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Irreversible inactivation of *Saccharomyces cerevisiae* fructose-1,6-bisphosphatase independent of protein phosphorylation at Ser[11]

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The fructose-1,6-bisphosphatase gene was used with multicopy plasmids to study rapid reversible and irreversible inactivation after addition of glucose to derepressed *Saccharomyces cerevisiae* cells. Both inactivation systems could inactivate the enzyme, even if 20-fold over-expressed. The putative serine residue, at which fructose-1,6-bisphosphatase is phosphorylated, was changed to an alanine residue without notably affecting the catalytic activity. No rapid reversible inactivation was observed with the mutated enzyme. Nonetheless, the modified enzyme was still irreversibly inactivated, clearly demonstrating that phosphorylation is an independent regulatory circuit that reduces fructose-1,6-bisphosphatase activity within seconds. Furthermore, irreversible glucose inactivation was not triggered by phosphorylation of the enzyme.

Carbohydrate metabolism; Fructose-1,6-bisphosphatase; Reversible inactivation; Phosphorylation; Irreversible inactivation; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

Carbohydrate metabolism is precisely regulated in *Saccharomyces cerevisiae*. As well as glucose repression (reviewed in [1,2]), which entails long-term adaptation of the yeast to growth on glucose, there is also glucose inactivation. This is a short-term regulatory mechanism, which by proteolytic degradation reduces the amount of enzyme available (reviewed in [3]). Although intensively investigated and observed as early as 1966 for cytoplasmic malate dehydrogenase [4], there is little information about the molecular mechanism of glucose inactivation. Other enzymes inactivate are FBPase [5] and PEP carboxykinase [6,7]. Inactivation is the result of irreversible proteolytic degradation, since the amount of immune-precipitable protein corresponded exactly with the decrease in enzyme activity. This was first demonstrated for cytoplasmic malate dehydrogenase (EC 1.1.1.37) [8] and also confirmed for FBPase [9] and PEP carboxykinase [10]. However, Lenz and Holzer [11], using glucose-grown cells in stationary phase, observed that FBPase inactivation remained reversible during the first few minutes of inactivation. During this time interval FBPase remained immune precipitable [12]. Reversible inactivation was also described subsequently for cells derepressed under carbon source limitation, but not in cells growing on ethanol [13], which were used in earlier experiments. Phosphorylation at a serine residue within the N-terminal part of the enzyme was shown to be responsible for the decrease in activity [14]. Phosphorylation of FBPase is clearly triggered by activation of adenylate cyclase, leading to

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Abbreviations: FBPase, fructose-1,6-bisphosphatase (EC 3.1.3.11); FBPase^{Ala}, mutated FBPase containing alanine instead of serine at position 11; PEP carboxykinase, phospho*enol*pyruvate carboxykinase (EC 4.1.1.38); YEP, yeast extract peptone; SC, synthetic complete

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a rise in cAMP concentration which, in turn, activates a serine kinase (reviewed in [15]). The mechanism by which adenylate cyclase is activated is still unknown.

Since rapid reversible inactivation preceded irreversible inactivation, phosphorylation was proposed as the triggering reaction that makes the enzyme accessible to the proteolytic machinery [16,17]. However, no reversible phase of inactivation was detected for cytoplasmic malate dehydrogenase [13] and atempts to demonstrate phosphorylation of this enzyme failed (Holzer, personal communication). In order to investigate the role of protein phosphorylation in irreversible inactivation, the FBPase structural gene has been isolated [18] and the putative serine residue, at which phosphorylation occurs, changed to alanine. Our results presented here showed unequivocally that irreversible inactivation of FBPase is independent of the phosphorylation reaction.

2. EXPERIMENTAL

For inactivation kinetics, isogenic strains ENY.WA-7C (*Mata leu2-3 leu2-112 MAL3 SUC3 MAL2-8^c*) and WAY.5-4A/1 (*Mata his3-Δ1 ura3-52 MAL3 SUC3 MAL2-8^c fbp1::HIS3*) were used. For genotypes, growth, derepression, inactivation, and reactivation conditions see [13,18]. The FBPase containing plasmid, pJS142, derived from YEp352, has been isolated after complementation of strain WAY.5-4A/1, and was kindly provided by H.-J. Schüller. Recombinant plasmids were amplified and purified as cited in [18]. One unit of enzyme activity is defined as μmol substrate converted per min.

