Changes in the activities of key enzymes of glycolysis during the cell cycle in yeast: a rectification

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Activities of glycolytic enzymes were determined in elutriation fractionated cultures of *Saccharomyces cerevisiae* grown on different carbon sources. Almost pure fractions of single cells at the G1 stage of cell division were obtained for some of the growth conditions tested, whereas other stages were enriched in particular fractions. Specific activities of glucose-6-phosphate dehydrogenase and alcohol dehydrogenase were found to be constant during the cell cycle, as reported by van Doorn *et al.* (1988*a*), *Journal of Bacteriology* 170, 4808–4815, and (1988*b*), *Journal of General Microbiology* 134, 785–790. In contrast to the earlier reports, the activities of hexokinase, phosphofructokinase, pyruvate kinase and trehalase were also constant in different stages of the cell cycle. For hexokinase and phosphofructokinase it was shown that the apparent specific activity in a cell-free extract strongly diminished when extracts contained less than 0.5–1 mg protein ml⁻¹. In the experiments of van Doorn *et al.* (1988*a*) the protein content of the outer fractions was up to 20 times lower than that of the central fractions, suggesting an alternative explanation for the observed changes in enzyme activities during the cell cycle. Therefore, we want to rectify the observations presented by van Doorn *et al.* (1988*a*), and conclude that the activities of the glycolytic enzymes do not vary greatly during the cell cycle of *S. cerevisiae*.

Introduction

Although growth of baker's yeast on glucose and other C₆ carbon sources has been studied extensively (for a review see Gancedo & Serrano, 1989), many aspects of the regulation of glucose metabolism are incompletely understood. Depending on the conditions used in the experiments, hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK) and glucose transport have been considered as steps that have a large control over the flux. For most of these glycolytic enzymes one or more mechanisms that control the activity of the enzyme have been established *in vitro*. For example, PFK activity was found to be influenced by (amongst others) NH₄⁺, phosphate, AMP and fructose 1,6-bisphosphate (Sols, 1981). Previous studies in our laboratory showed that the *in vitro* specific activity of these enzymes varied greatly when cells in different stages of the cell cycle were assayed. Using elutriation fractionation, a greatly re-

duced *in vitro* activity of several glycolytic enzymes was observed in the G1 phase (van Doorn *et al.*, 1988*a*). A similar change in activity was also observed for trehalase in a synchronously growing culture. This change could be eliminated by preincubation of the cell extract with a cAMP-dependent protein kinase, suggesting a dephosphorylation of the protein at the start of the G1 phase to be the cause of the observed fluctuations in specific activity (van Doorn *et al.*, 1988*b*). The mechanism for these changes in activity of the glycolytic enzymes has not been clarified. Studies with partly synchronized continuous cultures have shown significant changes in CO₂ production and O₂ consumption during the cell cycle (Küenzi & Fiechter, 1969). Although such results are not directly transferable to undisturbed, exponentially growing cultures they indicate that the flux through the glycolytic pathway is different in the various growth stages. From these results it seemed reasonable to propose that models describing the flux through glycolysis should include the cyclic changes of enzyme activities during the cell cycle, thus making them much more complicated.

This paper shows that the previously reported changes in enzyme activity were mainly due to differences in the

Abbreviations: PMSF, phenylmethylsulphonyl fluoride; KP_i, potassium phosphate buffer; HK, hexokinase; PFK, phosphofructokinase; PK, pyruvate kinase; ADH, alcohol dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase.

concentration of cells during extraction. When constant amounts of cell material were taken from all fractions, representing different stages of the cell cycle, no significant changes in the specific activities of glycolytic enzymes were observed, whether the cells were grown on glucose or on maltose or ethanol.

Methods

Organisms and cultivation. *Saccharomyces cerevisiae* strain DL1 was grown on YEPD [2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract] and *S. cerevisiae* strain Y185.1 (Creanor *et al.*, 1983) on Yeast Nitrogen Base (Difco) supplemented with maltose (0.5%) or ethanol (1%, v/v). Cells were cultivated at 28 °C in a rotary incubator at 180 r.p.m. for at least 20 generations in exponential phase and harvested at a density of $3-5 \times 10^6$ cells ml⁻¹.

Elutriation. Elutriation was performed as described by van Doorn *et al.* (1988a), except that a single 40 ml rotor cell was used. A Masterflex model 6523 (Cole Parmer Company, Chicago, USA) equipped with two pumpheads (pulsing out of phase) with tubing 16 was used to obtain the desired flow through the rotor. Pelleting of cells at the bottom of the chamber was prevented by using a small amount of Maxidens (Nycomed, Oslo, Norway) at the bottom (approx. 0.5% of the chamber volume).

