Changes in the activity of alcohol dehydrogenase during the cell cycle of the fission yeast Schizosaccharomyces pombe

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Summary

The activity of alcohol dehydrogenase (ADHase) was followed in synchronous cultures of Schizosaccharomyces pombe. In selection synchronised cultures of wild-type cells, it followed a linear pattern in which there was a constant rate of increase of activity followed by a doubling of this rate at the end of the cycle. The same pattern was also found in selection synchronised cells of wee mutants except that the point of rate change was shifted to 0.27 of the cycle. A similar linear pattern was also found in the shortened cell cycles produced by induction synchrony (block and release of the mutant cdc2.33) but the rate change point was at about 0.75 of the cycle. In the mutant cdc13.117, there was a marked fall in the rate of activity increase at 35°C but not at 37°C. In all these situations, the ADHase activity closely paralleled in pattern and timing the rate of production of CO₂ established in earlier papers. This suggests a coordinate control of the flux through glycolysis and the activity of the last enzyme in the glycolytic pathway in yeast. However, an interesting difference indicating a loss of the coordinate control occurred in synchronous cultures of cdc2.33 in which small cells had been selected but in which the DNA-division cycle had been blocked by a shift-up to the restrictive temperature. Rate changes both in CO₂ production and in ADHase activity continued in these blocked synchronous cultures but the timing was different. With ADHase activity the timing was 15% greater than that in a normal cell cycle whereas with CO₂ production it was 15% less. We suggest that these and other periodic events are subject to independent oscillatory controls in these blocked cultures with timings that differ from each other and from the normal cycle but in the normal cycle the oscillators are all entrained by one or more events of the DNA-division cycle.

Key words: alcohol dehydrogenase, cdc mutants, cell cycle, fission yeast, glycolysis, induction synchrony, oscillators, Schizosaccharomyces pombe, selection synchrony.

Introduction

It has been found that CO₂ production shows a periodic pattern in synchronous cultures of Schizosaccharomyces pombe prepared by selection in an elutriator (Novak and Mitchison, 1986). The rate of production follows a 'linear pattern' in which it shows a constant rate of increase through the cell cycle but this rate doubles at the end of the cycle. Acceleration (rate of the rate of production) therefore shows a stepwise pattern. This pattern persists, with slightly shorter timing, for a period after the DNA-division cycle has been blocked by raising to the restrictive temperature a 'synchronous' culture of the mutant cdc2.33. Synchronous culture is put in inverted commas, since, although the culture has been selected for small cells, there are no further cell divisions. It also persists, but with much shorter timing, after protein synthesis in a synchronous culture has been blocked with cycloheximide (Novak and Mitchison, 1987). Similar patterns occur during the short cell cycles produced by induction in which the DNA-division cycle is blocked for a period and then released (Novak and Mitchison, 1990a). The rate of CO₂ production is a measure of the in vivo flux through glycolysis. It is therefore of interest to see whether these patterns are reflected in the in vitro activity of the enzymes involved in the glycolytic pathway. We chose to concentrate at first on alcohol dehydrogenase (ADHase), since it is at the end of the pathway and its activity is not coordinated under these conditions.

Materials and methods

Strains

The wild-type (WT) strain 972h⁻ was originally obtained from Professor U. Leupold, Bern. The mutants cdc2.33 and cdc13.117 are temperature-sensitive conditionals that form colonies at 25°C but not at 35°C (Nurse et al. 1976). The cdc2.33 function is required both for mitosis and for an event in G₂ (Nurse and Bisset, 1981; Novak and Mitchison, 1989) but in normal growing cultures only the mitotic block is apparent in cdc2.33, since nearly all the cells are in S plus G₂ and they are arrested in G₂ after a shift-up to the restrictive temperature. The cdc13.117 function is required for...
mitosis. wee1.6 and wee1.50 are mutants that, at 35°C, are about half the size of WT cells (Thurau et al. 1978).

**Media**
The medium used throughout was EMM3, a minimal medium with phthalate buffer (Creanor and Mitchison, 1982).

**Synchronous cultures**
Selection synchronised cultures were prepared by selecting the smallest cells from an asynchronous culture with an elutriating rotor (Creanor and Mitchison, 1979). Asynchronous control cultures were prepared by collecting an asynchronous culture in the rotor, pumping it out without selection and then diluting it with conditioned medium collected during the loading of the rotor cell. Synchronous and asynchronous controls were separate experiments.

