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GLUCOSE-DEPENDENT METABOLIC INTERCONVERSION OF FRUCTOSE-1,6-BISPHOSPHATASE IN YEAST

Paolo TORTORA, Martina BIRTEL, Anke-Gabriele LENZ and Helmut HOLZER

Biochemisches Institut der Universität, D-7800 Freiburg im Breisgau (West-Germany) and GSF-Abteilung für Enzymchemie, D-8042 Neuherberg bei München (West-Germany)

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Summary: Addition of glucose to glucose-derepressed yeast cells causes disappearance of 60 % of the activity of fructose-1,6-bisphosphatase within 3 to 5 min. Reversibility of this "catabolite inactivation" reaction in a glucose-free medium is independent on de novo protein synthesis. The pH-optima of fructose-1,6-bisphosphatase activity in gel-filtrated crude extracts were shown to be 8.25 for the enzyme from derepressed cells and 8.8 for the enzyme from cells treated with glucose for 4 min. In studies with |^{3}H|-leucine labelled glucose-derepressed cells the protein cross reacting with antibodies against fructose-1,6-bisphosphatase did not disappear within the first 10 min after addition of glucose. These findings suggest that the glucose induced rapid inactivation of the enzyme is the result of a covalent modification which decreases the fructose-1,6-bisphosphatase activity and changes the pH-activity profile of the enzyme, but does not change its immunological reactivity to antibodies. It is concluded that the covalent modification renders the enzyme susceptible to proteinases and thereby initiates its selective proteolysis.

Introduction: In a preceding paper it was shown that the kinetics of the glucose induced catabolite inactivation of fructose-1,6-bisphosphatase (1,2) shows two phases. In the first phase, which we called "short term inactivation" about 60 % of the activity of the enzyme disappears rapidly within 3 min. A second phase, called "long term inactivation" follows which leads to an almost complete disappearance of the enzyme activity within 60 min (3). Transfer of the yeast cells incubated in glucose into a glucose-free, ace-

This paper is dedicated to Prof.Dr.Joachim KÜHNAU on the occasion of his 80th birthday.

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tate containing medium leads to reversibility of the "short term inactivation" in the presence of cycloheximide. Reactivation is therefore independent from de novo protein synthesis (3). In contrast to this , no reversibility of the inactivation reaction can be observed in the presence of cycloheximide after "long term inactivation" (1). These findings were discussed as an indication for a rapid glucose-dependent covalent interconversion of the active fructose-1,6-bisphosphatase to an only 40 % active form of the enzyme, followed by proteolytic degradation of the less active form. In the present paper, it is demonstrated that different forms of the enzyme exist, which exhibit different pH optima for activity. This is further evidence for the existence of two different stable forms of fructose-1,6-bisphosphatase. Furthermore it is shown, that the concentration of antibody cross reacting material of fructose-1,6-bisphosphatase does not decrease during the "short term inativation" reaction but only during the "long term inactivation" process. These findings are discussed as evidence for a glucose induced metabolic interconversion of the active form of fructose-1,6-bisphosphatase to a less active form, preceding the proteolytic degradation of the enzyme.

Materials and Methods: The reagents for the enzyme assays were obtained from Boehringer (Mannheim). All yeast growth media were obtained from Difco (Detroit, MI). Phenylmethylsulphonyl fluoride and Triton X-100 were from Serva (Heidelberg), DEAE-Sephadex A-50 from Pharmacia (Uppsala, Sweden), L-|4,5-^{3}H|leucine (48 Ci/mmole) and NCS tissue solubilizer from Amersham (Braunschweig), toluene scintillator Rotiszint 11 from Roth (Karlsruhe), pepstatin A and DL-dithiothreitol from Sigma (München). All other chemicals were obtained from Merck (Darmstadt).
The rabbit antiserum against fructose-1,6-bisphosphatase (cf. 4) was a gift of Drs. Carlos and Juana Maria Gancedo (Madrid).

