The Use of Step Enzymes as Markers During Meiosis and Ascospore Formation in *Saccharomyces cerevisiae*

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The activities of ornithine aminotransferase, sucrase and acid and alkaline phosphatases have been studied throughout sporulation in *Saccharomyces cerevisiae*. The same enzymes were monitored during synchronous vegetative growth. Each of these enzymes has been demonstrated to increase in a 'step' manner during both growth and sporulation. Alkaline phosphatase increased in a two-step manner whereas the others increased in a single step. The times of increase of these enzymes formed a similar sequence during both sporulation and growth. It has been proposed that these enzymes are under a common mechanism of control during growth and sporulation and that the sequence of enzyme appearance may be used as markers of the sporulation process.

INTRODUCTION

Since yeast cells growing exponentially maintain a constant chemical composition, the level of cellular components must double with each cell cycle. Several studies have indicated that the doubling of many cellular components occurs at a specific time in the cell cycle rather than continuously. The doubling of many enzymes occurs in a single step at a time in the cell cycle which is unique to each enzyme. The times of appearance of these 'step enzymes' have been used as markers for the progress of the cell cycle. Using such markers, a distinction has been made between events which appear to be associated with growth (markers of the 'growth cycle') and those associated with cell division (markers of the 'DNA-division cycle') (Mitchison, 1973; Halvorson *et al.*, 1971).

When diploid yeast cells are placed on acetate sporulation medium, vegetative growth and cell division are suppressed and ascus formation is initiated. During ascus formation, a single round of DNA replication and meiotic nuclear division occurs (Tingle *et al.*, 1973). In this study we have attempted to determine whether the suppression of vegetative growth and cell division is also accompanied by suppression of markers of the growth cycle, namely step enzyme formation. Although some increase in the protein content during the first stage of ascospore formation has been reported (Croes, 1967), it is not clear whether this represents normal growth or the formation of proteins which are unique to sporulation.

METHODS

Conditions for growth and sporulation. *Saccharomyces cerevisiae* strain K2nb was maintained on slopes of medium 2 containing 2% (w/v) agar. This strain was originally supplied by Professor E. A. Bevan, Queen Mary College, University of London.

Vegetative cells required for sporulation were grown in a medium containing 2% (w/v) glucose and

0.67 % (w/v) Difco Yeast Nitrogen Base without Amino Acids in distilled water (medium 1). Medium 2 was the same as medium 1 except that it also contained 0.1 % (w/v) yeast extract. All media were autoclaved at 121 °C for 15 min. Glucose was autoclaved separately and added prior to inoculation. The sporulation medium contained 1 % (w/v) potassium acetate.

For both growth and sporulation, *S. cerevisiae* was incubated in 250 ml conical flasks containing 100 ml medium. These were shaken at 250 rev. min⁻¹ on a Gallenkamp orbital shaker at 28 °C. Growth flasks were inoculated with 5×10^4 cells. Sporulation flasks were inoculated with cells from the late-exponential phase of growth to give a final concentration of 1×10^7 to 5×10^7 cells ml⁻¹. The number of cells inoculated in synchronous culture flasks was dependent on the efficiency of the yeast fractionation technique.

Growth was monitored using a nephelometer (EEL Unigalvo) in vegetative and sporulating cultures, but cell numbers were determined using an improved Neubauer haemocytometer in synchronous cultures.

Preparation of synchronous cultures. Yeast cells grown on medium 1 were centrifuged and washed with distilled water before being incubated in a dilute solution of gut juice of *Helix pomatia* to separate paired cells (Williamson & Scopes, 1960, 1962). The cells were then centrifuged three times from distilled water at 2000 rev. min⁻¹ for 2 min using an MSE Mistral 4L centrifuge to remove cells and debris. The remaining pellet was suspended in 5 ml 1 % (w/v) sucrose solution and layered on to a 15 to 40 % (w/v) sucrose gradient (Mitchison & Creanor, 1969). The gradient was centrifuged at 1500 rev. min⁻¹ for 5 min and fractionated using a Denisflow fractionator (Buchler, Fort Lee, N.J., U.S.A.). A middle fraction which contained uniformly large cells was collected, washed with distilled water to remove sucrose, then suspended in medium 2 and incubated at 28 °C.

Enzyme assays. Cells for analysis were harvested by centrifuging at 5000 *g* for 3 min and washed three times in ice-cold distilled water. Washed cells were suspended in the appropriate assay buffer, mixed with 5 ml of glass beads (0.45 to 0.5 mm diam.) and homogenized for 60 s in a Braun (Melsungen, W. Germany) homogenizer. This gave a 92 % release of protein. Cells from synchronous cultures could not be assayed immediately. These were filtered on to a Millipore membrane (0.45 µm pore size), washed with ice-cold distilled water, and the membrane was inserted in a disposable polystyrene tube and freeze-dried over phosphorus pentoxide. The assays for sucrase and acid and alkaline phosphatases were carried out directly on the freeze-dried cells (Mitchison & Creanor, 1969).