Routinely, fractionation experiments were done at 2 °C using 20 mM-potassium phosphate buffer (KP_i) pH 6 for elutriation. The culture was cooled quickly in ice/water, sonicated for 20 s (in 0.5 litre portions) and pumped into the rotor cell, spinning at 4500 r.p.m., at a flowrate of 60 ml min⁻¹. To prevent disturbance due to density or viscosity changes the last part of the culture was gradually mixed with elutriation buffer. After simultaneous decrease of flow rate and spinning speed to the preset values, fractionation was performed by stepwise increase of the flow rate as indicated for the different experiments. Fractions of 400 ml were collected and a suitable part was concentrated on 1.2 µm membrane filters (25 mm diam., 1.2 µm pore size). Cells were resuspended in 1 ml KP_i pH 6 in an Eppendorf tube, pelleted, frozen in liquid nitrogen and stored at -70 °C.

Cells were counted and studied morphologically as described previously (van Doorn *et al.*, 1988a).

Preparation of cell-free extract and enzyme assays. Extract was prepared by vortexing (2200 r.p.m.) with acid-washed glass beads at 4 °C on a Ika Vibrax VXR (Janke & Kunkel). On the basis of relative cell volume (calculated from the flow rate) and cell number, it was calculated how much of the frozen pellet should be used for the extraction procedure to obtain a similar protein content in each fraction. A linear relation between relative cell volume and protein content was assumed (Oehlen *et al.*, 1990). The calculated portion of the cells was resuspended in 200 µl disruption buffer (20 mM-KP_i, pH 7.0, 50 mM-NaF) and added to 0.7 ml Eppendorf tubes containing 200 mg glass beads (0.45 mm). Directly after disruption, 0.25% (v/v) of 400 mM-PMSF solution dissolved in dimethyl sulfoxide was added and cell debris was removed by centrifugation for 10 min in an Eppendorf centrifuge.

Enzyme activities were determined spectrophotometrically at 340 nm and 30 °C. Pyruvate kinase was assayed in 100 mM-potassium phosphate pH 6.0, containing MgSO₄, 30 mM; phosphoenolpyruvate, 5 mM; ADP, 2 mM; fructose 1,6-bisphosphate, 1 mM; NADH, 0.15 mM; lactate dehydrogenase, 1.4 U. Phosphofructokinase was measured in 50 mM-Tris/HCl, pH 7.2, according to Hofmann & Koppersläger (1982). Hexokinase was measured in 50 mM-triethanol-

amine/HCl pH 7.6, containing MgSO₄, 10 mM; ATP, 1 mM; NADP⁺, 0.4 mM; glucose-6-phosphate dehydrogenase, 0.6 U; phosphoglucose isomerase, 1 U and fructose, 10 mM. Glucose-6-phosphate dehydrogenase and alcohol dehydrogenase were measured as described by Bergmeyer *et al.* (1974b). Trehalase activity was determined in 20 mM-MOPS pH 6.8 containing PMSF, 1 mM; NaF, 50 mM; CaCl₂, 2.5 mM; trehalose, 20 mM. After 1 h the reaction was terminated by boiling the samples for 3 min and liberated glucose was determined as described by Bergmeyer *et al.* (1974a). For each sample a control was run without trehalose. All assays were tested for linearity with the quantity of extract. Usually analyses were performed on a Cobas Bio automatic analyser (Roche).

Protein was determined by means of the bicinchoninic acid reagent as described by the supplier (Sigma) using bovine serum albumin as a standard.

Results

Fractionation of S. cerevisiae Y185.1 grown on 0.5% maltose

Cells growing exponentially on maltose were fractionated according to cell size using the large elutriation cell as described in Methods. Fig. 1(a) shows the distribution of the different cell types. At flow rates up to 65 ml min⁻¹ almost pure fractions of single cells were obtained. At higher flow rates, fractions were enriched in cells with a bud, whereas at the highest flow rates binucleate cells were more abundant. In contrast to the single cells, the latter types were not obtained as pure fractions.

Fig. 1(b, c) shows the *in vitro* activity of some enzymes involved in glucose metabolism as found in cell extracts from the different fractions. The ADH and G6PDH activities did not vary significantly in the various fractions, in accordance with the results obtained for *S. cerevisiae* DL1 grown on YEPD by van Doorn *et al.* (1988a). However, in contrast to the earlier study no large changes in activity of PK were observed either, and for PFK and HK the differences in activities between the various fractions were much smaller than reported for strain DL1. Another feature that can be observed in Fig. 1(c) is the correlation between the patterns of HK activity and protein content. This might suggest that the observed changes are (partly) due to differences in protein content of the extracts.

