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Around the growth phase transition *S. cerevisiae*'s make-up favours sustained oscillations of intracellular metabolites

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Under a limited set of hitherto incompletely defined conditions, inhibition of respiration has been shown to cause transient oscillations in NAD(P)H fluorescence of yeast cells. In this paper, we apply a new method [1992, Anal. Biochem. 204, 118–132] for extraction of intracellular metabolites. This method involves spraying the cells into −40°C methanol; the neutral pH allows extraction of nearly all intracellular metabolites, including NADH. Close to the shift from glucose to ethanol as a growth substrate, the cells acquire a make-up amenable to sustained oscillations in intracellular concentrations of NADH and glycolytic intermediates such as glucose-6-phosphate. NADH was found to oscillate between 200 μM and 400 μM intracellular concentration. The cellular make-up determining the tendency to oscillate is 'remembered' by the cells after three hours of starvation.

Oscillation; Glycolysis; Intracellular metabolite; Yeast

1. INTRODUCTION

In the glycolytic pathway, oscillations in metabolite concentrations occur in different organisms under different conditions (for review see [1]). In yeast, such oscillations have been investigated extensively in cell-free extracts [2,3] but less so in intact cells and little is known about metabolite concentrations in the latter [4]. Oscillations in the NAD(P)H concentration in intact yeast cells can be induced when glucose is added to starved cells and, after that, respiration is inhibited. The oscillations in the various glycolytic intermediates differ in phase especially around the phospho-fructokinase. Accordingly in cell-free extracts, additions of glucose-6-phosphate and fructose-6-phosphate but not fructose-1,6-bisphosphate induced oscillations [2].

In addition to understanding the internal dynamics of glycolytic oscillations, it is important to understand which external condition determines whether or not the cells are prone to oscillatory glycolytic dynamics. And, if external conditions matter, is their effect immediate (in the sense of an effect on intracellular metabolite concentrations only), or is it imprinted onto the cells, e.g. involving sustained changes in their enzymatic set up? Until now, glycolytic oscillations in intact yeast cells have been measured mostly by inspecting NAD(P)H fluorescence.

The available extraction methods did not allow measurement of the cytosolic concentrations of NADH and NADPH. Recently, de Koning and van Dam [5] developed a procedure for rapid quenching of yeast cells, extraction and measurement of the glycolytic intermediates including NADH, NAD^+, NADPH and $NADP^+$. In this paper we employ this method and demonstrate that the oscillations do involve NADH in cells which show sustained oscillations.

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2. MATERIALS AND METHODS

The yeast *Saccharomyces cerevisiae* (X2180 diploid strain) was grown under semi-acrobic conditions at 30°C on a rotary shaker in a medium containing 10 g/l glucose, 6.7 g/l yeast nitrogen base (Bacto) and 100 mM potassium phthalate at pH 5.0. The cells were harvested by centrifugation or by filtration, washed twice with 100 mM potassium phosphate, pH 6.8, resuspended and starved in the same buffer for 3 h at 30°C on a rotary shaker. They were washed by filtration or centrifugation and stored on ice until use. The optical density was measured in a ten-fold diluted sample at 600 nm in a 10 mm cuvette in an LKB Novospec II spectrophotometer. An absorption of 1 corresponds to a protein concentration of 0.25 g/l. Protein was measured according to the method of Lowry et al. [6]. Glucose was measured with a commercial glucose kit (Sigma). Ethanol was measured in a glycine buffer reagent (Sigma) with 0.3 g/l NAD^+, by the change in NADH absorbance after addition of alcohol dehydrogenase. NAD(P)H fluorescence in intact cells was measured in an Eppendorf 1011M fluorimeter (excitation 316, emission 400–2,000 nm) in a stirred and thermostated (25°C) cuvette. Oscillations were induced by adding 20 mM glucose to the starved cells and after 4 min adding 10 mM KCN.

For quenching the cells, extraction of the metabolites and metabolite determination we followed the protocol of de Koning and van Dam [5]. For quenching we sprayed 5 ml of the cell suspension into 20 ml of 60% methanol at a temperature of −40°C. For the extraction experiment a protein concentration of 6 g/l was used. The cytosolic

concentrations were calculated assuming that 1 g of protein corresponds to 3.75 ml of cytosol [5].

