

Catabolite inactivation of fructose-1,6-bisphosphatase in yeast is mediated by the proteasome

Stefan M. Schork**, Gisela Bee**, Michael Thumm, Dieter H. Wolf*

Institut für Biochemie der Universität Stuttgart, Pfaffenwaldring 55, D-70569 Stuttgart, Germany

Received 3 June 1994

Abstract

Fructose-1,6-bisphosphatase, a key enzyme in gluconeogenesis, undergoes catabolite inactivation when glucose is added to gluconeogenetically active cells of the yeast *Saccharomyces cerevisiae*. Phosphorylation of the enzyme is followed by rapid degradation. To elucidate the cellular proteolytic system involved in catabolite-triggered degradation of fructose-1,6-bisphosphatase this event was followed in different protease-deficient yeast mutants. In a mutant defective in the proteolytic function of the vacuole the degradation rate of the enzyme is not diminished. In contrast mutants defective in the proteolytic activity of the proteasome exhibit a strongly reduced glucose-induced degradation of fructose-1,6-bisphosphatase as compared to their isogenic wild-type counterparts. Our studies suggest that catabolite inactivation of fructose-1,6-bisphosphatase occurs in the cytosol, the degradation event being mediated by the proteasome. An explanation is presented which tries to resolve the formerly conflicting results, which suggested glucose-triggered uptake of fructose-1,6-bisphosphatase into the vacuole followed by vacuolar proteolysis.

Key words: Catabolite inactivation; Fructose-1,6-bisphosphatase; Proteasome; Proteolysis; *Saccharomyces cerevisiae*

1. Introduction

Fructose-1,6-bisphosphatase (FBPase), a key enzyme in gluconeogenesis, is subject to catabolite inactivation in the yeast *Saccharomyces cerevisiae* [1]. Addition of glucose to cells grown on a non-fermentable carbon source causes a rapid inactivation of this enzyme due to phosphorylation and subsequent proteolytic degradation [2–4]. Previous studies focused predominantly on the role of vacuolar proteases in the degradation process, but contradictory results were reported [5–10]. The vacuolar proteolytic system consists of a variety of peptidases of which proteinase yscA, the *PRA1* (*PEP4*) gene product, occupies a central position. The enzyme is necessary for activation of its own and other inactive vacuolar peptidase precursors and it is highly active in vacuolar protein degradation [11,12]. Vacuolar proteolysis had been shown to be most active during stress and starvation of cells [9] and its involvement in degradation of long-lived proteins had been documented [13]. The central cytoplasmic protease in eukaryotic cells is the proteasome, a multisubunit enzyme complex harbouring several different proteolytic activities [14]. Up to now its involvement in vivo in the degradation of ubiquitinated [15,16] and short-lived proteins [17,18] had been shown. Of specific intracellular proteins the proteasome has been shown to be responsible for the degradation of ornithine decarboxylase [19], the α -subunit of fatty acid synthase, when unable to assemble to the $\alpha_6\beta_6$ complex [13] and the MAT α 2 transcriptional repressor (Richter-

Ruoff, B., Hochstrasser, M. and Wolf, D.H., in preparation). Here we present evidence that catabolite-triggered degradation of FBPase is not exerted by the proteolytic system in the vacuole but is mediated by the proteasome instead. The essential statement of this paper was the subject of a scientific correspondence letter in Nature [20]. Here we present the data which lead to our conclusion.

2. Materials and methods

2.1. Strains

Strains used were: wild-type strain WCG4 α (MAT α , *his* 3-11, 15 *leu*2-3, 112 *ura*3). The *Apra*1 mutant strain YMTA (MAT α , *his* 3-11, 15 *leu*2-3, 112 *ura*3, *pra1* Δ EN::HIS3) was generated by chromosomal replacement of the *PRA1* gene in strain WCG4 α with the deleted *pra1* allele. The *pra1* allele was constructed by eliminating the ORF between the *Eco*RI and *Nco*I sites and inserting the *Bam*HI fragment of the *HIS3* gene. Strains carrying the *pre1-1*, *pre1-1 pre2-1* mutations are isogenic derivatives of wild-type strain WCG4 α and are described elsewhere [6].

