Mg²⁺ is a necessary cofactor for the phosphatase reaction. Half-maximal activity was observed at about 5 mM MgCl₂ (Fig. 5). Half-maximal activation of the enzyme with Mn²⁺ leads to only about two-thirds of the activity of the maximal Mg²⁺-activated enzyme. Half-maximal activity was observed with 5 mM MnCl₂.

Dependence of phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase activity on the pH is shown in Fig. 6. Maximal activity was observed at pH 7.0.

To demonstrate dephosphorylation of phosphorylated fructose-1,6-bisphosphatase with the phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase contained in crude extract, ³²P-labeled phosphorylated fructose-1,6-bisphosphatase was prepared as substrate from yeast cells preincubated with [³²P]orthophosphate and then treated for 5 min with glucose as described previously (1). Immuno-precipitates with antibodies against fructose-1,6-bisphospha-
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Fig. 6. Dependence of phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase (FBPase(4P)ase) (activity) on pH. The reaction mixture contained in a final volume of 1.5 ml: 50 mM Hepes, 50 mM Mes, 50 mM Acet adjusted with NaOH to the indicated pH values, 10 mM MgCl₂, 0.6 units fructose isomerase, 0.6 units of glucose-6-phosphate dehydrogenase, 0.2 mM fructose-1,6-bisphosphatase, and 5 milliunits of phosphorylated fructose-1,6-bisphosphatase. An aliquot of 150 μl was removed and immunoprecipitated as described under "Materials and Methods." Units are recalculated according to assay 2 and given on the ordinate per ml of the phosphorylated fructose-1,6-bisphosphatase preparation used.

Fig. 7. Dephosphorylation of ³²P-labeled phosphorylated fructose-1,6-bisphosphatase. A crude extract, containing FBPase(4P)ase was prepared from yeast cells grown with KH₂³²PO₄ (see "Materials and Methods") and then treated for 10 min with glucose as described previously (1). The Sephadex G-25 filtrated crude extract, containing 0.45 unit/ml phosphorylated fructose-1,6-bisphosphatase was incubated in 50 mM imidazole/HCl, pH 7.0, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM benzamidine chloride with or without 25 mM MgCl₂ at 30 °C. Aliquots of 150 μl were removed and immunoprecipitated as described under "Materials and Methods." After SDS-polyacrylamide gel electrophoresis of the immunoprecipitated material, autoradiography was done as described under "Materials and Methods." Lanes 1, 3, 4, and 5, FBPase(4P)ase incubated at 30 °C 60 min without MgCl₂, for 0, 15, 30, and 60 min, respectively. Lane 1, fructose-1,6-bisphosphatase ³²P-labeled at 30 °C 60 min without MgCl₂. Lane 6, ³C-methylated marker proteins: phosphorylase b (M₀ = 92,500), bovine serum albumin (M₀ = 69,000), ovalbumin (M₀ = 46,000), carbonic anhydrase (M₀ = 30,000).

Parallelism of the time course of liberation of [³²P]orthophosphate and of activation of phosphorylated fructose-1,6-bisphosphatase as determined in the standard assay is shown in Fig. 8.

The phosphorylated and the nonphosphorylated forms of fructose-1,6-bisphosphatase exhibit characteristic differences in the pH optimum (5, 7) and in the dependence of activity on Mg²⁺ or Mn²⁺ (4, 7). When phosphorylated fructose-1,6-bisphosphatase is incubated with purified phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase in the presence of Mg²⁺, R(Mg/Mn) increases from 1.24 to 1.75 and Q(7/9) increases from 0.41 to 0.64 (Table II). With preparations of unphosphorylated fructose-1,6-bisphosphatase, R(Mg/Mn) = 2.6 and Q(7/9) = 0.62 had been obtained (7). Concentrations of inhibitors necessary for 50% inhibition of phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase activity are depicted in Table III. The inhibitors listed have also been found to inhibit protein phosphatases from mammalian tissues (11). In contrast to the inhibitory action on mammalian phosphatases no inhibitory effect on the yeast phosphatase was detected with spermine (10 mM), spermidine (10 mM), and poly-L-lysine (10 μM) (data not shown). Also a boiled extract from yeast did not inhibit. Incubation of the boiled yeast extract with ATP, Mg²⁺, and cyclic AMP in the presence of protein kinase from beef heart did not produce inhibitory activity. Evidence for a heat stable, proteinaceous inhibitor of yeast phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase similar to the one found in mammalian tissues (11) was therefore not obtained.

