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Yeast cells with a specific cellular make-up and an environment that removes acetaldehyde are prone to sustained glycolytic oscillations

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Abstract

Glycolytic oscillations can be induced by adding glucose to starved *Saccharomyces cerevisiae* cells and, after a steady state has been established, cyanide. Transient oscillations or limit-cycle oscillations can be induced depending on the growth phase in which the cells are harvested. To find what causes these differences in the dynamic behaviour, we analyzed glycolytic enzyme activities at different growth phases. The hexokinase activity increased by a factor of three after growth substrate transition from glucose to ethanol; the other measured activities remained constant. Cyanide was found not only to block respiration, but also to trap acetaldehyde. Both cyanide actions appear necessary for the occurrence of sustained glycolytic oscillations.

Key words: *S. cerevisiae*; Signalling; Yeast glycolysis; Flux control; Regulation; Limit cycle; Growth phase independence

1. Introduction

Oscillations in glycolytic metabolism occur in a variety of organisms and conditions (for reviews see [1–3]). After glucose has been added to intact starved cells and a steady state has been reached, addition of cyanide leads to oscillations in intracellular NADH (see also [4]). At high cell densities the oscillations last longer than at low cell densities [5,6]. Only at high cell concentrations, due to a synchronizing mechanism, the cells may remain in phase [7].

An other determinant of the duration of the oscillations is the phase of growth where the cells are harvested. Yeast cells that have been grown in batch culture, exhibit long-lasting oscillations when harvested around the growth phase transition from using glucose to using ethanol as a growth substrate. A short train of oscillations is observed when the cells are harvested during growth on glucose or during growth on ethanol [8,9]. Most probably these differences reflect differences in the cellular make-up, rather than differences in metabolite concentrations, since this behaviour was conserved over hours of starvation. By modulating cell density and time of harvesting, it was possible to demonstrate sustained oscillations of NADH in a population of yeast cells [10]. In the present work we elaborate on the differences in cellular make-up which decide between limit-cycle oscillations and macroscopic relaxation behaviour.

One role of cyanide in the oscillations is to simulate anaerobiosis at the level of cytochrome oxidase. However, we found that under anaerobic conditions in the absence of cyanide, oscillations were less pronounced. Moreover, other inhibitors of respiration or anaerobiosis led to a much shorter train of oscillations than cyanide [11]. In this paper we examine the possible side effects of cyanide. We report that its known reactivity with acetaldehyde is important for the persistence of the oscillations.

2. Materials and methods

The yeast *Saccharomyces cerevisiae* (X2180 diploid strain) was grown, harvested and starved as described [9].

The enzyme activities of the starved cells were determined as follows. Cells were resuspended at a protein concentration of 15 g/l in 100 mM PIPES (NaOH) pH 7.0 containing 1 μM phenylmethyl-sulfonyl fluoride (PMSF). 1 ml of this suspension was shaken with 1 g of glass pearls (diameter 0.4–0.5 mm) at 4°C for 15 min on a Vortex (VXR, Ika) and centrifuged for 5 min in a table centrifuge (Microliter, Hettich). The supernatant was diluted in the same buffer to protein concentrations between 1.5 mg/l to 3 g/l and kept on ice until the enzyme activities were measured in an automated analyzer (Cobas Bio, Roche).

For hexokinase activity the assay contained 45 mM PIPES (NaOH) pH 7.0, 8 mM $MgSO_4$, 0.15 mM $NADP^+$, 0.8 mM ATP, 0.45 U/ml glucose 6-phosphate dehydrogenase and 0.9 U/ml phosphoglucoisomerase. The reaction was started with 10 mM fructose. For phosphofruc-

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tokinase activity the extract additionally contained 1 g/l BSA and the assay contained 45 mM PIPES (NaOH) pH 7.0, 8 mM $MgSO_4$, 20 mM KCl, 1 g/l BSA, 0.6 mM phosphoenol pyruvate, 0.5 mM fructose 1,6-bisphosphate, 0.4 mM ATP, 0.12 mM NADH, 15 μM fructose 2,6-bisphosphate, 0.4 mM AMP, 4 U/ml pyruvate kinase and 4 U/ml lactate dehydrogenase. The reaction was started with 2.2 mM fructose 6-phosphate. For aldolase activity the assay contained 45 mM PIPES (NaOH) pH 7.0, 0.12 mM NADH, 80 mM KCl, 0.7 U/ml glycerol 3-phosphate dehydrogenase and 8 U/ml triosephosphate isomerase. The reaction was started with 2.3 mM fructose 1,6-bisphosphate. For pyruvate decarboxylase activity the assay contained 160 mM (Na)-citrate (pH 6.0), 0.1 mM NADH and 30 U/ml alcohol dehydrogenase. The reaction was started with 45 mM (Na)pyruvate. The alcohol dehydrogenase activity was assayed as described [12]. For all enzyme assays, titrations with the pure enzymes (Boehringer or Sigma) were performed to identify the range where enzyme concentration was proportional to the measured enzyme activity.