2.2. Media

Cells were grown either on YPD (1% yeast extract, 2% peptone, 2% glucose), YP ethanol (1% yeast extract, 2% peptone, 2% ethanol) or on mineral medium (0.67% Difco yeast nitrogen base without amino acids) containing 2% glucose or 2% ethanol as the carbon source and supplements (20 μ g·ml⁻¹ uracil, 20 μ g·ml⁻¹ histidine, 30 μ g·ml⁻¹ leucine). Radiolabeling was done in pulse medium (0.17% Difco yeast nitrogen base without amino acid and ammonium sulphate, 0.5% proline, 100 μ M ammonium sulphate, 2% ethanol and required supplements).

2.3. Antibodies

Rabbit antisera for FBPase were raised against a FBPase-MS2 fusion protein expressed in *E. coli* strain CGSC6769/pci857. For this purpose the ORF of the *FBP1* gene (derived from pRG6 [21]) was fused in-frame to the N-terminal part of the MS2-polymerase gene under the control of the λ PL promoter in vector pEx31b [22]. Expression and purification of the fusion protein was carried out as described [22]. Antibodies specific to CPY were described previously [23].

* Corresponding author. Fax: (49) (711) 685 4392.

** Both authors contributed equally to this study.

2.4. Enzyme assay

FBPase activity was assayed as in [24]. Cells were grown in YPD at 30°C until an absorbance at 578 nm (A_{578}) of 8 was reached. Cells were diluted to an A_{578} of 0.5 and transferred to YP ethanol to derepress FBPase. After an A_{578} of 3 was reached catabolite inactivation was induced by addition of glucose to a final concentration of 2%. Cells (10 A_{578} per sample) were washed, resuspended in 150 μ l 20 mM imidazole-HCl, pH 7, and broken by vigorous shaking with an equal volume of glass beads (10 min, 4°C). After centrifugation (15 min, 10,000 $\times$ g) the supernatant was used immediately for activity assays.

2.5. Pulse-chase experiments and immunoprecipitation

Wild-type and mutant cells were grown to an A_{578} of 5–6 in mineral medium containing 2% glucose and supplements. After harvesting (5 min, 500 $\times$ g) and washing cells were pre-incubated for 3.5 h in pulse medium at a density of 3 A_{578} $\cdot$ ml⁻¹. Radiolabeling was done by adding [³⁵S]methionine (Amersham, Braunschweig, Germany) to the cell suspension to a final concentration of 45 μ Ci $\cdot$ ml⁻¹ (pulse). After 1.5 h cells were shifted into mineral medium containing 2% glucose, 5 mM non-radioactive methionine and 0.5% ammonium sulphate (chase). Samples (3 A_{578} units) were taken at the times indicated and the chase period was terminated by addition of TCA (final concentration 5%). Precipitates were washed twice with ethanol (-20°C) and dried. Subsequently cells were lysed in 100 μ l breaking buffer by glass bead agitation as described [25]. Immunoprecipitation with antisera specific for FBPase (8 μ l), and as a control carboxypeptidase yscY (CPY) (4 μ l) was performed as outlined [26].