Fractionation of S. cerevisiae DL1 grown on YEPD

The differences in patterns of enzyme activities found in the present study for strain Y185.1 on maltose compared with previous findings for strain DL1 on YEPD, together with the possible effect of protein content of the extract on the activity of some enzymes, induced us to reinvestigate the results published by van Doorn *et al.* (1988a). In Fig. 2 the distribution of the different cell types and the activities of HK, PFK, ADH, PK,

Fig. 1. Elutriation fractionation of *S. cerevisiae* Y185.1 grown aerobically on mineral medium containing 0.5% maltose. (a) Distribution of the different cell types over the fractions: $\square$, single cells; $\triangle$, cells with bud; $\circ$, binucleate cells. (b, c) Activities [in $\mu\text{mol min}^{-1} (\text{mg protein}^{-1})$] of pyruvate kinase (PK, ∇), alcohol dehydrogenase (ADH, $\diamond$), glucose-6-phosphate dehydrogenase (G6PDH, $\circ$), hexokinase (HK, $\square$) and phosphofructokinase (PFK, $\triangle$) in the different fractions. One unit (U) is $1 \mu\text{mol min}^{-1}$. In (c) the protein content of the various samples is also plotted ($\bullet$).

G6PDH and trehalase in the different fractions after elutriation fractionation of strain DL1, grown on YEPD, are shown.

For G6PDH and PK only small, insignificant differences between the various fractions were observed. For the other enzymes a somewhat larger variation was measured, although the significance of the observed patterns may be disputed. In duplicate experiments (not

Fig. 2. Elutriation fractionation of *S. cerevisiae* DL1 grown on YEPD. (a) Distribution of the different cell types over the fractions; (b, c) enzyme activities. $\blacksquare$, Trehalase; the other symbols and units are as in Fig. 1.

shown) the patterns observed for these enzymes usually showed fluctuations of comparable magnitude, but without consistency between the duplicates. In no case did we find any clear evidence for a pattern as observed by van Doorn *et al.* (1988a).

Effect of cell density at extraction on specific activity

The similarity in the patterns for protein content and HK (and PFK) in Fig. 1 suggests that the protein content of the extract influences the specific activity. This relation was observed in most of the experiments. We therefore

Fig. 3. Effect of preparing extracts at different cell densities on the specific activity of several glycolytic enzymes. Extracts were prepared from a series of dilutions of a concentrated cell suspension of *S. cerevisiae* DL1, grown on YEPD. Portions (0.5 ml) of cell suspension were incubated with 0.5 g glass beads for 15 min in 1.5 ml Eppendorf tubes at 4 °C on a Vibrax at 2200 r.p.m. Cell debris was removed by centrifugation (10 min, 12000 r.p.m., 4 °C) and extracts were analysed on a Cobas Bio analyser (after appropriate dilution) for enzyme activities. Symbols and units are as in Fig. 1.

decided to investigate the relation between the cell density during extraction and the specific activity of some enzymes. The amount of protein extracted showed an almost linear relationship to the quantity of cells added; only at very low cell densities was somewhat less protein extracted (not shown). Extracts were diluted with extraction buffer to give approximately the same protein content in the assay. Fig. 3 shows the specific activities of HK, PFK, ADH, G6PDH and PK measured in the different extracts. For ADH and G6PDH the specific activity was nearly constant unless the extracts contained less than 0.05 mg protein ml⁻¹, whereas PK showed the highest specific activity around a protein content of 0.1–0.3 mg ml⁻¹, and was virtually constant above 0.3 mg protein ml⁻¹; below 0.1 mg ml⁻¹ the specific activity started to decrease. HK and PFK activities however, were reduced below 0.5–1 mg protein ml⁻¹ and were virtually zero at the lowest protein concentrations

Fig. 4. Elutriation fractionation of *S. cerevisiae* Y185.1 grown aerobically on mineral medium containing 1% ethanol. (a) Distribution of the different cell types over the fractions; (b, c) enzyme activities. Symbols and units are as in Figs 1 and 2.

investigated. This effect was caused by the extraction at low cell density and was not due to the dilution of the extract itself: dilution of a normal (2 mg ml⁻¹) extract to a protein content of about 50 µg ml⁻¹ in extraction buffer directly after the glass bead treatment did not influence the specific activity (not shown).