**Enzyme assay**
The activity of alcohol dehydrogenase (EC1. 1. 1. 1) was assayed by the method described by Racker (1955). Samples (20 µl) of cultured cells were mixed with 0.4 ml of Tris buffer (0.1 M, pH 8.5), containing 2.5 % (v/v) isomyl alcohol and then frozen at -20°C. For assay, each sample was mixed with 0.1 ml of the Tris buffer containing 20 mg ml⁻¹ NAD and 30 µl ml⁻¹ ethanol. The samples were incubated at 25–30°C and the reaction stopped by adding 0.5 ml methanol. After centrifuging at 7000 g for 3 min, the absorbance of the supernatant was measured at 340 nm.

To detect fine changes in enzyme activity, it is necessary to have a highly consistent assay. This assay method gave a coefficient of variation of less than 1 % on replicate samples. The two important factors were the use of cells permeabilised by freeze–thawing and by isomyl alcohol (rather than cell extract), and the reduction of pipetting and washing steps to a minimum.

1. Enzyme unit (e.u.) = 1 nmol substrate converted at 30°C by 10⁶ cells, min⁻¹

**Cell numbers**
Cell numbers were measured with a Coulter Counter (Industrial model D) with a 100 µm aperture.

**Analysis and smoothing**
Smoothed curves for the rate of change of enzyme activity were obtained from a computer programme described by Novak and Mitchison (1986). The smoothing was normally done over 15 data points, and, as with running means, data points were lost at the beginning and end of experiments.

When control asynchronous cultures were available, difference curves were calculated by subtracting the logarithmic values of the rate curve for a control culture from the logarithmic values of the rate curve for a synchronous culture. Otherwise, the pattern of a synchronous rate curve was made more conspicuous by computing the residuals from a smooth polynomial curve of best fit. This is equivalent to a difference curve but assumes that the rate curve for a control culture follows a smooth curve. If these two methods were applied to the same set of data, the results were substantially the same. The points of rate change were estimated from the half-rise points of the difference curve or of the residual curve.

**Results**

**Selection synchrony with wild-type cells**
Fig. 1 shows the increasing ADHase activity in a selection synchronised culture of WT cells (strain 972h⁻) and in an asynchronous control culture of the same strain that had been through the elutriating rotor but had not been size-selected. The synchronous culture showed a linear pattern with two rate changes, at about 2 and 4.3 h. The asynchronous culture showed a steady increase in activity without conspicuous rate change points. These patterns are shown more clearly in a plot of the smoothed first differential – the rate of change of activity (see Materials and methods). These are presented in Fig. 2 for the same data as presented in Fig. 1. The asynchronous control (Fig. 2B) had a smooth and continuous increase in rate whereas the synchronous culture (Fig. 2C) had two step rises. These two steps were nearly coincident with the steps in the cell number curve in Fig. 2E. Fig. 2A shows the oscillating difference curve between the rates for the two cultures.

A summary of four such experiments is shown as a cell cycle map in Fig. 3A, with the rate change points estimated from the half-rise points of the difference curves. The means are slightly, but not significantly, different from the means of cell division in the two cycles. It seems therefore that the rate of increase of activity doubles at the end of the cell cycle (0 or 1.0 of the cycle). Two similar experiments were done at 28°C and Fig. 3B shows that the rate change points maintained the same relationship to the cell cycle in the longer cycles at a slower growth rate.