2.6. Immunofluorescence

For growth conditions see section 2.4. After mixing with 4% formaldehyde for 1 h at 30°C cells were washed 4 times and resuspended in SP buffer (1.2 M sorbitol, 0.1 M KH₂PO₄, pH 7.25, 25 mM β -mercaptoethanol) to yield 6 A_{578} $\cdot$ ml⁻¹. Spheroplasts were formed by treating cells with 160 μ g $\cdot$ ml⁻¹ Zymolyase 100-T (Seikagaku Kyogo, Japan) at 30°C for 30 min. Cells were washed 3 times in 1.2 M sorbitol and finally resuspended in 500 μ l of the same solution. Fixed cell suspensions were applied to poly-lysine slides for 30 min in a humid chamber [27]. After washing with PBS/BSA/NP40 (10 mg $\cdot$ ml⁻¹ BSA, 0.05% NP40/PBS) spheroplasts were incubated with anti-FBPase antibody (1:20 in PBS/BSA/NP40) for 2 h at 25°C. Excess antibody was removed by washing 7 times and thereafter FITC-goat anti-rabbit antibodies (Sigma; 1:100) were applied for 1 h. After washing 7 times cells were mounted with 80% glycerol, 0.1% *p*-phenylenediamine PBS. Cells were visualized with a Zeiss Axioskop at a magnification of $\times$ 1000 and photographed using Kodak Tmax 400 film. Immunofluorescence performed with a FBPase deletion strain showed no background signal.

3. Results and discussion

We studied the glucose-induced inactivation of FBPase in yeast mutants defective in two different proteolytic systems, a cytoplasmic and the vacuolar system. A vacuolar participation in inactivation of FBPase was examined in a *pral*-deleted strain, which is deficient in most of the vacuolar proteolytic activity [7,9,28]. The involvement of the proteasome in the inactivation of FBPase was traced in two mutants, carrying missense mutations in essential genes, *PRE1* and *PRE2*, of the proteasome which both encode subunits necessary for its chymotrypsin-like activity [15,16]. *Pre1-1* mutant cells exhibit a dramatically reduced chymotrypsin-like activity, are hypersensitive to the amino acid analogue canavanine and accumulate proteins which undergo ubiquitination prior to degradation [15–18]. Introduction of the *pre2-1* allele into *pre1-1* mutant cells enhances the phenotypic disorders in the resulting double mutant cells [16]. To study

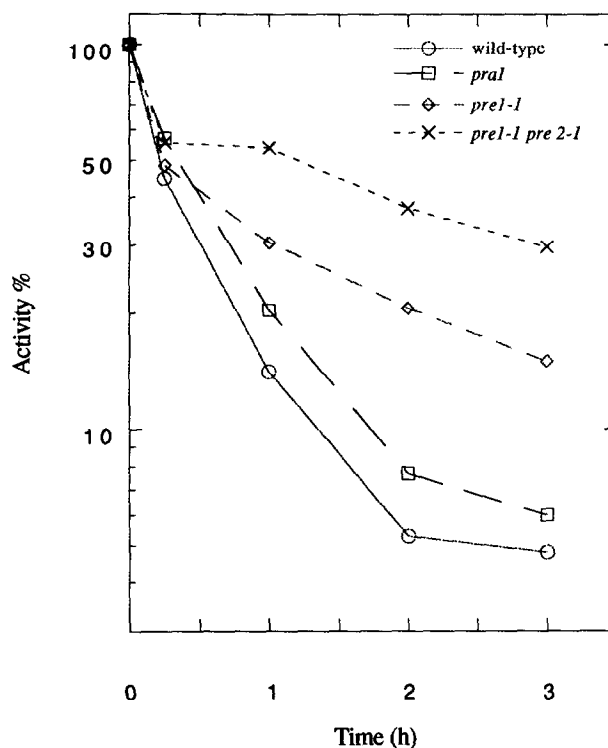


Fig. 1. FBPase activity pattern during catabolite inactivation in wild-type and protease mutants.

the in vivo degradation of FBPase, protease mutant and, for comparison, isogenic wild-type strains were grown in YPD medium and transferred to medium containing ethanol to derepress FBPase. Derepressed cells (half-life of FBPase 90 h [2]) were shifted into glucose medium, which leads to repression and FBPase synthesis and triggers inactivation and degradation of the enzyme (half-life about 30 min [2]). As can be seen in Fig. 1, in all strains a nearly 50% decrease of FBPase activity is observed during the first 15 min after glucose addition. This loss of activity is due to phosphorylation [3] and demonstrates that the glucose-induced signal is transduced equally efficiently in wild-type and all protease mutant strains. In wild-type and the proteinase *yscA* (*pral*)-deleted strain rapid disappearance of FBPase activity continues, leading to nearly complete inactivation of the enzyme after 3 h. In contrast, inactivation of FBPase is dramatically retarded in mutants defective in the chymotrypsin-like activity of the proteasome (*pre1-1*, *pre1-1 pre2-1*) (Fig. 1).

