*Biochimica et Biophysica Acta*, 972 (1988) 353–356
Elsevier



BBA 10225

**BBA Report**

# Occurrence of two phosphorylated forms of yeast fructose-1,6-bisphosphatase with different isoelectric points

Nedda Burlini, Patrizia Facheris, Paolo Tortora and Andrea Guerritore

*Dipartimento di Fisiologia e Biochimica Generali, Università di Milano, Milano (Italy)*

(Received 31 July 1988)

Key words: Fructose-1,6-bisphosphatase; Protein phosphorylation; Protein catabolism; (Yeast)

**Yeast fructose-1,6-bisphosphatase (EC 3.1.3.11) immunoprecipitated from glucose-derepressed wild-type cells and subjected to isoelectric focusing, appears as a unique peak, essentially homogeneous and devoid of incorporated phosphate. However, after cell incubation with glucose, two phosphorylated forms are detectable. The isoelectric point of one is higher and of the other is lower than that of the native form. In contrast, in the mutant ABYS1 which is deficient in several vacuolar proteinases (Achstetter, T., Emter, O., Ehmann, C. and Wolf, D.H. (1984) J. Biol. Chem. 259, 13334–13343), only the more acidic phospho form appears after cell incubation with glucose. However, sequence data rule out the possibility that limited proteolysis is the event responsible for the appearance of the more basic form of the phosphoenzyme. Nevertheless, time courses of glucose-induced inactivation of fructose-1,6-bisphosphatase show that the enzyme undergoes a substantially slower inactivation in the ABYS1 mutant as compared to the wild-type. These findings point to a degradative mechanism involving, besides the well-known phosphorylation, an additional as yet unknown modification which probably sensitizes the enzyme to proteolytic attack; furthermore, the enzyme responsible for such a modification seems to require one or more of the vacuolar proteinases missing in the mutant for its maturation.**

Glucose addition to yeast cells adapted to a sugar-free medium triggers inactivation of several enzymes [1]. The inactivation mechanism of fructose-1,6-bisphosphatase has been largely elucidated, and consists of a rapid phosphorylation [2,3] followed by a much slower proteolytic breakdown [4,5]. Enzyme phosphorylation is catalyzed by a cAMP-dependent protein kinase [6,7], which, in turn, is activated by a glucose-induced cAMP increase [8,9]; furthermore, the phosphorylative event represents an indispensable prerequisite for enzyme proteolysis, although other effectors evoked by glucose are required at the same time to trigger the degradative process [10].

The experiments described in this paper, were carried out in order to check the possible involvement of further modifications of the enzyme in the degradative process. To this end, fructose-1,6-bisphosphatase was immunoprecipitated from yeast cells before and after glucose-triggered phosphorylation, and analyzed by isoelectric focusing. For such experiments, two strains of *Saccharomyces cerevisiae* were employed: the diploid wild-type strain M1 (kindly supplied by Professor H. Holzer, University of Freiburg, F.R.G.) and the haploid

Abbreviations: Mes, 4-morpholineethanesulfonic acid; SDS, sodium dodecyl sulfate.

Correspondence: P. Tortora, Dipartimento di Fisiologia e Biochimica Generali, Università di Milano, Via Celoria 26, I-20133 Milano, Italy.

mutant ABYS1 [11], deficient of the four vacuolar proteolytic enzymes proteinase yscA, proteinase yscB, carboxypeptidase yscY and carboxypeptidase yscS (kindly supplied by Professor D.H. Wolf, University of Freiburg, F.R.G.). Yeasts were grown to the stationary phase in a low-$P_i$ YEPD medium [12] in the presence of [$^{32}$P]$P_i$ (50 $\mu$Ci/ml) and of [2,5-$^3$H]histidine (40 $\mu$Ci/ml). When using the strain ABYS1, adenine (0.5 mg/ml) was also added. At the end of growth, cells were collected, resuspended in 0.1 M potassium Mes buffer (pH 6.0) and incubated with 0.1 M glucose as previously reported [13]. At the indicated incubation times, cell samples were withdrawn, disrupted and centrifuged; quantitative immunoprecipitation of fructose-1,6-bisphosphatase was carried out on the supernatants, and the immunoprecipitates were subjected to SDS-gel electrophoresis. The whole procedure was performed as described [10]. The gel lanes were immediately cut into 2-mm slices and the radioactivity was extracted from each slice by grinding it in a Potter homogenizer in the presence of 1.2 ml of 7 mM Tris-HCl (pH 8.0). After removal of gel debris by centrifugation, suitable aliquots of each supernatant were counted. The enzyme-containing supernatant was then concentrated in a Centricon-30 microconcentrator (Amicon, Danvers, MA) and subjected to isoelectric focusing according to O'Farrell [14], using a mixture of 1% Bio-Lyte 4/6, 1% Bio-Lyte 6/8 (Bio-Rad, Richmond, VA) as ampholytes. The gel rod was cut into 3-mm slices, which were extracted with 0.7 ml of NCS tissue solubilizer from Amersham International (Amersham, U.K.) containing 10% (v/v) water and counted in a two-channel liquid scintillation counter for separate estimate of $^3$H and $^{32}$P.