The oscillations were induced by adding 20 mM glucose to a stirred and thermostated (25°C) yeast cell suspension and, after 4 min, 3 mM KCN. Oscillations in NADH were monitored in a fluorimeter [9].

The acetaldehyde and lactonitril was measured after Shachar-Nishri and Freeman [13] modified for smaller concentrations. 100 μl of a Millipore filtrate was added to an equal amount of Tris-HCl (500 mM, pH 8.0), followed by 750 μl of dinitrophenyl hydrazine (0.3 g/l) in 4 N HCl. The mixture was incubated overnight at 4°C, centrifuged (Microliter, Hettich), the supernatant absorption measured at 405 nm, and referred to a calibration using acetaldehyde standards.

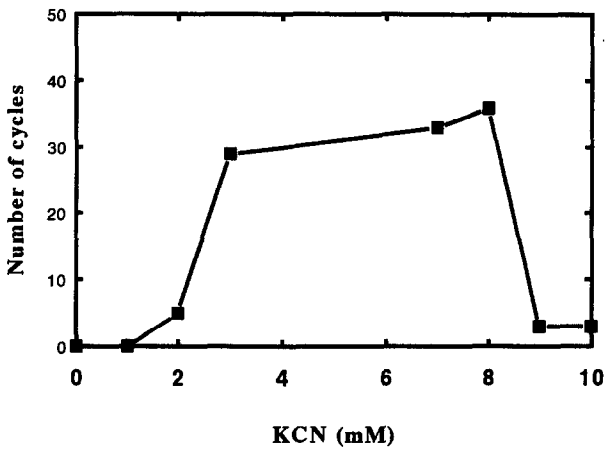

Fig. 2. Dependence of the duration of glycolytic oscillations on the concentration of cyanide. Shown are the number of cycles in NADH oscillation after addition of different concentrations of cyanide to starved cells (13.8 g protein/liter) provided with 40 mM of glucose. Cells were harvested at the transition from using glucose to using ethanol as a growth substrate.

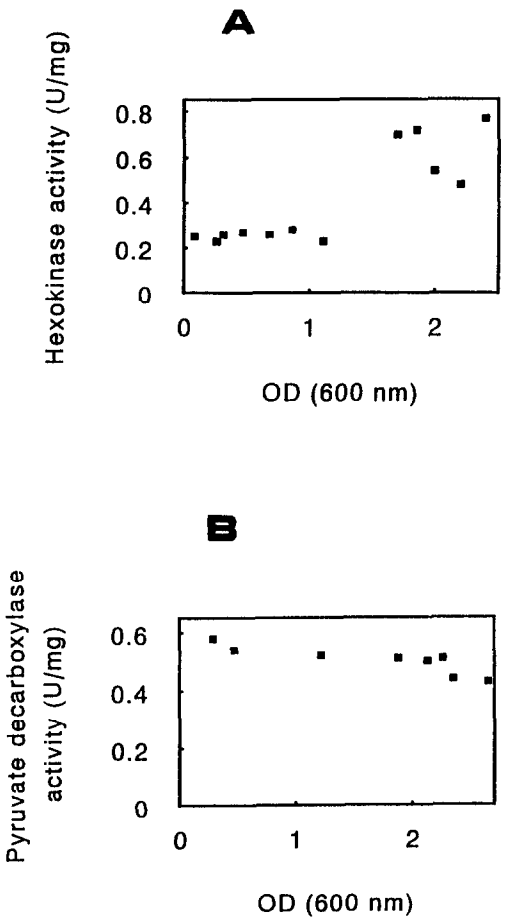

Fig. 1. Variation of enzyme activities in *Saccharomyces cerevisiae* during aerobic batch growth on glucose. (A) hexokinase, (B) pyruvate decarboxylase. Activities are given per total protein. At an optical density of 1.8, glucose was exhausted and growth continued, using the generated ethanol as a substrate.