This observation indicates that the proteolytic step of catabolite inactivation of FBPase is disturbed in the proteasome mutants but not in the mutant defective in proteinase *yscA* of the vacuole. To confirm this we measured the protein levels of FBPase in wild-type and mutant cells during the inactivation process. During incubation in ethanol medium cells were labeled with [³⁵S]methionine and thereafter transferred to glucose medium containing

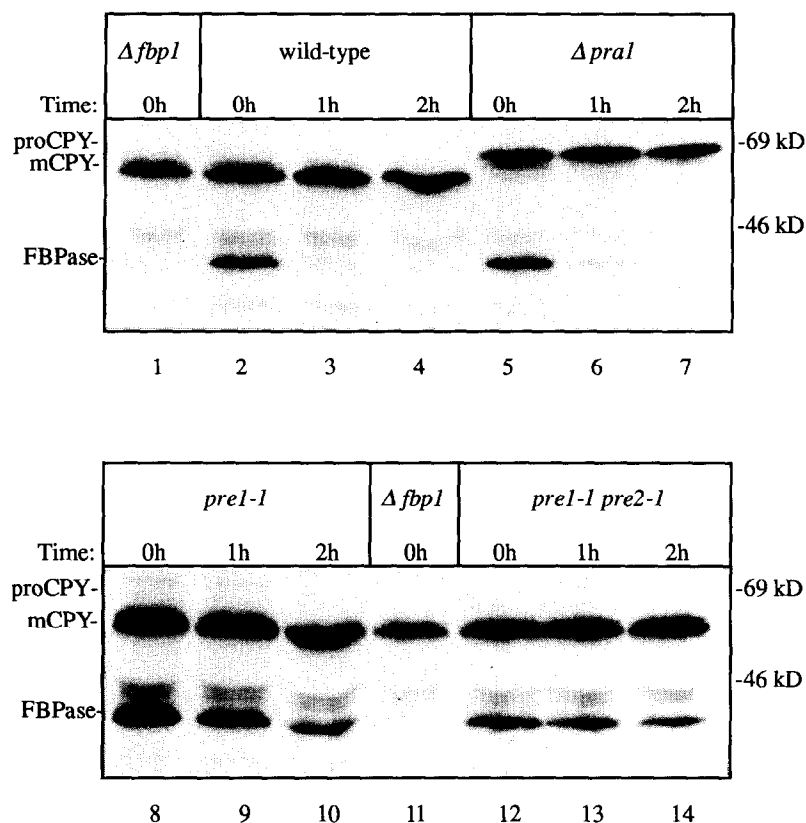


Fig. 2. Glucose-triggered degradation of FBPase is not vacuolar but depends upon an intact proteasome. Lanes 1 and 11, FBPase null mutant (*fbp1 Δ ::LEU2*) shifted to glucose for 0 h (control). Lanes 2–4, wild-type cells shifted to glucose for 0, 1 and 2 h. Lanes 5–7, *$\Delta pral$* cells shifted to glucose for 0, 1 and 2 h. Lanes 8–10, *pre1-1* cells shifted to glucose for 0, 1 and 2 h. Lanes 12–14, *pre1-1 pre2-1* cells shifted to glucose for 0, 1 and 2 h.