The diploid yeast Saccharomyces cerevisiae M_1 (supplied by Dr.A.W. Linnane, Monash University, Clayton, Australia) was used in these experiments. Growth conditions, cell collection and resuspension in media were done as previously reported (3). For quantitative immunoprecipitation of radioactive fructose-1,6-bisphosphatase

protein was labelled by adding 15 µCi L-[4,5-^{3}H]leucine per ml of culture fluid to a cell culture undergoing release from repression by glucose (7 h of growth). Inactivation of the enzyme by 2 % glucose was performed at 30°C using a cell suspension (2 % wet wt/vol) in 0.1 M potassium phosphate buffer pH 6.0 which had been preincubated for 5 min without glucose. At different times of inactivation 13.6 ml samples were collected, washed, resuspended (12 % wet wt/vol) and passed through a French pressure cell as previously described (3). The cell extract supernates obtained from the homogenate after 30 min centrifugation at 120,000 x g were used for determination of enzyme activity, protein and for quantitative immunoprecipitation. Fructose-1,6-bisphosphatase activity was assayed as described in (5) except that fructose-1,6-bisphosphate was 0.2 mM. Protein was determined according to Lowry et al. (6) using bovine serum albumin as standard.

Antiserum against fructose-1,6-bisphosphatase precipitated unidentified labelled proteins in addition to fructose-1,6-bisphosphatase from crude cell extracts. Thus, to remove contaminating antibodies, 0.8 ml portions of the antiserum were mixed several times with 0.8 mg of protein of a concentrated extract from cells in which fructose-1,6-bisphosphatase was glucose-repressed. After each addition the antiserum was incubated 24 h at 4°C in the presence of 2 mM NaN_3 and the precipitate that had been formed was discarded. Additions of protein to the antiserum were continued until no further precipitation was seen any more (usually about 15 times). The IgG fraction of the antiserum was subsequently purified by ammonium sulfate precipitation and DEAE-Sephadex A-50 chromatography as described in (7). Such treatment of the antiserum remarkably improved the specificity of the immunoprecipitation, as shown in fig. 1.

For quantitative immunoprecipitation 0.2 - 0.3 ml samples of the crude extracts (equivalent to 0.25 units of fructose-1,6-bisphosphatase at zero time of glucose inactivation) were incubated 2 h at 4°C in 0.15 M NaCl with a twofold excess of purified antiserum with respect to the zero time sample. 1.5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride and 5 µg/ml pepstatin A were added in addition. The final volume of the incubation mixture was about 1.2-times of the sample volume. The immunoprecipitation of fructose-1,6-bisphosphatase was complete under these conditions (not shown). Collection of fructose-1,6-bisphosphatase-CRM, washing, solubilization and SDS-disc electrophoresis of the immunoprecipitates on rod gels, Coomassie staining and destaining as well as slicing of the gels and radioactivity counting were carried out as reported in (7).

Results and Discussion: The dependency of fructose-1,6-bisphosphatase hydrolysing activity of pH was measured in diethylamine/HCl-buffer using extracts of derepressed cells and "short term" (4 min) glucose-inactivated cells. The results are shown in fig. 2. The activity profiles show distinct differences. The enzyme of derepressed cells exhibits its maximum of activity at pH 8.25 whereas