For in vitro mutagenesis the *Hin*dIII/*Eco*RV fragment of FBPase was subcloned into pBR328. After digestion with *Nde*I and *Eco*RV the resulting 57 bp fragment was removed by gel electrophoresis and a double-stranded synthetic piece of DNA was inserted instead. For construction steps see section 3.3, fig.2. Established protocols were followed as for standard techniques of recombinant DNA [19]. Synthetic oligonucleotides were synthesized on a 380B DNA synthesizer (Applied Biosystems; D-6108 Weiterstadt, FRG).

3. RESULTS

3.1. *Multicopy expression of FBPase*

As reported recently, the cloned *Hin*dIII/*Xho*I fragment, containing the FBPase open reading frame, had a promoter size of 318 bp and gave only low level expression even with episomal shuttle vectors [18]. This was indicate of an upstream activation element as target site for positive regulation, which is assumed to be involved in the release of cells from glucose repression [20]. This interpretation was confirmed when the 3.8 kbp *Xba*I fragment, including a 2.2 kbp promoter region (pJS142), gave high level expression (table 1). On glucose FBPase activity was completely repressed and after transfer to derepression medium the activity started to increase after 3 h and reached a maximum after 7 h. The specific activity on ethanol was increased about 13-fold relative to the single-copy wild type and could be further increased when cells were also transferred to derepression medium (table 1).

3.2. *Glucose inactivation of multicopy expressed FBPase*

The high level of expression of FBPase made it practical to test the capacity of the glucose inactivation system. Transformant cells pre-grown on glucose were derepressed for various times before glucose was added. After 4 h of derepression, when the cells had reached about the maximum level of the wild type, rapid reversible and irreversible inactivation occurred as in the wild type. This was also true with cells having five-fold increased activity (derepression time: 5 h). Even after complete derepression (derepression time: 6 h) inactivation kinetics were totally comparable to those of the single-copy wild type (fig.1). The same observation was made when cells, pre-grown on ethanol, were derepressed and inactivated, although the specific activity was 580 mU/mg, compared with 30

Table 1

Specific activity of FBPase in wild type, short promoter FBPase (plasmid pRV28, promoter size: 0.3 bp), long promoter FBPase (plasmid pJS142, promoter size: 2.2 kbp) and FBPase^{Ala} (plasmid pRV44, promoter size: 2.2 kbp) after growth on different carbon sources and after derepression with SC Na-acetate

		Specific activity (nmol/min · mg)			
		SC glucose (− uracil)		YEP ethanol	
Strain	Plasmid	0 h[a]	6 h	0 h	6 h
ENY.WA-7C	none	2	30	25	45
WAY.5-4A/1	pRV28	1		9	
WAY.5-4A/1	pJS142	3	380	230	581
WAY.5-4A/1	pRV44	2	280	180	375