non-radioactive methionine as a chase. Radiolabeled FBPase antigenic material was immunoprecipitated using specific antibodies, separated by SDS-PAGE and visualized by autoradiography. As a control protein not affected by catabolite inactivation, but at the same time monitoring the *pral* mutant phenotype showing a defect in vacuolar peptidase processing, carboxypeptidase yscY (CPY) was co-precipitated (Fig. 2). While already after 1 h on glucose the FBPase protein is nearly completely lost in wild-type (Fig. 2, lane 3) and the *pral*-deleted strain (Fig. 2, lane 6), a large amount of the enzyme is still detectable in the proteasome mutants (Fig. 2 lanes 9 and 13). Even after 2 h on glucose a considerable amount of FBPase antigen is found in the *pre1-1* single mutant (Fig. 2, lane 10), and moreover in the *pre1-1 pre2-1* double mutant a decrease of labeled FBPase protein is hardly detectable during this time period (Fig. 2, lanes 12–14). Similar results were obtained when the fate of FBPase was followed via immunoblot analysis (not shown). These experiments confirm previous results [7–9] showing that endoproteinase yscA and vacuolar proteolysis are not involved in glucose-triggered degradation of FBPase. They furthermore provide strong evidence for a non-vacuolar proteolytic process which is

mediated by the proteasome. As measured by immunofluorescence microscopy under the conditions of glucose-induced degradation of FBPase, there is also no visible import of FBPase into the vacuole (Fig. 3), a phenomenon which would have been expected if glucose-triggered degradation were of vacuolar origin. Neither after growth of cells on ethanol (Fig. 3a,b) nor after 1 h (Fig. 3c,d) and 3 h (Fig. 3e,f) of glucose-induced inactivation is a substantial amount of FBPase detectable in vacuoles.

In an attempt to trace the different components involved in glucose-triggered degradation of FBPase we screened for mutants unable to inactivate FBPase upon glucose addition. Several of these were defective in proteolytic activities of the proteasome; none of the mutants found carried an obvious defect in the proteolytic system of the vacuole (not shown).

Several authors have reported a vacuolar proteinase requirement for catabolite inactivation of FBPase [5,6]. In the most recent report Chiang and Schekman [10] suggested a selective import of FBPase into the vacuole prior to degradation by the vacuolar proteolytic system under these conditions. It has recently been shown that nutrient starvation or prolonged growth of yeast cells on