Two points can be made here. The first is that these results closely resemble those obtained on CO₂ production (Novak and Mitchison, 1986) where the rate change points were also at the end of the cycle. However, what is doubling here is the rate of increase of ADHase activity (first differential) whereas with CO₂ it was the acceleration or rate of the rate of increase of CO₂ (second differential). Second, at first sight the rate curves for the synchronous culture in Fig. 2C suggest that the rate change may be a slow process occupying an appreciable part of the cell cycle. But two factors will increase the apparent time of the rate change. The culture is not perfectly synchronous, as shown by the cell number curve, and the data have gone through a smoothing process. It is likely therefore that the rate change is a relatively abrupt process at the level of the single cell.
The results from three such 'synchronous' cultures are summarised in the cell cycle map in Fig. 3D. The average timing between the rate change points is 172 min. It is not strictly fair to compare this with the WT selection synchrony at 35°C, since the temperature was somewhat higher at 36.5°C and there was a temperature shift that might have affected the timings. Three experiments were therefore carried out on WT cells that had been shifted from 28°C to 36.5°C after selection. Curves are not presented, since they were very similar to those in Fig. 2. The results are summarised in the cell cycle map in Fig. 3E. The first division and rate change point is delayed by about 33 min by the temperature shift. The timing to the next division and rate change point is 148–149 min. Growth is slightly slower at 36.5°C. This was checked in asynchronous cultures of WT cells at the two temperatures (data not shown) where the doubling time was 9% greater at the higher temperature. The timing between the rate change points of 172 min in the blocked cultures should therefore be compared with the 149 min in Fig. 3E: it is 15% longer.

CO₂ production also shows continuing periodic changes in blocked 'synchronous' cultures (Novak and Mitchison, 1986) but their timing is about 15% shorter than in the normal cycle. To bring out this point, the mean CO₂ acceleration points are shown as diamonds below the line in Fig 3D. The second CO₂ point lies almost halfway between the two ADHase activity points.

Induction synchrony with cdc2 cells

In induction synchrony, all the cells in a normal asynchronous culture are induced to divide synchronously by a variety of techniques. An effective way in S. pombe is to block the DNA-division cycle for a period of several hours by raising a culture of the mutant cdc2 to the restrictive temperature and then releasing the block by shifting down to the permissive temperature (Creanor and Mitchison, 1989; Novak and Mitchison, 1990a). The oversize cells produced during the block then divide synchronously but with a cycle time that is shorter than normal (down to 50%).
Fig. 3. Cell cycle maps of rate of increase of ADHase activity. Triangles show the time of rate change points for individual experiments. The upper arrow is the mean time of rate change points, with cross bar for the standard error. The upper number in parenthesis is the time between the mean rate change points. The lower arrow is the mean time of cell division, with cross bar for the standard error. The lower number in parenthesis is the time between cell divisions (cell cycle time). Map A, selection synchrony with WT cells at 35°C. Map B, selection synchrony with WT cells at 28°C. Map C, selection synchrony with wee cells at 35°C. Filled triangles, wee1.6 and empty triangles wee1.50. Map D, 'synchronous' culture of cdc2.33 grown at 28°C, selected, and then shifted up to 36.5°C at time zero. The lower diamonds show the acceleration points for CO₂ production in similar 'synchronous' cultures, from Novak and Mitchison (1986). Map E, selection synchrony with WT cells grown at 28°C, selected and then shifted up to 36.5°C at time zero. Map F, induction synchrony with cdc2.33. Asynchronous culture shifted up from 28°C to 35°C for 4h and then shifted down to 28°C at time zero. Rate change points were determined from difference curves in maps A and C, and from residuals in maps B, D and E.

been transferred from 28°C to 35°C for 4h and then transferred back to 28°C. The rate curve for activity (Fig. 5D) showed stepwise increases that were rather less marked than with selection synchrony and the residuals showed oscillations with cell cycle timings (compare Fig. 5B with F). It is not possible to make a strict control with induction synchrony but it is possible to do a partial control test on the effect of temperature changes on WT cells. The results are also shown in Fig. 5 for a WT culture that had been through the same temperature regime as the cdc2 culture. The residuals (Fig. 5A) showed an initial fall associated with the slight perturbations in the cell number curve (Fig. 5E). But thereafter there were only small fluctuations in the residuals and these did not have cell cycle timings. The collected results from four induction synchrony experiments are shown in the cell cycle map in Fig. 3F.

This association of the activity steps with division after induction synchrony is similar to that with the rate changes in CO₂ production described by Novak and Mitchison (1990a). The timings of the activity steps were rather earlier than cell division in both cases. This may, at any rate in part, be due to an earlier S period in induction synchrony (Novak and Mitchison, 1990a). With ADHase activity the mean timing of the first step was 20 min before division and with CO₂ production it was 25 min — a difference that is probably not significant. The steps in CO₂ acceleration were however more marked than those in ADHase activity.

