Changes in Respiratory Activities during the Cell-Cycle of the Fission Yeast *Schizosaccharomyces pombe* 972h⁻ Growing in the Presence of Glycerol

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1. The specific activities of cytochrome *c* oxidase, catalase, succinate dehydrogenase, succinate-cytochrome *c* oxidoreductase, NADH-cytochrome *c* oxidoreductase, and NADPH-cytochrome *c* oxidoreductase in mid-exponential-phase batch cultures of glycerol-grown *Schizosaccharomyces pombe* indicated that the organisms were catabolite-repressed. 2. In cultures growing synchronously in the presence of glycerol as sole carbon source, the respiration rate showed two abrupt increases at about 0.45 and 0.95 of the cell-cycle and remained constant in the periods between successive rises. 3. Catalase, succinate dehydrogenase, NADH-cytochrome *c* oxidoreductase and acid *p*-nitrophenyl-phosphatase all showed peak patterns of expression in synchronous cultures. 4. Cytochrome *c* oxidase and cytochromes *a*+*a*₃ both showed step patterns of expression with two rises per cell-cycle. 5. Cytochromes *c*₅₄₈, *b*₅₅₄ and *b*₅₆₀ all followed similar time-courses in step patterns of expression, but these were distinct from, and more complex than, that of cytochromes *a*+*a*₃. 6. These results are compared with those previously obtained with glucose-grown cultures, and the part played by catabolite repression in the expression of respiratory activities in the cell-cycle is assessed.

Previous work on the development of respiratory activity during the cell-cycle of the fission yeast *Schizosaccharomyces pombe* has been with cells growing in the presence of 1% glucose. Under these conditions many of the enzymes of terminal respiration are catabolite-repressed (Poole & Lloyd, 1973), and the respiration rates of cell suspensions from synchronous cultures show an oscillation with a periodicity of 0.5 of a cell-cycle (Poole *et al.*, 1973). All enzymes assayed exhibited peak patterns of expression during the cell-cycle (Poole & Lloyd, 1973) as did cytochromes *b*₅₆₃ and *a*+*a*₃ (Poole *et al.*, 1974).

In the present study the effects of catabolite repression produced by the glucose-containing medium have been eliminated by utilization of glycerol as the carbon source. We report that, under these conditions, several enzymes including succinate dehydrogenase and NADH-cytochrome *c* oxidoreductase are still expressed in peak patterns, whereas cytochrome *c* oxidase and cytochromes *a*+*a*₃, as well as cellular respiration, all increase periodically exhibiting two 'steps' per cell-cycle. The implications of these results in terms of possible mechanisms of regulation of synthesis of mitochondrial enzymes are discussed.

Materials and Methods

Maintenance, growth and harvesting of the organism

S. pombe 972h⁻ (kindly supplied by Dr. Urs Leupold, Institute of General Microbiology, University of Bern, Switzerland) was maintained and grown

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on defined medium as described by Poole *et al.* (1973) except that 1% (w/v) glycerol was employed instead of glucose as the carbon source, and the pH of the medium was adjusted to 4.5 with 1M-HCl.

Preparation and harvesting of synchronous cultures for the determination of enzyme amounts and cellular contents of cytochromes

Synchronous cultures were prepared by a velocity sedimentation size-selection method (Mitchison & Vincent, 1965) on sucrose gradients in an MSE HS zonal rotor as described previously (Poole *et al.*, 1973); gradient solutions were made up in growth medium containing 1% glycerol. The degree of synchrony was assessed by the method of Blumenthal & Zahler (1962). Harvesting of synchronous cultures and the subsequent preparation of cell-free extracts for determination of enzyme amounts by using a French press (Milner *et al.*, 1950) was as described by Poole & Lloyd (1973).

For the determination of cytochrome contents of intact cells, culture samples (200ml during the first 3h of synchronous growth, and subsequently 100ml) were divided into two equal portions and cells harvested by acceleration to 5000rev./min followed by immediate deceleration in the 6×250ml rotor of the MSE 18 centrifuge at 4°C. Each pellet was resuspended quantitatively in 50mM-potassium phosphate-0.4M-mannitol (pH 7.4) containing either 2% (w/v) glucose (for reduction of cytochromes) or 3.3% (v/v) H₂O₂ (for oxidation of cytochromes) respectively to a total volume of 1ml and the suspensions were transferred to 2ml glass centrifuge tubes. Cells were

pelleted by centrifugation for 2 min at 1000 rev./min in a BTL Bench Centrifuge and resuspended to total volumes of 0.2 ml in the same tubes by using the respective buffer. These cell suspensions were used for the recording of cytochrome difference spectra.