[a] Depression time with SC Na-acetate

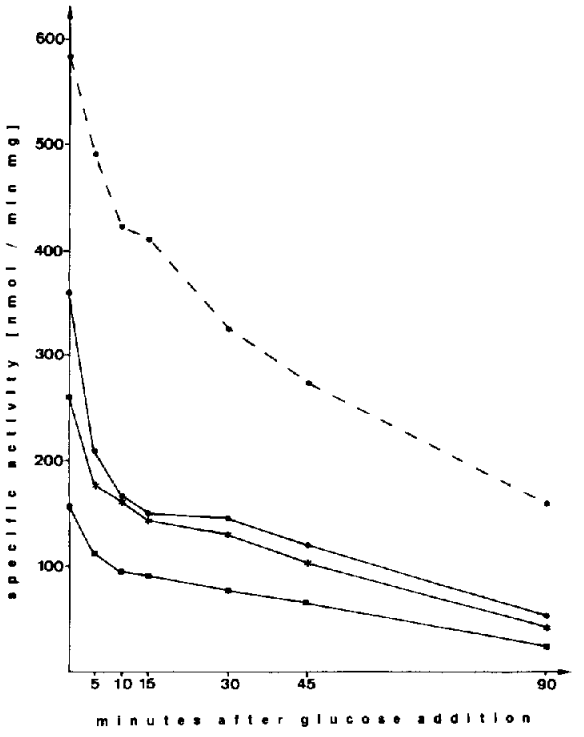

Fig. 1. Inactivation of FBPase transformants after different derepression times. Cells were grown on SC 2% glucose medium, lacking uracil (——), and YEP 3% ethanol (– – –), respectively. In late logarithmic phase cells were washed twice, and resuspended in derepression medium (SC 0.3% Na-acetate). Inactivation was started after 4 h derepression (■), 5 h derepression (*) and 6 h derepression (●).

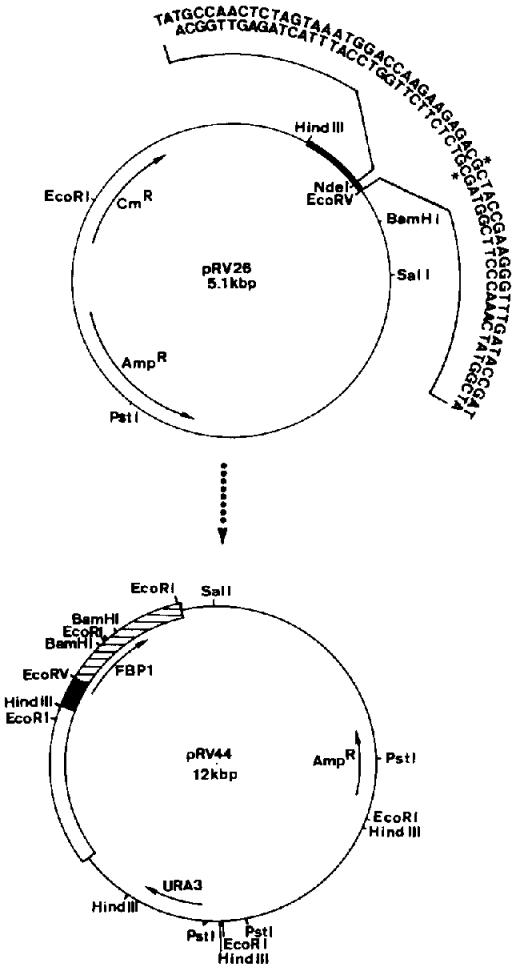

Fig. 2. Construction of FBPase^{Ala}. Part of the FBPase open reading frame was replaced by the DNA sequence given above. The changed bases with respect to wild type are marked by an asterisk. The resulting FBPase^{Ala} fragment (■) was recombined with the respective 5′- (□) and 3′-sequences (▤) by several cloning steps (••••▶). Finally, the resulting YEp24-derived yeast/*E. coli* vector, pRV44, was obtained.

mU/mg in single-copy wild-type cells (fig.1). Hence, the glucose inactivation system by far exceeded the capacity necessary to inactivate the amount of FBPase present in wild-type cells.

3.3. *In vitro mutagenesis of FBPase*

The site of FBPase phosphorylation was located by peptide mapping at Ser^{11} [14]. In order to elucidate the role of enzyme phosphorylation in glucose inactivation, Ser^{11} was mutagenized in vitro to Ala^{11}, by replacing the native DNA sequence between restriction sites *Nde*I and *Eco*RV [18] with two appropriate oligonucleotides (fig.2). To manifest the correct in vitro mutagenesis, the newly synthesized FBPase^{Ala} fragment was sequenced in the final construct (fig.2). The specific activity of the resulting FBPase^{Ala} was only slightly reduced, relative to that of the FBPase (table 1).