3. RESULTS AND DISCUSSION

During growth of a batch culture of *Saccharomyces cerevisiae* with an initial glucose concentration of 10 g/l, the glucose was consumed and ethanol was produced. The cell concentration increased rapidly during glucose consumption. When the glucose had been consumed the cells switched to ethanol oxidation and the growth rate slowed down. We harvested cells at different points on the growth curve. After adjusting the cells to equal concentrations of 2 g protein per liter, they were starved and then glucose and cyanide were added to test for oscillations. The changes in NAD(P)H fluorescence were measured. The oscillatory behaviour was found to be different in cells harvested at different points on the growth curve as indicated in Fig. 1. With cells harvested in the period of glucose consumption as well as during ethanol consumption we observed damped oscillations; only 4–6 cycles were observed. In cells harvested at the transition from using glucose to ethanol, i.e. during the first hour after glucose has been consumed, we found oscillations that were essentially continuous.

The undamped train of oscillations as shown in Fig. 1, lasted for 40 min and then suddenly died down. We conjectured that, in essence, the oscillations were sustained, but that some factor important for the oscillation had been depleted. Indeed, when we measured the extracellular concentrations of glucose during oscillation experiments with cells harvested at the growth transition, we observed that the oscillation died down at the moment glucose was exhausted. Further additions of glucose led to further oscillations [8].

Pye [7] studied *transient* oscillations of NAD(P)H fluorescence in *S. carlsbergensis* and observed that the duration of these oscillations increased when the cells were harvested at the transition from glycolysis to ethanolysis. However, in his experiments, the end of the oscillations was probably not caused by depletion of glucose, as more than sufficient was added. Consequently, either *S. carlsbergensis* is unable to support sustained glycolytic oscillations under these conditions, whereas *S. cerevisiae* is, or some other factor limited the oscillations in the experiments of Pye [7].

It should be noted that the oscillation experiments were performed after the cells were starved for 3 h in phosphate buffer. Nevertheless, the cells seemed to 'remember' whether they had been harvested close to or away from the growth transition point. We would expect that after 3 h the metabolite concentrations would be constant. Effects of differences in cell cycle phase are unlikely because of the long starvation period. In the oscillation experiment, the cells were suspended at equal concentrations, excluding the effects of cell concentration [9]. The differences in the dynamics of glucose fermentation must be due to differences in the cellular make up caused by different environmental conditions, and these differences must have been conserved during starvation.

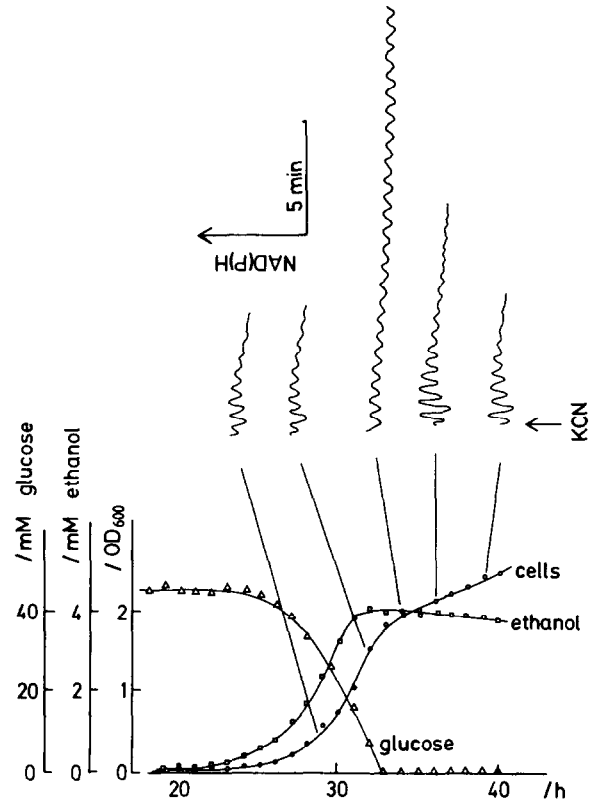

Fig. 1. *Lower part.* Growth curve of *S. cerevisiae*. Triangles show the glucose concentration, the squares the ethanol concentration and the circles the optical density of the culture. *Upper part:* NAD(P)H fluorescence traces of the cells harvested at the indicated points of the growth curve, starved and then supplied with glucose and cyanide.