Enzyme assays

Details of enzyme assays were as described by Poole & Lloyd (1973). Assays were performed within 12 h of preparation of the extracts. Enzyme units are expressed as nmol of substrate transformed/min or as nmol of measured product formed/min.

Other determinations

Protein. Protein was measured in cell-free extracts by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Total cell protein was determined in 0.5 M-NaOH extracts as described by Herbert *et al.* (1971) with the same standard.

Glycerol and ethanol. Glycerol was measured by the method of Garland & Randle (1962). Ethanol was determined by using Sigma reagent kit 330-UV.

Oxygen uptake. Respiration of cell suspensions was measured polarographically by using the apparatus described by Lloyd & Brookman (1967).

Cytochrome spectra. Difference spectra were traced by using a Unicam SP. 1800 recording spectrophotometer fitted with an attachment (constructed in this laboratory) to allow recordings to be made at the temperature of liquid N₂. The spectral band width was 1 nm and the scanning speed 0.5 nm/s. Quantification of cytochrome amounts was achieved by using the wavelength pairs, extinction coefficients and low-temperature intensification factors described previously (Poole *et al.*, 1974).

Results

Changes in enzyme activities during growth of an asynchronously dividing culture

In an asynchronously dividing culture, exponential growth continued until a stationary phase population (10^8 cells/ml) was attained (Fig. 1a). The mean generation time was 6 h throughout the period of exponential growth. Over the first 40 h of incubation, glycerol was utilized rapidly and ethanol accumulated. The specific activities of the enzymes assayed declined so as to reach minimal values at the end of this period (Figs. 1b–1d). Subsequently, glycerol was utilized at a slower rate and the concentration of ethanol fell rapidly. Activities of catalase and acid *p*-nitrophenylphosphatase (Fig. 1b) and NADH-cytochrome *c* oxidoreductase (Fig. 1d) increased continuously during this phase, whereas those of cytochrome *c* oxidase (Fig. 1b), NADPH-cytochrome *c* oxidoreductase (Fig. 1d), malate dehydrogenase, succinate

dehydrogenase and succinate-cytochrome *c* oxidoreductase (Fig. 1c) increased at first but declined as the stationary phase of growth was attained. The specific activities of all enzymes [except that of acid *p*-nitrophenylphosphatase, which remains at repressed values throughout (Mitchison, 1970; Mitchison & Creanor, 1969)] during the phase of rapid glycerol utilization were 4–20-fold higher than corresponding activities during glucose-repressed growth, but were similar to those observed after glucose de-repression (Poole & Lloyd, 1973). Organisms used for the establishment of synchronous cultures were harvested before the population reached 5×10^7 cells/ml.

Cellular contents of cytochromes in glycerol-grown cells were higher than those in cells grown under conditions of glucose repression (Poole *et al.*, 1974); whereas the contents of *b*- and *c*-type cytochromes were increased only by a factor of 1.5–2.0, the cellular content of cytochromes *a*+*a*₃ was increased 14-fold. Cytochrome *P*-450 is present in glucose-grown cells (Poole *et al.*, 1974), but was not detected in CO difference spectra (dithionite-reduced+CO minus dithionite-reduced) of glycerol-grown cells (R. K. Poole, D. Lloyd & B. Chance, unpublished work).

O₂-uptake of cell suspensions from a synchronous culture

Cell counts increased in a stepwise fashion through two doublings in numbers (Fig. 2a). The duration of the first full cycle of a typical synchronous culture growing in the presence of 1% glycerol was 5 h. Synchrony indices for the first and second cell divisions (*F*₁ and *F*₂) varied between 0.75 and 0.85. Respiration rates of undiluted samples from the culture vessel also increased in a stepwise fashion (Fig. 2b), i.e. O₂-uptake rates rose abruptly twice during each cell-cycle and remained constant in the periods between successive rises such that the respiration rate increased overall by a factor slightly greater than 2 during the cell-cycle. The first abrupt increase in respiration rate occurred during the phase of cell elongation before cell division; this was followed by a period when respiration rate remained constant for approx. 2 h. A further abrupt increase in respiration rate coincided with cell division. This pattern was then repeated during the next cell-cycle. Each of the two plateaux in respiration rate occurring during one cell-cycle were approximately equal in duration (1.5–2.0 h). Addition of an uncoupler of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenylhydrazone, to samples from cultures of cells growing exponentially with glycerol (4.1×10^7 cells/ml) indicated that maximum stimulation of respiration by this compound was produced at a concentration of 0.3 μM. The addition of 0.3 μM-carbonyl cyanide *m*-chlorophenylhydrazone produced between 3 and 13% acceleration of respiration during the periods