Effects in blocked cultures of cdc13

Although cdc13 was originally isolated as a mitotic mutant, recent experiments have shown that its gene product has homologies with cyclins and is involved in an important complex with the cdc2 + gene product (references cited by Novak and Mitchison, 1990a). At 37°C the mitotic block is tight but at 35°C it is much less tight and the cells leak through to a terminal phenotype with septa and condensed chromatia present. This effect at these two restrictive temperatures is very similar to that found with different glucose concentrations by Hagan et al. (1988). CO₂ acceleration is maintained at 37°C (though with a fluctuation) but at 35°C it drops by at least fourfold over a
A

Time (h)

3 4

Fig. 4. ADHase activity in a 'synchronous' culture and in an 'asynchronous' control of cdc2.33 grown and selected at 28°C and then shifted up to 36.5°C at time zero. Curve A, difference between rate curves ('synchronous'-'asynchronous'). Curve B, rate of activity increase in 'asynchronous' culture; 1 a.u. = 1.87x10^-2 enzyme unit (e.u.) min^-1. Curve C, rate of activity increase in 'synchronous' culture; 1 a.u. = 2.06x10^-2 e.u. min^-1. Curve D, cell number in part of synchronous culture not shifted up to 36.5°C but kept at 28°C; 1 a.u. = 10^6 cells ml^-1. Curve E, cell number in 'asynchronous' culture; 1 a.u. = 10^6 cells ml^-1. Curve F, cell number in 'asynchronous' culture; 1 a.u. = 1.33x10^6 cells ml^-1.

period of 300 min (Novak and Mitchison, 1990a). We therefore examined ADHase activity to see whether or not there was a similar striking effect.

Fig. 6 shows the rate curves for ADHase activity in an asynchronous culture of cdc13.117 after shifting up from 28°C to 35°C and 37°C. The results are similar to those from experiments with CO₂ acceleration (Fig. 9, see Novak and Mitchison, 1990a). The ADHase rate curve at 37°C increases for the first 300 min whereas at 35°C it drops (after the first 100 min) to one-fifth of the value at 37°C by the end of the experiment.

Effect of a cycloheximide block
Oscillations in CO₂ production continue in the presence of a block to protein synthesis produced by the addition of cycloheximide (Novak and Mitchison, 1987). Also, in an earlier analysis of S. pombe enzymes with linear patterns of activity increase, it was found that two out of the three enzymes showed a delay between the addition of cycloheximide and the cessation of activity increase (Mitchison and Creanor, 1969). The delay was 30 min with alkaline phosphatase, 15 min with sucrase and 0 min with acid phosphatase. This 'precursor delay' was attributed to a

Fig. 5. ADHase activity in induction synchrony and in a partial control. For the synchronous culture, an asynchronous culture of cdc2.33 was shifted up from 28°C to 35°C for 4 h and then shifted down to 28°C at time zero. For the control, WT cells were subjected to the same temperature shifts. Curve A, residuals of the rate of activity increase in control. Curve B, residuals of the rate of activity increase in synchronous culture. Curve C, rate of activity increase in control; 1 a.u. = 1.72x10^-2 enzyme unit (e.u.) min^-1. Curve D, rate of activity increase in synchronous culture; 1 a.u. = 2.08x10^-2 e.u. min^-1. Curve E, cell number in control; 1 a.u. = 0.71x10^6 cells ml^-1. Curve F, cell number in synchronous culture; 1 a.u. = 1.05x10^6 cells ml^-1.

Fig. 6. Rate of ADHase activity increase in asynchronous culture of cdc13.117 grown at 28°C and shifted up to 37°C (upper curve) and to 35°C (lower curve) at time zero.
pause between the synthesis of the enzyme protein and its activation. We therefore examined the effect of adding cycloheximide on the ADHase activity in an asynchronous culture. Fig. 7 shows that the activity ceased to rise as soon as the cycloheximide was added and that there was therefore no precursor delay. The fractional increase in cell numbers after the addition of cycloheximide gives a transition point of 0.75 of the cycle at about the start of mitosis. A cell will therefore complete mitosis and cell division without the synthesis of any new protein.

