Fructose 2,6-Bisphosphate Activates the cAMP-dependent Phosphorylation of Yeast Fructose-1,6-bisphosphatase in Vitro*

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Fructose-1,6-bisphosphatase purified from Saccharomyces cerevisiae is phosphorylated in vitro by a cAMP-dependent protein kinase. The phosphorylation reaction incorporates 1 mol of phosphate/mol of enzyme and is greatly stimulated by fructose 2,6-bisphosphate. Fructose 2,6-bisphosphate acts upon fructose-1,6-bisphosphatase, not on the protein kinase. The phosphorylation of fructose 1,6-bisphosphatase lowers its activity by about 50%. The characteristics of the phosphorylation reaction in vitro show that this modification is responsible for the inactivation of fructose-1,6-bisphosphatase observed in vivo.

Addition of glucose to a culture of Saccharomyces cerevisiae grown on a gluconeogenic carbon source triggers an activation of the gluconeogenic enzyme fructose 1,6-bisphosphatase (1, 2). In certain conditions, within 1 to 3 min after glucose addition, about 50% of the fructose-1,6-bisphosphatase activity is lost (3) and a concomitant phosphorylation of the enzyme is observed (4-6). Although the temporal correlation between inactivation and phosphorylation of fructose-1,6-bisphosphatase suggests that the phosphorylation is the underlying cause of the inactivation, this might not hold true. Indeed, there are cases in which phosphorylation and inactivation of one enzyme occur simultaneously without an obvious role for phosphorylation in the inactivation phenomenon (7).

In this communication, we show that the in vitro phosphorylation of purified yeast fructose-1,6-bisphosphatase by a cAMP-dependent protein kinase leads to a decrease of the activity of the enzyme. This phosphorylation is greatly stimulated by fructose 2,6-bisphosphate.

EXPERIMENTAL PROCEDURES

Materials—Auxiliary enzymes were from Sigma. Material for electrophoresis was from Bio-Rad. [γ-32P]ATP was from The Radiochemical Centre (Amersham, Great Britain). Bovine heart cAMP-dependent protein kinase and protein kinase inhibitor were from Sigma. Fructose 2,6-bisphosphate, prepared as described by Van Schaftingen and Hers (8), was a gift from these workers (Université Catholique de Louvain, Belgium). Fructose-1,6-bisphosphatase was purified as described by Ponarama et al. (9) by A. Alcina in this laboratory.

Assay of Fructose-1,6-bisphosphatase Activity—The enzyme was assayed spectrophotometrically as previously described (10).

Inactivation of Fructose-1,6-bisphosphatase—The inactivation mixture contained in a final volume of 0.2 ml: 50 mM potassium phosphate, pH 7, 10 mM magnesium sulfate, 5 mM dithiothreitol, 20 μM cAMP, 50 μM [γ-32P]ATP, 1 μM fructose 2,6-bisphosphate, 100 millimiliters of fructose-1,6-bisphosphatase, and 0.1 millimicron of cAMP-dependent protein kinase. The mixture was incubated at 30 °C. At appropriate time intervals, samples were taken and assayed for fructose-1,6-bisphosphatase as described.

Phosphorylation Assays—The incubation mixture was described for the inactivation but [γ-32P]ATP was used with a specific activity of 500 μCi/μmol. The assay was started by the addition of protein kinase. At appropriate times after incubation at 30 °C, 10-μl samples were taken for assay of enzymatic activity and 50-μl samples for assay of phosphorylation. The latter samples were rapidly mixed with 50 μl of NaDodSO4 buffer (5% NaDodSO4, 0.1 M Tris-HCl, pH 6.8, 20% glycerol, 10% mercaptoethanol, 0.002% bromphenol blue) and immediately boiled for 7 min. The samples were subjected to NaDodSO4-gel electrophoresis or slab polyacrylamide gels (11). The gels were stained for protein with Coomassie brilliant blue R-250, destained, dried, and exposed to X-Omat S-5 x-ray film for 15 h. The incorporation of 32P into fructose-1,6-bisphosphatase was measured by counting the corresponding dry gel slice in a liquid scintillation counter.