Oscillations in yeast glycolysis have been mainly investigated in cell-free yeast extracts. Such a system has the advantage that it can be easily manipulated and absolute concentrations are readily determined. A disadvantage is that it is an in vitro system and its correspondence to the in vivo situation might be limited. Most studies with intact cells have employed NAD(P)H fluorescence as a monitor of glycolytic oscillations, e.g. [7,9]. This method has the disadvantage that it is not quite certain that the oscillations in NAD(P)H *fluorescence* imply also oscillations in concentrations of NADH and glycolytic intermediates. We wanted to quantify the oscillations in NADH concentration and establish that also the glycolytic intermediates show sustained oscillations.

To investigate the metabolite concentrations under conditions of sustained glycolytic oscillations we used cells harvested at the growth phase transition and started sampling 5 min after inducing the oscillations. We took samples every 5 s and immediately quenched

the reaction by spraying the cells into cold (−40°C) methanol. The cells were then extracted and analyzed for the metabolites. This technique for quenching and extraction, developed by de Koning and van Dam [5] has several advantages. One is that the reaction is immediately stopped and another that the cells can be separated from the extracellular medium. The separation allows discrimination between intracellular and extracellular phases and thereby the determination of the cytosolic metabolite concentrations. The usual procedure for quenching is to mix the cells with trichloroacetic acid or perchloric acid. With this procedure quenching is slower and, because the cells are disrupted, it is impossible to discriminate between the intracellular and extracellular phase.

Our extraction procedure uses neutral pH which enables also the extraction of compounds which are unstable in acid or base, like NADH or NADPH. Fig. 2B shows the result for NADH. The figure gives the concentration of cytosolic NADH during steady oscillations. The frequency and phase were the same as in the fluorescence measurement. With the extraction method one can discriminate between NADH and NADPH and it gives the additional information that the cytosolic NADH concentration is oscillating between 200 μM and 400 μM. NADPH and NADP were found to be constant. NAD oscillated with a lower amplitude relative to its average concentration. The sum of NAD and NADH was found to be constant at 1 mM. Fig. 2A shows the oscillations of glucose-6-phosphate under the same conditions as the NADH. Glucose-6-phosphate oscillates between 2 mM and 8 mM and its phase was shifted with respect to the NADH.

Fig. 2 is the first demonstration of sustained ('limit-cycle') oscillations in the intracellular concentrations of glycolytic intermediates, in this case NADH and glucose-6-phosphate. Thus it extends the observations of Betz and Chance [4], who monitored concentrations of glycolytic intermediates other than NADH, did not discriminate between intracellular and extracellular medium and observed only transient oscillations (in these earlier studies, the cells were not harvested at the growth phase transition and the acid extraction method did not allow the measurement of NADH).

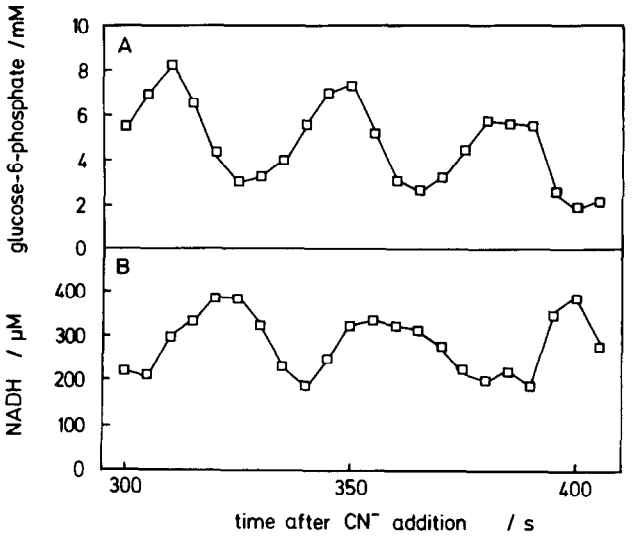

Fig. 2. (A) Cytosolic glucose-6-phosphate concentration during sustained glycolytic oscillations. (B) Cytosolic NADH concentration under the same conditions as in Fig. 2A.

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