Sucrase (EC 3.2.1.26). This was assayed using the method of Dahlquist (1964) in which the glucose released by sucrase activity is assayed spectrophotometrically by the glucose oxidase method. Extract (0.5 ml) was added to 1 ml of reaction mixture containing 5 % (w/v) sucrose in 0.05 M-citrate buffer pH 4.5. The reaction was stopped after 20 min by adding a few drops of 13.4 M-NaOH. Then 0.5 ml of the incubation mixture was transferred to a second tube containing Tris/glucose oxidase reagent (Sigma). The colour development was measured at 420 nm on a Pye Unicam SP600 spectrophotometer.

Ornithine aminotransferase (EC 2.6.1.13). This was assayed using the method of Middelhoven (1963, 1964). Extract (0.5 ml) was added to 1 ml of reaction mixture containing 20 µmol disodium α-ketoglutarate, 1.0 µmol pyridoxal 5-phosphate, 10 µmol *o*-aminobenzaldehyde, 10 µmol MgCl₂ and 100 µmol phosphate buffer pH 7.4. The reaction was stopped by adding 0.1 ml 15 % (v/v) perchloric acid; then, after removal of denatured protein by centrifugation, absorbance was read at 450 nm.

Acid phosphatase (EC 3.1.3.2). This was determined by the method of Schmidt *et al.* (1963). Extract (1 ml) was added to 9 ml of reaction mixture containing 0.05 M-sodium acetate pH 4.0 and 0.005 M-sodium phenyl phosphate. After incubation for 10 min at 27 °C, the reaction was stopped by adding 5 ml 5 % (w/v) trichloroacetic acid. After centrifugation, the phosphate content of the supernatant was determined by the method of Sumner (1944).

Alkaline phosphatase (EC 3.1.3.1). This was determined using a Biochemica Test (Boehringer), which contained 5.5 mm-*p*-nitrophenyl phosphate in 50 mm-glycine buffer as the substrate.

Proteinase A (EC 3.4.23.8). Washed whole cells were incubated with 3 ml 2 % (w/v) acid-denatured haemoglobin for 60 min at 27 °C (Lenney, 1956). Incubation was stopped by centrifugation to remove the cells, and then 4 ml 5 % (w/v) trichloroacetic acid was added. The protein precipitate was removed by centrifuging and the trichloroacetic acid-insoluble material released by the enzyme was determined using the Folin Ciocalteu assay.

Chemical assays. DNA was determined by the diphenylamine reaction (Burton, 1956) and protein by the Folin Ciocalteu method (Lowry *et al.*, 1951). Analytical chemicals were obtained from either BDH or Sigma.

Fig. 1. Changes in dry weight (□), protein (Δ) and the time of appearance of mature asci (○) during sporulation of *S. cerevisiae*.

RESULTS

Enzyme formation during sporulation

High levels of sporulation, in excess of 70%, have been consistently obtained using the techniques described. During sporulation there was no increase in the cell number and no bud formation was observed; however, a twofold increase in the turbidity of the cell suspension occurred which could be attributed to a doubling of the dry weight of the cells (Fig. 1). The degree of synchrony of sporulation was not very high since ascospores appeared between 16 and 30 h. This is usual for cells grown on a glucose-based growth medium (Croes, 1967).

DNA biosynthesis occurred between approximately 5 and 15 h with the mid-point in different experiments falling between 7 and 8 h. The increase in DNA varied between 55 and 70% (Fig. 2). If all the cells which undergo DNA replication also produce ascospores then cultures exhibiting 70% sporulation should also show a 70% increase in the DNA level. The protein content of the developing asci increased during the first 5 h then decreased between 5 and 10 h in a manner similar to that reported by Croes (1967) (Fig. 1).

Studies on step enzyme formation should be carried out when the cells are either fully repressed or fully induced for the enzyme concerned, and transitions from one state to the other should be avoided (Sebastian *et al.*, 1973). During sporulation, the absence of phosphate should favour a derepressed level of acid phosphatase (Mitchison & Creanor, 1969) and a constitutive level of alkaline phosphatase (Masters & Donachie, 1966). Sucrase activity should be derepressed in the absence of glucose (Dodyk & Rothstein, 1964), and ornithine aminotransferase should be present at a basal level since arginine was absent from the medium (Middelhoven, 1964; Sebastian *et al.*, 1973). For each experiment, data for DNA have been included since there was some variation between rates of sporulation in different experiments.