RESULTS

Incubation of purified fructose-1,6-bisphosphatase with a cAMP-dependent protein kinase in the presence of fructose 2,6-bisphosphate resulted in a loss of activity of fructose-1,6-bisphosphatase (Table I). Addition of protein kinase inhibitor produced an inhibition of the inactivation reaction. Although the rate of inactivation was very low if fructose 2,6-bisphosphate was omitted, this compound is not strictly required. Inactivation could be observed in the absence of fructose 2,6-bisphosphate using a 5 times higher ratio of protein kinase to fructose-1,6-bisphosphatase. In these conditions, 50% inactivation is reached in 10 min in the presence of 1 μM fructose 2,6-bisphosphate, whereas in its absence, 2 h are required for the same degree of inactivation. Maximal rate of inactivation was observed at about 5 μM fructose 2,6-bisphosphate, the half-maximal rate being reached at 1 μM. Fructose 2,6-bisphosphate appears to act upon fructose-1,6-bisphosphatase since the phosphorylating activity of the protein kinase upon histones remained unaffected by this compound. Inactivation did not exceed 50 to 60% even with longer periods of incubation (up to 3 hr).

In samples taken before and after inactivation, fructose-1,6-bisphosphatase was assayed at 2 mM Mn2+ and at 2 mM Mg2+ (12). The quotient of the activities measured in the two conditions increased from 0.6 to 1.3 in the course of the inactivation.

When the incubation was performed in the presence of [γ-32P]ATP, an incorporation of 32P in the fructose-1,6-bisphosphatase was observed (Fig. 1A). Only a slight incorporation was detectable in the absence of fructose 2,6-bisphosphatase. The incorporation of 32P can be correlated with the loss of activity (Fig. 1B). From the radioactivity incorporated in the

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1 The abbreviation used is: NaDodSO4, sodium dodecyl sulfate.
fructose-1,6-bisphosphatase is phosphorylated in vitro by a cAMP-dependent protein kinase. This phosphorylation brings about a partial inactivation of the enzyme. The effects produced in vitro by the phosphorylation are similar to those produced in vivo by the addition of glucose to a yeast culture, namely about a 50% decrease in activity (3) and a 3-fold increase in the stoichiometry of the enzyme assayed with 2 mM Mn⁴⁺ and 2 mM Mg²⁺ (12). In addition, the stoichiometry of the phosphorylation is the same in vivo (5) and in vitro. These facts unambiguously show that phosphorylation is responsible for the inactivation observed in vivo.

There have been recently two independent reports describing inactivation in vitro of yeast fructose-1,6-bisphosphatase using crude extracts (13, 14); however, no experiments on phosphorylation were reported. We could not achieve inactivation in crude extracts of our yeast strain. It cannot be excluded that in one case (14) the reported inactivation was simply an inhibition of fructose-1,6-bisphosphatase by AMP produced in the incubation mixture. The discrepancy between our results and those of the other report (13) could be due to differences in the strain or growth conditions.

The fact that fructose-2,6-bisphosphate activates the phosphorylation of fructose-1,6-bisphosphatase in vitro has its counterpart in the in vivo inactivation. Addition of glucose to a yeast culture produces an immediate sharp increase of the levels of fructose 2,6-bisphosphate, reaching concentrations of about 2 μM in 1 min(15). The concentration needed in vitro for activation of the phosphorylation reaction is, therefore, well within the physiological range of concentration of this metabolite. Also, cAMP increases in vivo very rapidly upon addition of glucose (14, 16, 17). Moreover, other treatments that cause inactivation of this enzyme in vivo produce an increase in cAMP and fructose 2,6-bisphosphate concentration. Therefore, it may be concluded that in vivo the inactivation of fructose-1,6-bisphosphatase is the consequence of a phosphorylation which is triggered by the simultaneous increase of cAMP and fructose 2,6-bisphosphate. The rise in cAMP would activate a cAMP-dependent protein kinase, whereas that of fructose 2,6-bisphosphate would make fructose-1,6-bisphosphatase a better substrate for phosphorylation.

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The results presented in this work demonstrate that yeast
Fructose 2,6-bisphosphate activates the cAMP-dependent phosphorylation of yeast fructose-1,6-bisphosphatase in vitro.
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