Elutriation fractionation of ethanol-grown cells

Although no important changes in the activity of glycolytic enzymes were observed after growth on glucose or maltose, we wished to investigate the possibility that changes occur under conditions where

glycolysis is not active. We therefore decided to test strain Y185.1 grown on 1% ethanol. Growth on ethanol is slow and as a result the number of single (G1) cells was increased compared with growth on maltose (compare Fig. 4a with Fig. 1a). The levels of most glycolytic enzymes were lower than during growth on maltose or glucose (Fig. 4b, c). Not surprisingly, ADH was increased significantly, as was trehalase. Large changes in activities during the cell cycle were, however, not observed.

Discussion

In most metabolic studies on yeast, effects of the cell cycle on metabolism have not been taken into account. The reason is presumably the experimental difficulty in obtaining sufficient cells at a certain stage of the cell cycle without applying an external stress. Experiments with synchronized continuous cultures, however, show that the flux through glycolysis is not constant during passage through the cell cycle (Küenzi & Fiechter, 1969). Using elutriation fractionation it is possible to obtain samples from an exponentially growing culture enriched in a particular stage of the cell cycle (see Figs 1a, 2a and 4a). However, only G1 cells are obtained in an almost pure form; the other stages are always mutually contaminated, which can obscure possible changes.

The studies of van Doorn *et al.* (1988a, b) on cells fractionated by elutriation indicated that in an exponentially growing culture cells contain an enzymic machinery that is dependent on the cell cycle. The quantity of cells obtained was very low, especially in the outer fractions. Consequently, unless special precautions are taken, the outer fractions tend to contain (much) less protein than the fractions in the middle (as was observed in the crude data of van Doorn *et al.*, 1988a). From Fig. 3, it can be seen that the apparent specific activities of the enzymes HK and PFK were very sensitive to the amount of protein in the extract when protein contents fell below 0.5 mg ml⁻¹. Although G6PDH and ADH were also influenced by the protein content, these enzyme activities were almost constant down to 0.05 mg protein ml⁻¹. The reason for the protein-dependent decrease in specific activity of HK and PFK is unknown but in a control experiment it was shown that dilution of the extract had no effect on the measured specific activity. Because it is quite unusual to prepare extracts with such a low protein content, further investigations on the background of this peculiar phenomenon were not undertaken.

The dependence of the specific activity on the concentration of cells during extraction probably ex-

plains why the activities of the enzymes HK and PFK in the experiments of van Doorn *et al.* (1988a) appeared to change dramatically as a function of the cell cycle whereas those of ADH and G6PDH did not. For PK and trehalase the observed patterns are less easily explained, although PK sometimes showed an irreproducible behaviour and trehalase activity was low, making it very difficult to assay in dilute extracts. The pattern found by van Doorn *et al.* (1988b) for trehalase in synchronized cultures cannot be explained by differences in protein content. In those experiments, cells were lysed with lyticase and possibly small, unbudded cells may be somewhat more resistant to the lytic enzyme treatment. Prolonged incubation for the activation of trehalase by protein kinase presumably then resulted in total lysis of all samples. This might explain why trehalase, as it was assayed by van Doorn *et al.* (1988b), seemed to be activated by protein kinase treatment. Because of the low pH of the assay mixture, however, the vacuolar trehalase was measured in these experiments whereas only the cytosolic trehalase is activated by protein kinase (Londesborough & Varimo, 1984).

In the elutriation experiments described in this paper, more cells per fraction were obtained by the use of a large elutriation cell and calculations were done to ensure that the protein content of the various fractions did not differ more than twofold. Under these conditions the specific activity of glycolytic enzymes was found to vary at most by a factor of two and the variations that showed up in a single experiment were not found in the repeats. The reason for these intra- and interexperimental variations is not known. Duplicate assays of the same extract on the Cobas Bio automatic analyser resulted, whenever tested, in differences of less than 2%. All experiments were repeated at least twice. Taking averages of the repeats was not feasible, however, because the flow rate was not a set constant but was determined experimentally.

Preliminary results with synchronous cultures obtained by further cultivation of a single-cell fraction from an elutriation showed that under these conditions also large changes in activity are absent. We therefore conclude that the activities of HK, PK, PFK, trehalase, G6PDH and ADH do not vary to a large extent during the cell cycle of exponentially growing *S. cerevisiae*. However, due to the limited accuracy of the methods employed, more subtle cell-cycle-dependent differences in enzyme activities like the 'linear patterns' found by Creanor *et al.* (1983) cannot be excluded.

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