3. Results

We have measured the activities of the glycolytic enzymes hexokinase, phosphofructokinase, aldolase, pyruvate decarboxylase and alcohol dehydrogenase as a function of growth of the cells. Fig. 1A shows how hexokinase activity varied with the optical density of the culture at the time of harvesting the cells. Glucose was exhausted at an optical density of 1.8. During growth on glucose the activity of hexokinase was about 0.25 U/mg of total protein. Close to the transition from using glucose to ethanol as a growth substrate, the activity suddenly increased 3-fold to approximately 0.7 U/mg. Analyzing the glycolytic intermediates during the glycolytic oscillations we have found a very high pyruvate concentration [14], which could be due to pyruvate decarboxylase being a bottleneck in the pathway. The pyruvate decarboxylase activity was, however, constant around 0.5 U/mg at all stages of growth (Fig. 1B). We have also analyzed phosphofructokinase, aldolase and alcohol dehydrogenase and could not detect any variation in activity during growth. Phosphofructokinase had a constant activity of approximately 0.4 U/mg, aldolase of 0.4 U/mg and that alcohol dehydrogenase activity amounted to 2.4 U/mg.

We then measured the influence of the cyanide concentration on the duration of the oscillations. To yeast cells with a protein concentration of 13.8 g/l, 40 mM glucose was added and 4 min later various amounts of KCN. The oscillations in NADH were monitored using a fluorimeter and the number of cycles was counted until the amplitude was smaller than 10% of the initial amplitude. In Fig. 2 this number of cycles is plotted as a function of

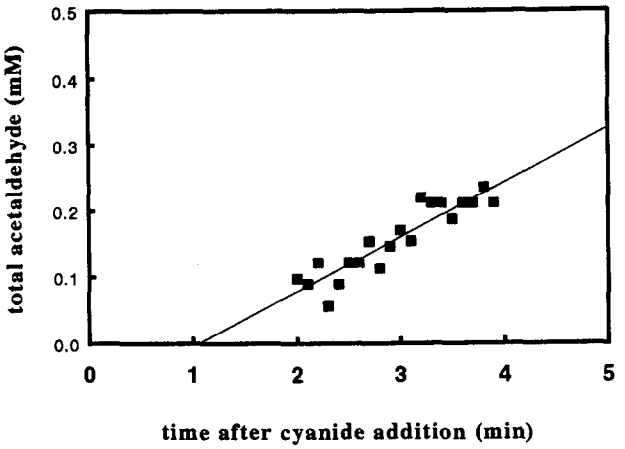

Fig. 3. Production of lactonitrile by oscillating yeast cells. Starved cells at 12 g protein/liter were given 40 mM of glucose and (4 min later) 7 mM of cyanide. Shown is the 'total acetaldehyde' concentration, including acetaldehyde and lactonitril. Free acetaldehyde concentrations were below 100 μM at all times.

the cyanide concentration. Cyanide concentrations of 2 mM or lower led to fewer than 6 whereas cyanide concentrations between 3 and 8 mM led to more than 30 cycles. Above 8 mM the number of cycles decreased to 3 or less. At 3 mM of cyanide, cellular respiration was inhibited for more than 95%. Consequently Fig. 2 suggested that cyanide had a second action that became important at cyanide concentrations above 3 mM. As cyanide is known to react with aldehydes [15], we asked if the second action might be condensation with acetaldehyde to lactonitril.

Fig. 3 shows the results of a measurement of 'total acetaldehyde' defined as free acetaldehyde plus lactonitril (or at least acid-labile adducts of acetaldehyde) [13]. In parallel experiments we found that the free acetaldehyde concentrations remained below 0.1 mM (manuscript in preparation). Clearly, lactonitril was produced at a significant rate after cyanide addition, apart from a lag phase of 1 min.

Continuous removal of acetaldehyde from the system will leave an excess of redox equivalents because the basal anaerobic glycolysis from glucose to ethanol and CO_2 is redox-neutral. Fig. 4B shows that the cells cope with the excess redox equivalents by increasing the production of glycerol. The glycerol production rate increased monotonically with increasing concentrations of cyanide, suggesting that acetaldehyde trapping did the same. Significantly, with increasing cyanide concentrations, the ethanol production first increased, then decreased (Fig. 4A), again reflecting a dual action of cyanide; at low cyanide concentrations inhibition of respiration and the concomitant Pasteur effect stimulates glycolysis to ethanol, at higher concentrations the increasing trapping of acetaldehyde competes with ethanol formation.