Discussion

Linear patterns of enzyme activity during the cell cycle of *S. pombe* were first shown over 20 years ago for sucrase, acid phosphatase and alkaline phosphatase (Mitchison and Creanor, 1969). The experiments on ADHase were easier both because of much more accurate assays on permeabilised cells and because of better methods of analysis. In addition, techniques are available now for following activity in blocked 'synchronous' cultures and after induction synchrony.

ADHase is the last enzyme in the glycolytic pathway in yeast and CO₂ production is a measure of the *in vivo* flux through the pathway. If there is a rate-limiting enzyme in the pathway, then the rate of production of CO₂ might be proportional to the activity of this enzyme. In this case, a linear pattern in activity would produce a linear pattern in the rate of CO₂ production and the rate change points would be at the same time in the cell cycle. The results here show that there is indeed such a coincidence between ADHase activity and CO₂ production after selection synchrony of WT and *wee* cells and after induction synchrony with *cdc2* or *cdcl3*. This does not imply that ADHase is necessarily the rate-limiting step in glycolysis or that there is a direct connection between its *in vitro* activity and the *in vivo* flux. But it is possible that there is a coordinate control of the steps in glycolysis (including glucose uptake) that includes ADHase as well as other parts of the pathway that may be rate-limiting.

This then raises the question of what is happening in the synchronous cultures after a *cdc2* block to the DNA-division cycle. The difference in timing between the rate change points for ADHase and CO₂ makes it clear that there is not a coordinate control when the DNA-division cycle is blocked or at least that the coordination is not the same as in the normal cycle.

At this point it is worth mentioning very briefly the timings in other situations where there is a continuing oscillatory control after a *cdc2* block. 'Oscillatory' is used here in a very broad sense to refer to any repetitive timing control. The timing for ADHase activity is about 15% longer than the normal cycle. It is the same as the normal cycle in the steps in nucleoside diphosphokinase activity (Creanor and Mitchison, 1986). In most of the cases, however, it is shorter than the normal cycle – by 5–10% for enzyme potential of sucrase and arginase (Benitez et al., 1980), by 15–20% for CO₂ production (Novak and Mitchison, 1986), by 26% for oxygen consumption (Novak and Mitchison, 1990b) and by about 40% for changes in the rate of length growth (Miyata et al. 1988). The wide spread in these values argues very strongly against any overall timer or oscillator controlling all the events and suggests instead a series of independent oscillators that are entrained and brought into cell cycle timing by one or more signals from the DNA-division cycle.

In the case of CO₂ production, there is evidence from single and double-block experiments in induction synchrony that such an entrainment signal can come from 'start' and from mitosis, which in the normal cell cycle are close together (Novak and Mitchison, 1990a). It was also suggested that this signal is a 'stop acceleration' followed shortly afterwards by a 'recommence acceleration'. In the case of the dramatic fall in acceleration in *cdcl3* at 35°C, the stop signal could have occurred without the recommence signal.

The close parallels between ADHase activity and CO₂ production in selection synchrony, induction synchrony and blocked *cdcl3* suggest that the same entrainment signals could also apply to ADHase. Two points, however, should be made. First, stop followed by recommence is a way of lengthening a short free-running CO₂ oscillator to a longer DNA-division cycle. It cannot operate in the same way to shorten the long free-running ADHase oscillator. Second, the signal in the blocked *cdcl3* is not a general one affecting all growth and synthesis, since the rate of total protein and rRNA synthesis is not altered substantially in *cdcl3* maintained at 35°C (Novak and Mitchison, 1990a). In addition, whereas the rate change points for ADHase activity and CO₂ production are both shifted coordinately in *wee* cells, that for total protein synthesis is not (Creanor and Mitchison, 1982).

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Fig. 7. Effect of cycloheximide on ADHase activity and cell numbers in an asynchronous culture of WT cells at 35°C. Cycloheximide (100 μg ml⁻¹) added to part of culture at 1 h.

(D) ➤ ADHase activity; 1 arbitrary unit (a.u.)=7.0 enzyme unit. (O, □) cell numbers; 1 a.u. =10⁶ cells ml⁻¹.

Arbitrary units (a.u.)

Time (h)

CH

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