Under these conditions, a unique, essentially homogeneous enzyme peak was extracted from wild-type cells before incubation with glucose (Fig. 1A); furthermore, the peak was devoid of any incorporated phosphate as expected, and displayed an isoelectric point of 5.8, which is fairly close to the previously reported value of 5.5 [15]. By contrast, two phosphorylated peaks of comparable size and ratio $^{32}$P/$^3$H were obtained from yeast cells incubated for 3 or for 20 min with glucose (Fig. 1B and C); in addition, a residual amount of the unphosphorylated form repre-

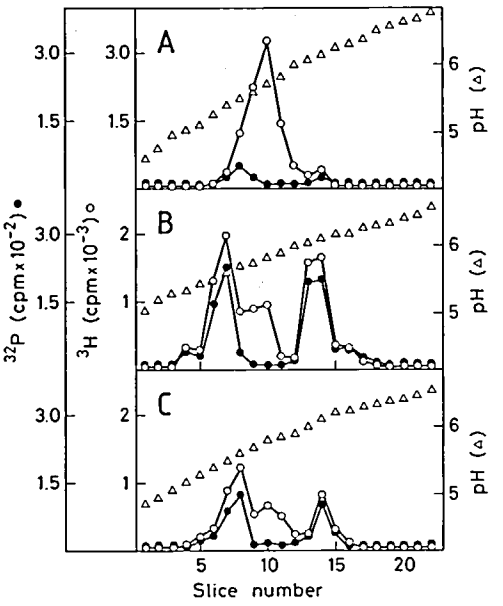


Fig. 1. Isoelectric focusing of fructose-1,6-bisphosphatase immunoprecipitated from yeast strain M1 grown to the stationary phase in the presence of [$^3$H]histidine and [$^{32}$P]$P_i$. Excess antibody was added to extracts from cells incubated 0 min (A), 3 min (B) and 20 min (C) with glucose.

senting about 20% of the total enzyme protein was also detected in both samples. The more acidic phosphorylated peak displayed an isoelectric point of 5.6, which is lower than that of the native form; this is an obvious consequence of enzyme phosphorylation. However, the additional phospho form displayed an isoelectric point of 6.1, thus proving to be even more basic than the native one. This suggests that the enzyme undergoes a further modification besides phosphorylation.

We therefore took into consideration the possibility that the enzyme encountered limited proteolysis after phosphorylation: in fact, in support of this idea, we found that in the mutant ABYS1, only the more acidic phosphorylated peak was evidenced after cell incubation with glucose (Fig. 2). However, the phosphorylated and the unphosphorylated forms from wild-type cells showed identical patterns in a gradient SDS-gel electrophoresis (data not shown), although under these conditions, a proteolytic event removing as little as ten amino acids should have been detected as an increase in electrophoretic mobility. Furthermore, the amino acid sequence indicates that the