3.4. *Inactivation of FBPase^{Ala}*

When glucose was added to derepressed transformants containing FBPase^{Ala}, no inactivation was observed within the first 10 min, whereas wild type FBPase lost about 40% of its initial activity (fig.3). Clearly, rapid reversible inactivation was not expressed. Hence, Ser^{11} is the only site of phosphorylation which reduces FBPase activity. Despite the lack of rapid reversible inactivation, FBPase^{Ala} activity started to decrease 15 min after

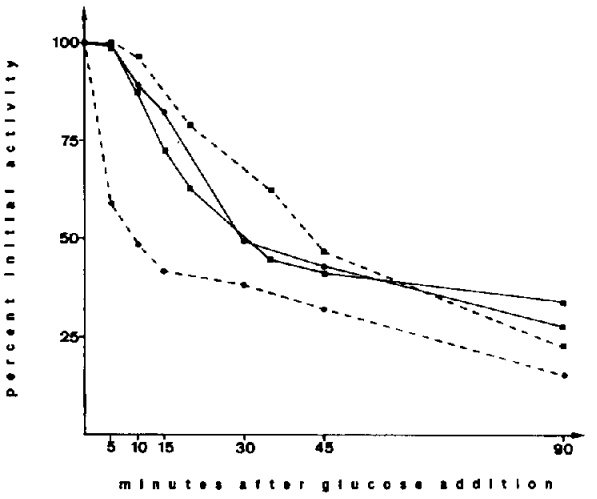

Fig. 3. Inactivations experiments with transformants containing plasmid pJS142 (FBPase) (●) or pRV44 (FBPase^{Ala}) (■), respectively. FBPase (– – –) (initial activities 359 and 262 nmol/min · mg), malate dehydrogenase (——) (initial activities 252 and 208 nmol/min · mg, respectively). Control experiments without addition of glucose showed no change in FBPase activity during the experimental interval.

adding glucose and, although almost no growth occurred, within 45 min, the specific activity fell below 40% of the initial activity. Similar kinetics were obtained when ethanol grown cells were derepressed and inactivated (not shown). As an additional control, malate dehydrogenase activity was followed (fig.3). Of the two malate dehydrogenase isoenzymes present only the cytoplasmic enzyme is irreversibly degraded [21,22]. Within the first 45 min, the inactivation kinetics were similar to those of FBPase^{Ala}; thereafter, malate dehydrogenase inactivation slowed down, as mitochondrial malate dehydrogenase activity interfered with the process.

4. DISCUSSION

From the above results, phosphorylation of FBPase appears to be an independent regulatory mechanism and rapid reversible inactivation is not the event that triggers irreversible inactivation. Hence several independent control systems exist, that adapt the yeast cell to glucose as carbon source. First, as an immediate reaction, FBPase is phosphorylated to reduce enzyme activity. Secondly, irreversible inactivation follows, degrading most of the FBPase activity, and thirdly, transcription of FBPase is repressed simultaneously.

By replacing Ser^{11} by Asp which mimics phosphorylation, Marcus et al. [23] showed recently, that one effect of phosphorylation is to increase sensitivity of FBPase for the low molecular mass inhibitors fructose 2,6-bisphosphate [24] and AMP [26], hence, giving an allosterically reduced activity. Fructose 2,6-bisphosphate also acts as an activator of phosphofructokinase [25], and so stimulates glycolysis. Hitherto, it was unclear whether only FBPase was phosphorylated and not cytoplasmic malate dehydrogenase, too. This report shows phosphorylation to be an independent process. These findings, and the very similar inactivation kinetics of FBPase^{Ala} and cytoplasmic malate dehydrogenase give some support to the view that all gluconeogenic enzymes subject to glucose inactivation are degraded by a common mechanism. This conclusion was also consistent with the finding that, under conditions that blocked irreversible degradation of cytoplasmic malate dehydrogenase, PEP carboxykinase behaved similarly, whereas FBPase inactivation remained reversible for at least 2 h [13].