Incubation with a cyclic AMP-dependent protein kinase
The indicated times, samples were assayed for fructose-1,6-bisphosphatase dephosphorylated trehalase from yeast (1.4 units/ml) (data not shown). The incubation mixture contained in a final volume of 2.2 ml, 99 milliliters of FBPase(4P), 50 mM imidazole/HCl, pH 7.0, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM diithiothreitol, and 25 mM MgCl2. FBPase(4P) was partially purified (see "Materials and Methods") from 32P-labeled yeast (see legend to Fig. 6). Incubation at 30°C was started by addition of 0.8 milliunits of phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase and at the indicated times, samples were assayed for fructose-1,6-bisphosphatase activity and counted for 32P radioactivity as described under "Materials and Methods."

**TABLE II**

| Ratio of activity with Mg²⁺ or Mn²⁺, R(Mg/Mn), and quotient of activity at pH 7.0 or 9.0, Q(7/9), of phosphorylated fructose-1,6-bisphosphatase after incubation with and without phosphorylated 1,6-bisphosphatase dephosphorylating protein phosphatase |  |
|---|---|
| The incubation mixture (total volume 120 μl) contained 27 milliunits of phosphorylated fructose-1,6-bisphosphatase (FBPase(4P)) in 50 mM imidazole/HCl, pH 7.0, 25 mM MgCl₂, 0.5 mM diithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Incubation was started with addition of 0.3 milliunits of phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase or incubation buffer, respectively. After 60 min at 30°C, fructose-1,6-bisphosphatase activity was measured in the presence of 10 mM Mg²⁺ or 2 mM Mn²⁺ and at pH 7.0 or 9.0 according to Noda et al. (6). |  |

![Figure 8. Liberation of [32P]orthophosphate from FBPase(4P) parallel to the activation of the FBPase(4P).](image)

**TABLE III**

| Inhibition of phosphorylated fructose-1,6-bisphosphatase protein phosphatase activity |  |
|---|---|
| Phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase was incubated with the inhibitors for 10 min in the mixture of assay 1 (cf. "Materials and Methods"). In contrast to the routine start with fructose-1,6-bisphosphatase the assay was started with phosphorylated fructose-1,6-bisphosphatase. |  |

**DISCUSSION**

During studies on phosphorylation/dephosphorylation of proteins in yeast, evidence was obtained for enzymes dephosphorylating phosphorylated glycogen phosphorylase (12), glycolen synthase (13, 14), trehalase (15), NAD-glutamate dehydrogenase (16), and fructose-1,6-bisphosphatase (1, 2). Partially purified and characterized were the protein phosphatases which dephosphorylate NAD-glutamate dehydrogenase in Candida utilis (16) and glycogen phosphorylase in baker’s yeast (17). The fructose-1,6-bisphosphatase dephosphorylating enzyme from Saccharomyces cerevisiae described in the present paper is probably different from these two previously characterized protein phosphatases from the following reasons. The NAD-glutamate dehydrogenase dephosphorylating activity elutes from DEAE-ion exchange columns at 0.2-0.35 M KCl (16), whereas the protein phosphatase described here elutes at 0.1 M NaCl. The glycogen phosphorylase dephosphorylating activity elutes at 0.35 M KCl (17) and is therefore also very probably not identical with our enzyme eluting at 0.1 M NaCl. Furthermore, the seervalfold activation of glycogen phosphorylase by incubation with ATP and Mg²⁺ observed with the glycogen phosphorylase phosphatase (17) is not seen with the phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase described here. Under conditions where 4 μg of phosphorylated fructose-1,6-bisphosphatase are dephosphorylated in 1 min, the purified phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase does not dephosphorylate, i.e., inactivate, 2 μg of phosphorylated trehalase purified from baker’s yeast (17).