4. Discussion

Yeast cells exhibit different dynamic behaviour depending on the growth phase in which they are harvested. When the cells had been harvested around the transition from using glucose to ethanol as a growth substrate, limit-cycle oscillations were observed, whereas when they had been harvested at another growth phase they showed damped oscillations [9]. It is unlikely that these differences in the dynamics are due to differences in metabolite concentrations since the cells were starved for 3 h before the oscillations were evoked. Therefore we have investigated the enzyme activities of the glycolytic enzymes during growth, to check whether the enzymatic make-up might be responsible for the different dynamics. Starting with hexokinase we found large changes in the activity at the transition from using glucose to using ethanol as a growth substrate. A V_{max} of 0.25 U/mg during growth on glucose is comparable to the rate of glucose uptake which is approximately 0.14 U/mg under these conditions [10]. A threefold increase of hexokinase activity around the growth phase transition seems to be significant since the maximal glucose uptake rate does

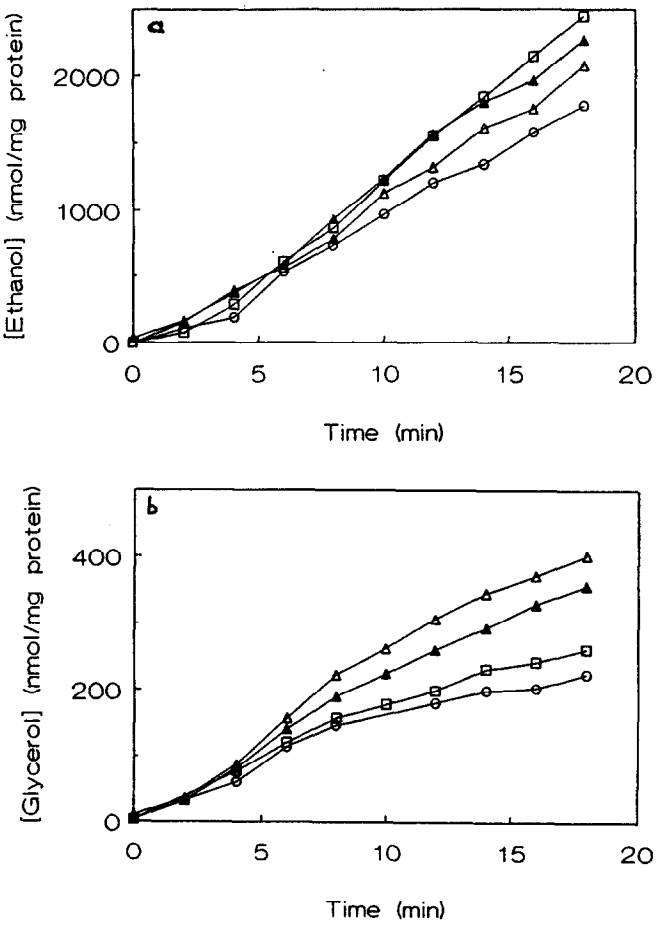

Fig. 4. A high KCN concentration inhibits ethanol production (A) and concomitantly stimulates glycerol production (B) by yeast cells. Cell density, 6.6 g protein/liter; and [glucose], 20 mM. [KCN], 0 (○), 0.5 (□), 2 (▲), or 8 (△) mM.

not change [16]. On the other hand, hexokinase is not the only glucose phosphorylating enzyme. Glucokinase, which phosphorylates glucose but not fructose, may catalyze up to 50% of the glucose flux [17]. Phosphofructokinase did not change in activity during growth. Between 20% and 50% of the glucose that is consumed under these conditions is converted to storage carbohydrates [18] so that the average glycolytic flux through phosphofructokinase does not exceed 0.1 U/mg, while the maximal velocity of phosphofructokinase was 0.4 U/mg. The constant aldolase V_{max} of 0.4 U/mg was twice the flux through the lower part of glycolysis, 0.2 U/mg. The same holds true for pyruvate decarboxylase and alcohol dehydrogenase. Most importantly, from the fact that for none of the investigated enzymes except for hexokinase V_{max} changed, we conclude that the shift from transient to sustained oscillations of cells harvested around the growth phase transition is not determined by changes in enzyme activities other than hexokinase. The dynamic behaviour might also be influenced by the glucose uptake system. Although the maximal uptake rate does not change, the affinity of transport for glucose changes from a low affinity (K_m = 25 mM) when growing on glucose to a high affinity (K_m = 1.5 mM) after the transition [16].

Compared to other respiratory inhibitors or a shift to anaerobiosis, cyanide addition yielded longer series of oscillations [11]. This suggested already that cyanide does not only act as an inhibitor of respiration. At 40 mM glucose, we found limit-cycle oscillations only in the range from 3 mM to 8 mM cyanide, although respiration was fully inhibited at a cyanide concentration of both 1 mM and 5 mM (not shown), further suggesting that there is another effect of cyanide. We identified this effect as the removal of acetaldehyde. The cells maintained their average redox balance by producing glycerol.

Our results suggest that for sustained glycolytic oscillations to occur it is not only required that the cells are anaerobic but also that acetaldehyde is continuously removed at a certain rate. In addition, a specific cellular make-up is required which includes an increased activity of hexokinase.

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