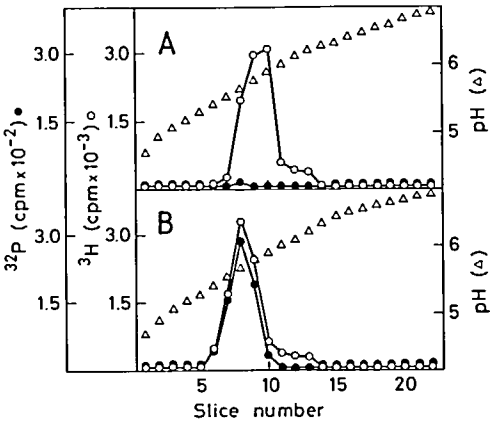


Fig. 2. Isoelectric focusing of fructose-1,6-bisphosphatase immunoprecipitated from yeast mutant ABYS1 grown to the stationary phase in the presence of [$^{3}$H]histidine and [$^{32}$P]$P_i$. Excess antibody was added to extracts from cells incubated 0 min (A) and 20 min (B) with glucose.

removal of the first acidic amino acid starting from the N-terminus, i.e., Asp-10, requires a simultaneous loss of the two basic residues Arg-8 and Arg-9 [16]. Likewise, further sequence data (Marcus, F., personal communication) show that proteolytic removal of the acidic amino acid closest to the C-terminus also involves the loss of an arginine and of a histidine residue. Therefore, these data demonstrate that a limited proteolysis can not be responsible for the appearance of the more basic phospho form. On the other hand, the results reported in Fig. 1B and C, show that the relative areas of the two phosphorylated peaks did not appreciably change during enzyme degradation, although after 20 min of incubation, the residual radioactivity represented about 50% of that detected at 0 and at 3 min. This suggests a prompt metabolic equilibration of the two phospho forms like in the case of phosphorylative step, which is reversed in vivo by a phosphatase activity [17]. Furthermore, evidence that this additional modification is a covalent one is provided by the fact that the isoelectric focusing procedure employed here is carried out in the presence of urea [14], which should dissociate non-covalently bound molecules.

Also, the possibility that the heterogeneity of the phosphoprotein described represents an artifact arising in crude extracts of the wild-type strain could be ruled out on the basis of the following experiment: the ABYS1 cells were grown in the presence of [2,5-$^{3}$H]histidine and incubated with glucose; crude extracts were prepared and mixed, prior to immunoprecipitation, with extracts from wild-type cells grown in the absence of radioactivity and also incubated with glucose. Nevertheless, under these conditions, the radioactive pattern detected in isoelectric focusing was the same as that reported in Fig. 2B (data not shown).

On the whole, these data suggest that one or more of the proteinase activities missing in the mutant ABYS1 play a role in the inactivation mechanism of fructose-1,6-bisphosphatase, although a direct action of these proteolytic enzymes on fructose-1,6-bisphosphatase can not be postulated. A plausible hypothesis might be that they promote the maturation of an enzyme catalyzing a covalent modification of the phosphatase. However, the nature of such modification has still to be established.

The involvement of the aforementioned proteinases in the degradation mechanism of fructose-1,6-bisphosphatase is also supported by a substantially slower inactivation in the mutant as compared to the wild type: indeed, glucose-induced inactivation carried out with cells collected after 24 h of growth, showed that the wild-type M1 underwent a complete disappearance of the immunologically reactive protein within 2 h (Fig. 3); this result compares well with those reported for other wild-type strains [4,18]. In contrast, in the mutant ABYS1 about 40% of the immunologically reactive protein was retained after 3 h of incubation (Fig. 3). On assuming a first-order degradation kinetics, these data indicate a 6-fold lower degradation constant in the mutant with respect to the wild type. Furthermore, such a pattern was not substantially changed if the cells were collected at 16 or at 48 h of growth (data not shown).

Based on these results, we speculate that the additional modification shown in this paper might involve a sensitization of the phospho form to proteolytic attack with resulting stimulation of enzyme degradation.

A major question about the inactivation mechanism of fructose-1,6-bisphosphatase concerns the

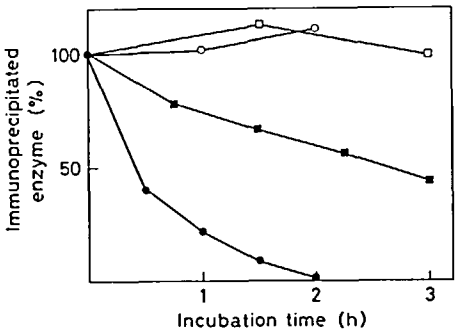


Fig. 3. Time course of glucose-induced degradation of fructose-1,6-bisphosphatase in the yeast strain M1 (●, ○) and in the mutant ABYS1 (■, □). Cells grown in the presence of [$^3$H]histidine were resuspended in 0.1 M potassium Mes (pH 6.0). At zero time, 0.15 M glucose was added; at the indicated times, cell samples were collected, the enzyme was immunoprecipitated and subjected to SDS-gel electrophoresis. The radioactivity incorporated into the enzyme band was then quantified and expressed as relative to zero time. Open symbols indicate no glucose added.