From the phosphatase activity of the crude extract shown in Table I it is calculated that 1.6 μg of phosphorylated fructose-1,6-bisphosphatase can be dephosphorylated per min and per g, wet weight, of yeast. With a content of 48 μg of fructose-1,6-bisphosphatase/g, wet weight (6), dephosphorylation of the total amount of fructose-1,6-bisphosphatase in yeast could take place in 48:1.6 = 30 min. In vivo the dephosphorylation of fructose-1,6-bisphosphatase takes about 120 min after transfer of glucose-treated yeast to a glucose-free acetate containing medium (1, 2, 7). An explanation for the discrepancy of the time calculated for the dephosphorylation in vitro and in vivo may be that in intact cells the conditions for dephosphorylation of phosphorylated fructose-1,6-bisphosphatase are not as optimal as they are in the in vitro assay. Furthermore, in vivo phosphorylated fructose-1,6-bisphosphatase dephosphorylating protein phosphatase may be inhibited under the conditions where dephosphorylation is observed, i.e., at incubation of glucose-treated cells in a glucose-free, acetate containing medium (7). The presence of inhibiting metabolites, such as orthophosphate, pyrophosphate, succinate (cf. Table III), or of inhibiting macromole-

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4 A. Manhart and A. Noshiro, unpublished data.

5 H. App, unpublished data.
cules, as well as the absence of activating ions, such as free
Mg\(^{2+}\), may be considered among other possibilities in this
context.

Acknowledgments—We are grateful to Dr. Bernd Mechler, Dr. Matthias Müller, and Prof. Dr. Dieter H. Wolf for many helpful
discussions. We thank Dr. Matthias Müller for critical reading of
the manuscript and Wolfgang Fritz and Ulrike Kopas for help with the
figures and for typing the manuscript.

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Yeast Fructose-1,6-bisphosphatase Dephosphorylating Phosphatase

Fructose-1,6-bisphosphatase(4P) Dephosphorylating Protein-Phosphatase

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Materials and Methods

Chemicals - All chemicals were purchased from Sigma Chemical Company (St. Louis, MO). E. coli, E. coli (Lythgoe, USA), DEAE-Sephrose, phenyl-Sepharose and heparin-Sepharose were obtained from Pharmacia. All other, chemicals were of the highest grade available.

Preparation of Enzyme - Yeast cells were disrupted by ultrasonic treatment (Misonix Sonicator 3000) as previously described (4). The resulting cell-free extract was prepared and stored at -20°C as described by Müller. The enzyme was obtained by centrifugation at 27,000 rpm for 10 min. The supernatant was diluted to 150 ml with buffer A for 12 hours. The protein was removed by centrifugation at 27,000 rpm for 10 min. The supernatant was diluted to 150 ml with buffer A.

Fru-2,6-P2-Sepharose - The fructose-2,6-bisphosphatase-Sepharose was immobilized by applying a linear decreasing gradient of NaCl in buffer A from 1 M to 0.1 M. The fractions of highest activity were pooled and dialysed against buffer A.

Phenyl-Sepharose Chromatography - The phenyl-Sepharose column was eluted with a 1 M phosphate buffer (pH 7.5) containing 0.5 M NaCl.

Immunoglobulin - Immunoglobulin was prepared by immunizing rabbits with the enzyme.

Definition of units - One unit of FBPase is defined as the amount of enzyme that catalyses the dephosphorylation of 1 pmol of fructose-1,6-bisphosphate per min. (0.1 pmol of fructose-1,6-bisphosphate per min). Because FBPase activity is subject to regulation by a variety of factors, the specific activity of FBPase (ml units/mg protein) is used to express the activity of FBPase. One unit of FBPase activity is defined as the amount of enzyme that catalyses the dephosphorylation of 1 pmol of fructose-1,6-bisphosphate per min. (0.1 pmol of fructose-1,6-bisphosphate per min).

Purification of FBPase - All operations were performed at 4°C.

Purification of FBPase by affinity chromatography - The affinity column was prepared by immobilizing the enzyme on Sepharose-4B. The enzyme was purified by affinity chromatography on a 1 ml column of Sepharose-4B. The enzyme was eluted with a 1 M phosphate buffer (pH 7.5) containing 0.5 M NaCl.

Sedimentation and ultracentrifugation - Sedimentation and ultracentrifugation were performed as described by Müller. The enzyme was purified by ultracentrifugation at 200,000 g for 12 hours. The supernatant was diluted to 150 ml with buffer A.

Purification of FBPase - All operations were performed at 4°C.

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