The finding that FBPase is normally inactivated, even if strongly overproduced, contrasts with those recently obtained, when the FBPase open reading frame was fused to nonglucose-repressible promoters [27] yielding high activity even on glucose. From this observations these authors concluded that the glucose inactivation is only of marginal importance. An explanation for these conflicting results might be that a component of the inactivation system could be repressed by glucose. In this case, no glucose inactivation would occur if the cells were grown on glucose. One such component might be the glucose repressible glucose carrier encoded by the *SNF3* gene [28]. The ability of uncouplers to inactivate [29] lends some support to this suggestion, as they might mimic a proton driven glucose uptake, so far unrecognized.

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REFERENCES

[1] Entian, K.-D. (1986) Microbiol. Sci. 3, 366–371.
[2] Gancedo, J.M. and Gancedo, C. (1986) FEMS 32, 179–187.
[3] Holzer, H. (1976) Trends Biochem. Sci. 1, 178–181.
[4] Witt, I., Kronau, R. and Holzer, H. (1966) Biochim. Biophys. Acta 118, 522–537.
[5] Gancedo, C. (1971) J. Bacteriol. 197, 401–405.
[6] Haarasilta, S. and Oura, E. (1975) Eur. J. Biochem. 52, 1–7.
[7] Gancedo, C. and Schwerzmann, N. (1976) Arch. Microbiol. 109, 221–225.
[8] Hägele, E., Neeff, J. and Mecke, D. (1978) Eur. J. Biochem. 83, 67–76.
[9] Funayama, S., Gancedo, J.M. and Gancedo, C. (1980) Eur. J. Biochem. 109, 61–66.
[10] Müller, M., Müller, H. and Holzer, H. (1981) J. Biol. Chem. 256, 723–727.
[11] Lenz, A.-G. and Holzer, H. (1980) FEBS Lett. 109, 271–274.
[12] Tortora, P., Birtel, M. Lenz, A.-G. and Holzer, H. (1981) Biochem. Biophys. Res. Commun. 100, 688–695.
[13] Entian, K.-D., Dröll, L. and Mecke, D. (1983) Arch. Microbiol. 134, 187–192.
[14] Rittenhouse, J., Harsch, H.B., Kim, J.N. and Marcus, F. (1986) J. Biol. Chem. 261, 3939–3943.
[15] Holzer, H. (1984) in: Enzyme Regulation by Reversible Phosphorylation – Further Advances (Cohen, P ed.) pp. 143–154, Elsevier, Amsterdam, New York.
[16] Müller, D. and Holzer, H. (1981) Biochem. Biophys. Res. Commun. 103, 926–933.
[17] Mazon, M.J., Gancedo, J.M. and Gancedo, C. (1982) J. Biol. Chem. 257, 1128–1130.
[18] Entian, K.-D., Vogel, R., Rose, M., Hofmann, L. and Mecke, D. (1988) FEBS Lett. 236, 195–200.
[19] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, NY.
[20] Schüller, H.-J. and Entian, K.-D. (1988) Gene 67, 247–257.
[21] Witt, I., Kronau, R., Holzer, H. (1966) Biochim. Biophys. Acta 128, 63–73.
[22] Entian, K.-D., Fröhlich, K.-U. and Mecke, D. (1984) Biochim. Biophys. Acta 799, 181–186.
[23] Marcus, F., Rittenhouse, J., Moberly, L., Edelstein, I., Hiller, E. and Rogers, D.T. (1988) J. Biol. Chem. 263, 6058–6062.
[24] Francois, J., Van Schaftingen, E. and Hers, H.G. (1984) Eur. J. Biochem. 145, 187–193.
[25] Hers, H.G. and Van Schaftingen, E. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17, 343–349.
[26] Gancedo, C., Salas, M.L., Giner, A. and Sols, A. (1965) Biochem. Biophys. Res. Commun. 20, 15–20.
[27] Rogers, D.T., Hiller, E., Mitsock, L. and Orr, E. (1988) J. Biol. Chem. 263, 6051–6057.
[28] Bisson, L.F., Neigeborn, L., Carlson, M. and Fraenkel, D.G. (1987) J. Bacteriol. 169, 1656–1662.
[29] Mazon, M.J., Gancedo, J.M. and Gancedo, C. (1982) Eur. J. Biochem. 127, 605–608.