intracellular compartment in which it takes place. This paper and one from Funaguma et al. [19] suggest that the vacuolar proteolytic machinery is involved in the proteolysis of the enzyme. This, however, does not provide conclusive evidence in support of an intravacuolar degradation, if one assumes, as proposed above, that the vacuolar proteinases are responsible for the maturation of an enzyme which in turn modifies fructose-1,6-bisphosphatase, and that this latter event occurs in the cytoplasmic compartment. Moreover, a recent report from Schäfer et al. [20] proposes that the proteolytic degradation of the enzyme occurs extravacuolarly, since its incubation with an extract from isolated vacuoles leads to the accumulation of a 32-kDa immunologically reactive intermediate, which instead is not detected in intact cells. Thus, further studies are required to gain a better understanding of the mechanism of enzyme proteolysis; nevertheless, our results strongly suggest an involvement in the process of the vacuolar proteinases.

We thank Professor D.H. Wolf for the generous gift of the mutant strain ABYS1. We thank Professor F. Marcus for having communicated sequence data of the C-terminus of fructose-1,6-bisphosphatase. This work was supported by a grant from the Italian Ministero della Pubblica Istruzione, DPR 382/80, art. 65.

## References

1 Holzer, H. (1976) Trends Biochem. Sci. 1, 178–181.
2 Müller, D. and Holzer, H. (1981) Biochem. Biophys. Res. Commun. 103, 926–933.
3 Mazòn, M.J., Gancedo, M.J. and Gancedo, C. (1982) J. Biol. Chem. 257, 1128–1130.
4 Funayama, S., Gancedo, J.M. and Gancedo, C. (1980) Eur. J. Biochem. 109, 61–66.
5 Tortora, P., Birtel, M., Lenz, A.G. and Holzer, H. (1981) Biochem. Biophys. Res. Commun. 100, 688–695.
6 Pohlig, G., Wingender-Drissen, R., Noda, T. and Holzer, H. (1983) Biochem. Biophys. Res. Commun. 115, 317–324.
7 Pohlig, G. and Holzer, H. (1985) J. Biol. Chem. 260, 13818–13823.
8 Purwin, C., Leidig, F. and Holzer, H. (1982) Biochem. Biophys. Res. Commun. 107, 1482–1489.
9 Tortora, P., Burlini, N., Hanozet, G.M. and Guerritore, A. (1982) Eur. J. Biochem. 126, 617–622.
10 Lamponi, S., Galassi, C., Tortora, P. and Guerritore, A. (1987) FEBS Lett. 216, 265–269.
11 Achstetter, T., Emter, O., Ehmann, C. and Wolf, D.H. (1984) J. Biol. Chem. 259, 13334–13343.
12 Matsumoto, K., Uno, I. and Ishikawa, T. (1984) Genetics 108, 53–66.
13 Tortora, P., Burlini, N., Caspani, G. and Guerritore, A. (1984) Eur. J. Biochem. 145, 543–548.
14 O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
15 Noda, T., Hoffschulte, H. and Holzer, H. (1984) J. Biol. Chem. 259, 7191–7197.
16 Rittenhouse, J., Harrsch, P.B., Kim, J.N. and Marcus, F. (1986) J. Biol. Chem. 261, 3939–3943.
17 Horn, D. and Holzer, H. (1987) J. Biol. Chem. 262, 2056–2061.
18 Gancedo, C. (1971) J. Bacteriol. 107, 401–405.
19 Funaguma, T., Toyoda, Y. and Sy, J. (1985) Biochem. Biophys. Res. Commun. 130, 467–471.
20 Schäfer, W., Kalisz, H. and Holzer, H. (1987) Biochim. Biophys. Acta 925, 150–155.