The activity of ornithine aminotransferase increased in a stepwise manner between 4 and 12 h and the mid-point of the rise, i.e. the time at which a 50% increase in enzyme activity had taken place, occurred about 60 min later than the mid-point of the rise in DNA synthesis (Fig. 2). Acid phosphatase activity increased between 10 and 14 h with a mid-point at 12 h, 5 h after the mid-point of the DNA increase (Fig. 2). Sucrase also exhibited a single step increase between 5 and 10 h, its mid-point occurring about 90 min after the mid-point of DNA increase (Fig. 3). The increase in alkaline phosphatase occurred in two steps,

Fig. 2. Increases in ornithine aminotransferase activity (□), acid phosphatase activity (■) and DNA (●) during sporulation of *S. cerevisiae*.

the first between 4 and 8 h and the second between 10 and 14 h; the mid-points of these increases occurred 60 min earlier and 6 h later than the mid-point of DNA biosynthesis (Fig. 3). Whereas acid phosphatase, ornithine aminotransferase and alkaline phosphatase showed an approximate doubling of activity, the increase in sucrase activity was much greater.

Enzyme formation in vegetative controls

The results obtained with sucrase, which showed only one period of enzyme formation in contrast to the two steps reported previously (Gorman *et al.*, 1964), indicated that it would be necessary to carry out control experiments on synchronous vegetative cells using this strain. The synchrony in the cultures prepared for these experiments persisted for two cell cycles (Fig. 4). The degree of synchrony was estimated using the technique of Scherbaum (1964) and Synchronization Index values of 0.57 and 0.49 were obtained for the first and second cell cycles, respectively. These values are in close agreement with those reported by Scherbaum (1964) for a number of synchronous microbial systems. The increase in DNA and the time of bud appearance are also shown in Fig. 4. The initiation of DNA biosynthesis correlated well with the time of bud initiation as reported by Williamson (1974). Taking the time of first bud appearance as the start of the cell cycle, the mid-points of increase in enzyme activities were expressed as a fraction of the cell cycle. Sucrase showed a sharp doubling with a mid-point at 0.4 in the first cell cycle (Fig. 5). Acid phosphatase also doubled in activity with a mid-point at 0.6, but alkaline phosphatase showed a two-step increase during the first cell cycle with mid-points at 0.12 and 0.45 (Fig. 5).

Proteinase A formation during sporulation

Proteinase A increased to a peak at 14 h and then decreased (Fig. 6). A similar increase in proteinase A activity has been reported by Klar & Halvorson (1975). However, in their experiments the peak of activity occurred at 20 h and the loss of activity at the time of sporulation was much less than we have observed. They reported that the increase in activity was dependent upon protein biosynthesis and that a further stimulation could be obtained by heat activation of the samples. Since this increase was spread over the whole

Fig. 3. Increases in sucrase activity (○), alkaline phosphatase activity (▲) and DNA (●) during sporulation of *S. cerevisiae*.

Fig. 4. Increases in cell number (□), protein (Δ) and DNA per ml (●) during synchronous growth of *S. cerevisiae*. The arrows indicate the times of bud initiation.

of the first 12 h of the fermentation it is probably not acceptable to consider it to be the result of a periodic increase in an unstable enzyme (Mitchison, 1971). However, the timing of the increase in proteinase activity, from a low point prior to the initiation of DNA biosynthesis to a peak immediately before ascospore formation, shows some similarity to the pattern of proteinase activity in vegetative yeast. It has been reported that proteinase activity is low before the S phase and high immediately before cell division (Lenney *et al.*, 1976). Since proteinase A occurs in vacuoles (Matile & Wiemken, 1967), the changes in proteinase A activity may be associated with changes in the organization of the vacuoles which occur during sporulation (Heywood & Magee, 1976).

Fig. 5

Fig. 6

Fig. 5. Increases in sucrose activity (○), acid phosphatase activity (■), and alkaline phosphatase activity (▲) during synchronous growth of *S. cerevisiae*. The scale represents the duration of the first cell cycle and the bar represents the duration of DNA biosynthesis; arrows indicate the mid-points of enzyme increase.

Fig. 6. Changes in proteinase activity during sporulation of *S. cerevisiae*.

Fig. 7. Diagrammatic comparison of the sequence of enzyme 'steps' during the vegetative cell cycle and during sporulation. The period from one bud initiation to the next was taken as one vegetative cell cycle. During sporulation the meiotic cycle was measured from the time of transfer into sporulation medium to the time of appearance of ascospores. For each enzyme, the duration of the period of increase is indicated by the bar and the mid-point of the increase by the triangle.