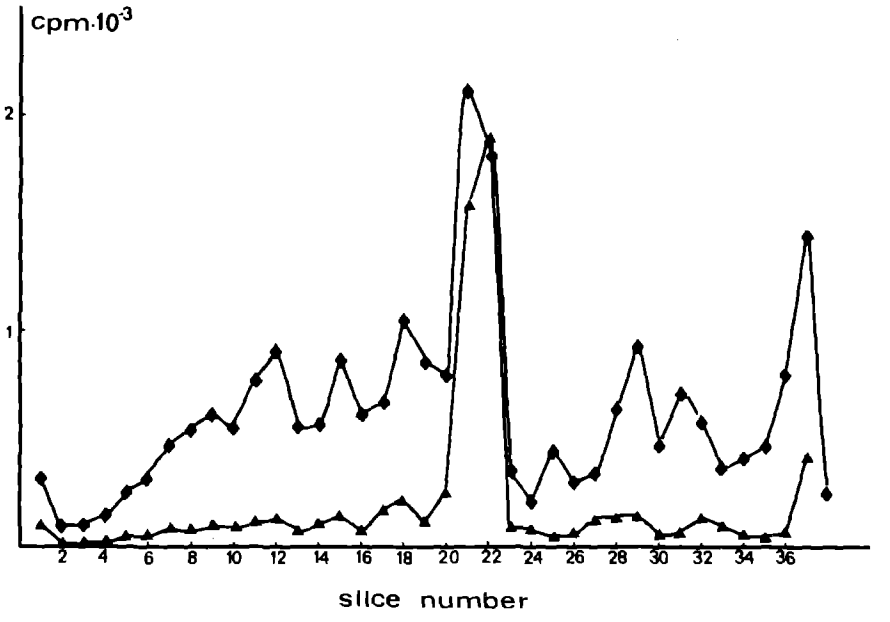

Figure 1: SDS-polyacrylamide gel electrophoresis of precipitates formed by addition of antiserum against fructose-1,6-bisphosphatase to extracts of $|^3H|$leucine labelled derepressed yeast. Cell extract pretreated (▲-▲) and untreated (◆-◆) antiserum were used in this experiment. The location of the peak of radioactivity with pretreated antiserum was identical with the peak obtained when purified fructose-1,6-bisphosphatase was precipitated and submitted to gel electrophoresis (not shown). Preparation of extracts, immunoprecipitation, solubilization, electrophoresis, gel slicing and radioactivity counting were carried out as described in Materials and Methods. Ordinate: cpm per slice.

the enzyme from inactivated cells shows its maximum of activity at pH 8.80. Therefore the glucose initiated "short term inactivation" of fructose-1,6-bisphosphatase can only be observed if one compares the enzyme activity at pH values below 8.9. At higher pH values no "inactivation" of fructose-1,6-bisphosphatase would be observed. When measuring the pH activity profiles of the active and the short term inactivated fructose-1,6-bisphosphatase one has to take into account that these profiles strongly depend on the buffer used (not shown). The enzyme preparations used for pH optimum measurements have been prepared by gel-filtration of extracts on Sephadex G-25. Thus the characteristic differences of the activity

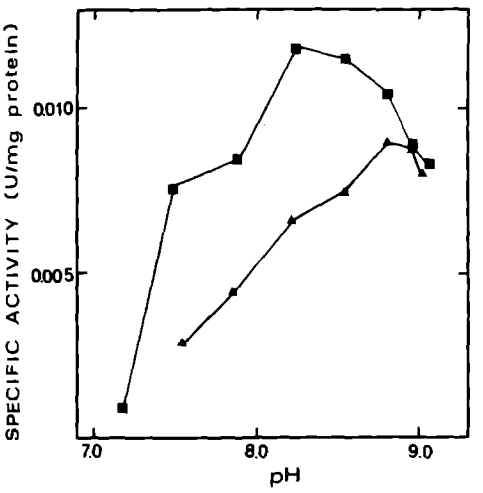

Figure 2: Activity vs. pH profiles of derepressed (■) and 4 min glucose inactivated (▲) fructose-1,6-bisphosphatase. Cells were suspended in 0.1 M diethanolamine buffer pH 6.0 at 28°C and an aliquot was inactivated for 4 min by addition of glucose as described previously (3). Crude extracts were prepared from 50 % cell suspension (wet wt/vol) in 0.1 M diethanolamine buffer pH 7.0 using a French pressure cell as described in (3). The centrifuged (27,000 x g) extract was filtrated through Sephadex G-25. The pH was adjusted to the desired values by addition of 1 N HCl. The optical tests for activity at λ = 366 nm and d = 1 cm were carried out in a reaction mixture (total vol. 1.5 ml) which contained: 50 mM diethanolamine buffer, 10 mM $MgCl_2$, 0.76 mM $NADP^+$, 1.17 units phosphoglucose-isomerase, 0.58 units glucose-6-phosphate dehydrogenase, 0.2 mM fructose-1,6-bisphosphate and 10 or 20 µl of extract, respectively.