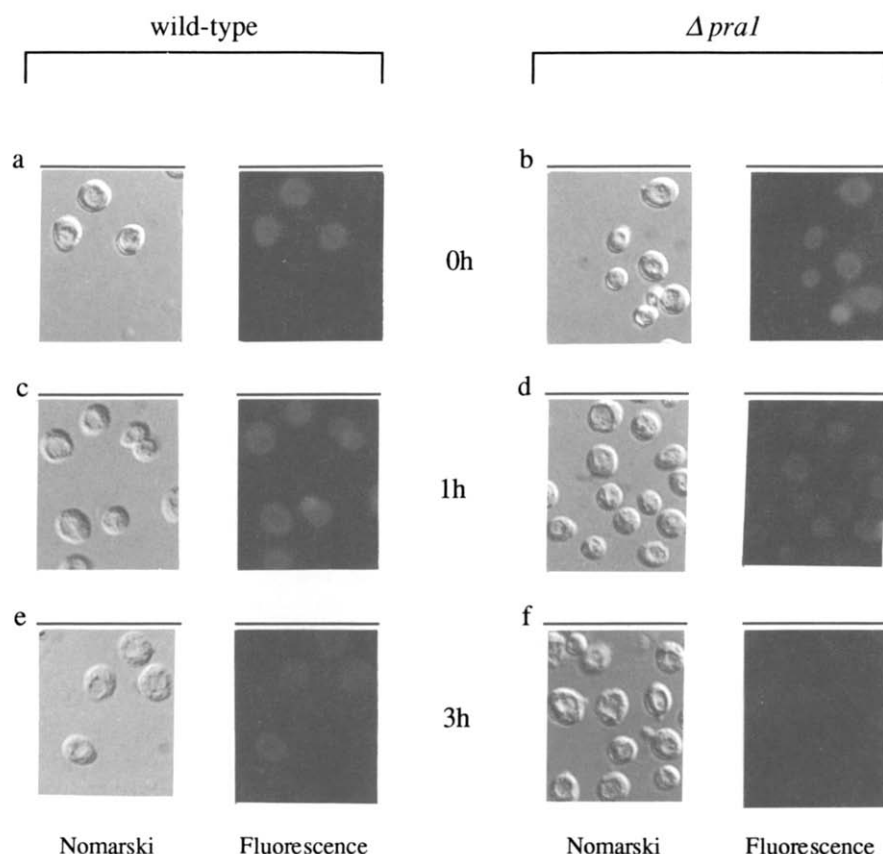


Fig. 3. Intracellular location of FBPase during catabolite inactivation was monitored by indirect immunofluorescence in wild-type and $\Delta pral$ cells. Panels a,c,e, wild-type cells shifted to glucose for 0, 1 and 3 h. Panels b,d,f, $\Delta pral$ cells shifted to glucose for 0, 1 and 3 h.

poor carbon sources, such as acetate or ethanol, induce autophagy [13,29,30], leading to bulk uptake of cytoplasmic proteins into the vacuole where they are degraded in a vacuolar proteinase-dependent fashion [13]. When yeast cells were grown on acetate [13] or ethanol medium (not shown) for a longer time (18 h on YPD, then transferred for 24 h on acetate) FBPase is found in the vacuole without addition of glucose. In their reply to our scientific correspondence letter [20] Chiang and Schekman report several derepression conditions of FBPase. Here,

also in some cases (derepression for 48 h on acetate) FBPase is degraded, however with reduced kinetics, upon glucose addition in *pep4* (*pral*) mutant cells. One would not expect this phenotypic behaviour if proteinase yscA and vacuolar proteolysis were the inactivating principle under these catabolite inactivation conditions. Our explanation for the partial stabilization of FBPase in *pep4* (*pral*) mutant cells found by Chiang and Schekman [20] under their catabolite inactivating conditions is as follows. Due to their conditions of prolonged derepression, a certain amount of FBPase enters the proteolytically inactive vacuole via starvation-induced autophagocytosis. Inside the vacuole this amount is protected from proteasome-mediated degradation naturally occurring upon glucose addition.

Over-expression of the enzyme on a multicopy plasmid, conditions used in [10], also leads to its appearance in the vacuole (Fig. 4). These findings complement earlier observations which show that the influence of proteinase yscA on degradation of FBPase is dependent on the cultivation conditions of cells [8]. In accordance with our results another group (C. and J.M. Gancedo, personal communication) found that a *pral* disruption had no effect on catabolite inactivation of FBPase.

Our finding that glucose-induced degradation of FBPase is mediated by the proteasome poses the ques-

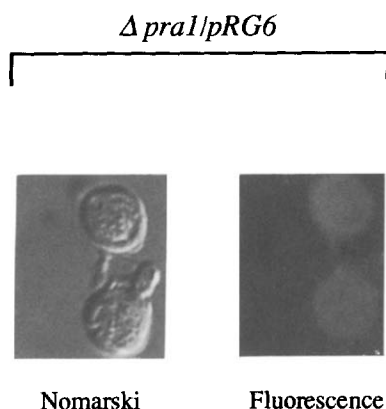


Fig. 4. Intracellular location of FBPase in $\Delta pral$ cells transformed with the multicopy plasmid pRG6 [22], containing the *FBP1* gene, after derepression in ethanol medium.

tion about the mechanism and signals triggering hydrolysis of FBPase. The proteasome has been shown to be involved in degradation of ubiquitinated proteins in vivo. In vitro studies point to the possibility that the proteasome is also able to degrade non-ubiquitinated proteins [31]. The isolated mutants defective in glucose-induced FBPase degradation will most likely allow us to unravel the degradation mechanism of this enzyme.

Acknowledgements: We thank J.M. and C. Gancedo for the gift of the *FBP1* gene. This work was supported by a grant of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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