DISCUSSION

The enzymes investigated in this study were selected because they have been previously described as step enzymes in *Saccharomyces cerevisiae*. We are not aware of any specific role for these enzymes in sporulation. In *S. cerevisiae*, sucrase and alkaline phosphatase have been reported to increase in two steps in the cell cycle, whereas ornithine aminotransferase and acid phosphatase were synthesized in the more typical single-step manner (Gorman *et al.*, 1964; Tauro & Halvorson, 1966). The times of appearance of step enzymes have been recognized as markers of the 'growth cycle'. During exponential growth, the doubling of each cellular component is essential to maintain balanced growth (Bu'lock, 1975). During sporulation, growth is restricted to a limited period during the early stages, and cell division involves a special kind of nuclear division – meiosis – which gives rise to four haploid nuclei which are incorporated into the four ascospores. Thus there is no obvious reason why a pattern of step enzymes should be found during sporulation.

The results obtained in this study suggest, however, that step enzyme formation does occur during sporulation. Ornithine aminotransferase, acid phosphatase and sucrase increased in activity in a single step and alkaline phosphatase exhibited a two-step increase. The single-step increase in sucrase activity was particularly unexpected since it has been reported to be a two-step enzyme. However, the results obtained in synchronous vegetative cells (Fig. 5) suggest that the difference between our results and those of Halvorson and co-workers (Gorman *et al.*, 1964) can probably be attributed to strain differences, rather than differences between the vegetative cell cycle and sporulation. It has been proposed that two or more steps occur when multiple non-allelic genes for the same enzyme are present in the same genome (Tauro & Halvorson, 1966). Acid and alkaline phosphatases and ornithine aminotransferase approximately double in activity during sporulation.

The periodic or step increase in activity in these enzymes suggests that they may be under a similar control mechanism to that operating during the vegetative cell cycle. This view is also supported by the observation that the sequence of appearance of alkaline phosphatase (step 1), DNA, sucrase and acid phosphatase is the same in the vegetative cell cycle and in sporulation. The appearance of alkaline phosphatase (step 2) appears to be delayed during sporulation relative to sucrase and acid phosphatase (Fig. 7).

Since the biosynthesis of DNA occupies a relatively longer period during sporulation than in the vegetative cell cycle, more of the step enzyme increases fall during the S phase. However, this may not be significant since the formation of many step enzymes during the vegetative cell cycle has been shown to be independent of DNA biosynthesis. Similar results have been obtained during sporulation (Matur, 1977).

Two hypotheses have been presented to explain step enzyme formation: the oscillatory repression model (Masters & Donachie, 1966) and the linear transcription model (Halvorson *et al.*, 1971). We have not been able to distinguish between these models on our present data and the restrictions imposed by the conditions required for sporulation limit the controls which could be carried out using this system. However, since phosphate, arginine, sucrose and glucose are absent from the sporulation medium there is no obvious reason why the induction state of the enzymes studied should alter during the experiments. If control by oscillatory repression was occurring, it would be necessary to postulate that fluctuations in the levels of endogenous intermediates followed the same temporal sequence during both growth and sporulation.

The difficulties of explaining multi-step enzyme formation using the oscillatory repression model have already been discussed (Mitchison, 1971). The observation that sucrase increases in a single step in our strain of yeast but in two steps in other strains (Gorman *et al.*, 1964) is perhaps easier to explain in terms of changes in the number of structural genes for sucrase, rather than differences in the pattern of intermediates between the two strains. The existence of a similar enzyme pattern during both growth on a rich medium and sporulation on a

minimal acetate medium suggests that step enzyme formation is tightly controlled rather than subject to transient changes in the metabolic state of the cell.

We are aware that sporulation of yeast cannot be considered as a true cell cycle since it involves a reduction division, which has the characteristics of two nuclear divisions, giving rise to four haploid nuclei. However, we feel that it is valuable to consider the early stages of sporulation as a modified cell cycle which involves a G1 phase, an extended S phase, a G2 phase and a modified nuclear division. An improved understanding of the genetic control of meiosis has been obtained by studying the effect of *cdc* mutations (which are selected by their effect on mitosis) on meiosis (Hartwell, 1974; Simchen, 1974).

The results of Simchen (1974) indicate that the *cdc* mutations which affect meiosis are those which affect nuclear division and DNA replication in the vegetative cycle, whereas those which affect bud emergence, cytokinesis and cell separation do not affect meiosis. In the vegetative cell, growth is associated with bud formation which appears to be genetically independent of the events of the DNA-division cycle (Hartwell, 1974). Since step enzymes are considered to be markers of the growth cycle, it is surprising that these are not suppressed during sporulation as are the morphological markers associated with vegetative growth, i.e. bud emergence and growth, nuclear migration and cytokinesis and cell separation.

There seems little doubt that many of the nuclear events of mitosis and meiosis are under common genetic control.

We propose the hypothesis that the vegetative cell cycle and meiospore development are under common biochemical control. Thus the process of sporulation in yeast can be considered as a growth cycle, in which step enzymes can be used as markers, and a DNA-division cycle, which includes such events as DNA and histone biosynthesis and commitment to recombination and meiosis itself.

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