profiles are most likely not the result of influences of loosely bound low molecular weight effectors on the enzyme, but indicate the existence of different stable forms of fructose-1,6-bisphosphatase. Differences in the profiles of activity vs. pH have been observed previously for the different forms of glutamine synthetase from Escherichia coli and for other enzymes with different covalently modified forms (8). It should be emphasized, however, that interaction with a high molecular weight "modifying protein" which firmly binds to the fructose-1,6-bisphosphatase and which is degraded in vivo in the presence of glucose could also explain the differences in the pH/activity profiles shown in fig. 2.

The results shown in fig. 3 clearly demonstrate that the "short

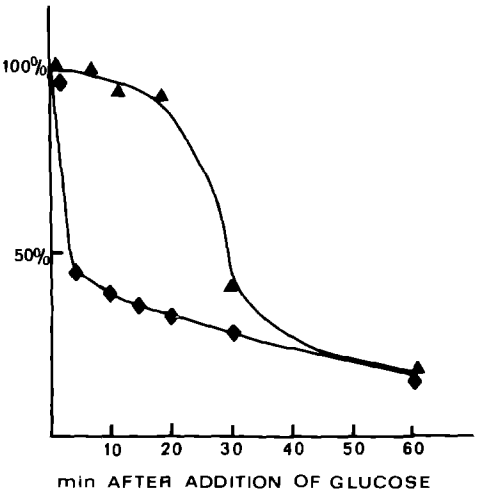

Figure 3: Immunological cross reacting material (cpm per slice, ▲) and specific catalytic activity of fructose-1,6-bisphosphatase (U/mg protein, ◆) after addition of glucose to derepressed yeast cells. [^{3}H]leucine-labelled cells were washed and resuspended in 0.1 M potassium phosphate buffer pH 6.0. At zero time glucose at a final concentration of 2 % was added and the cells were further incubated at 30°C. At the indicated times yeast samples were collected by centrifugation, extracts prepared and specific catalytic activity and radioactivity in cross reacting material determined as described in Materials and Methods. The values at zero time were set to 100 %.

term" (5 min) glucose-inactivated fructose-1,6-bisphosphatase protein, which is the "less active enzyme" still exhibits full antigenic reactivity. This represents strong evidence for the idea that "short term inactivation" does not consist in a proteolytic degradation of the enzyme but only in a minor change in structure. This change leads to a decrease of about 60 % of the fructose-1,6-bisphosphatase activity, when assayed either at pH 8.25 in diethanolamine buffer or at pH 7.0 in imidazole/HCl buffer, which can be reversed in the presence of cycloheximide, i.e. without de novo protein synthesis (3). When preparing fructose-1,6-bisphosphatase from cells grown on a medium containing ^{32}P-orthophosphate, preliminary experiments show that in contrast to the "active form" the "less active form" of the enzyme contains firmly bound ^{32}P,

which is not released into the supernatant by treatment with trichloroacetic acid ((9), Doris Müller: unpublished experiments). This points to a covalent modification by a phosphorous containing group (phosphorylation, adenylation etc.) as the chemical mechanism of "short term inactivation".

It may be seen from fig. 3 that about 10 min after the addition of glucose a rapid decrease of the antigenic reactivity of fructose-1,6-bisphosphatase is observed. In accordance with the observation from Funayama et al. (10) this finding indicates proteolytic degradation of the enzyme. We assume that the covalent modification of the enzyme occuring in the first minutes after addition of glucose renders the enzyme susceptible to proteinases and thereby initiates selective proteolysis. Covalent modification of enzymes by the respective modifying enzymes upon the occurence of metabolic effectors is known to be highly specific under biological conditions (8). It might therefore well be that the initiation of proteolysis by covalent modification as proposed in the present paper for fructose-1,6-bisphosphatase represents a more generally occuring mechanism for selective proteolysis (11).

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