Fig. 1. Changes in cell population, glycerol and ethanol concentrations in the medium, and specific activities of enzymes in cell-free extracts from *S. pombe* during growth in glycerol-containing medium

(a) shows the cell concentration (cells/ml, ●), glycerol concentration (□) and ethanol concentration (■). (b) shows activities of cytochrome c oxidase (●), catalase (○) and acid p-nitrophenylphosphatase (Δ). (c) shows activities of succinate dehydrogenase (▽), succinate-cytochrome c oxidoreductase (□) and malate dehydrogenase (●). (d) shows activities of NADH- (■) and NADPH- (▲) cytochrome c oxidoreductases. The enzyme activities are all expressed as specific activities (nmol/min per mg of protein).

Fig. 2. O_2 uptake of yeast suspensions from a glycerol-containing synchronous culture of *S. pombe* and effect of carbonyl cyanide *m*-chlorophenylhydrazone

F_1 and F_2 denote the synchrony indices of the first and second doublings in cell numbers (●) respectively. O_2 -uptake measurements on culture samples removed at frequent intervals from a synchronous culture were made in the absence (○) or the presence (■) of $0.3 \mu M$ -carbonyl cyanide *m*-chlorophenylhydrazone.

when the respiration rate remained constant, but between 13 and 34% acceleration of O_2 consumption during the periods of increasing respiration rate (Fig. 2c). The stimulatory effect of carbonyl cyanide *m*-chlorophenylhydrazone decreased throughout the experiment as cell numbers increased.

Changes in enzyme amounts

Assays of total protein indicated that this increased exponentially through the cell-cycle of a synchronous culture (Fig. 3a). Assays of protein released in the cell-free extracts (Fig. 3a) indicated that there was no marked variation in the susceptibility of organisms to disruption at different times in the cell-cycle. Between 45 and 65% of the total cell protein was recovered in the extracts, and the measured efficiencies of cell breakage for each sample were used in the calculation of total enzyme units in the culture. Total amounts of cytochrome *c* oxidase increased in stepwise fashion, rising abruptly twice during each cell-

cycle, once during cell division, and once at approx. 0.5 of the cell-cycle (Fig. 3b). The amounts of other enzymes assayed exhibited 'peak' patterns of expression (Fig. 3b). For catalase and succinate dehydrogenase, two maxima were observed per cell-cycle; one occurred during cell division and the other at approx. 0.5 of the cell-cycle. Similar patterns of expression of acid *p*-nitrophenylphosphatase and NADH-cytochrome *c* oxidoreductase were also evident, but the timings of occurrence of maximal amounts of these two enzymes were not clearly defined as these were observed as broad plateaux rather than as clearly defined 'peaks'.

Changes in the cellular content of cytochromes

Cell numbers increased in a stepwise manner through one complete doubling, and a satisfactory synchrony index was obtained (Fig. 4a). Cytochromes $a+a_3$ (measured at 603–630 nm) increased in a stepwise pattern; the mid-points of increase occurred during cell division and at approx. 0.5 of the cell-cycle (Fig. 4b); the timings of these increases are similar to those observed for cytochrome *c* oxidase activity (Fig. 3b). The changes in total amounts of cytochromes c_{548} , b_{554} and b_{560} also occurred in stepwise fashion; the expression of these three cytochromes all followed a similar time-course. The timing of the increase observed at approx. 0.5 of the cycle was similar to that observed for cytochromes $a+a_3$, but at other times in the cell-cycle the patterns of variation were more complex.

Discussion

Studies of the repressive effects of growth with fermentable carbon sources such as glucose have been of value in studying mitochondrial biogenesis in yeast (see review by Linnane *et al.*, 1972). However, superimposition of the complex phenomena resulting from glucose-repression may complicate interpretation of the effects on biogenesis of other parameters under investigation. Thus it is of value to assess the contribution of the effects of glucose-repression on the observed changes in respiratory activities during the cell-cycle in glucose-grown cultures.

Previous results have indicated that when *S. pombe* is grown in the presence of 1% glucose the activities of several of the enzymes involved in terminal respiration (e.g. malate dehydrogenase, succinate dehydrogenase, NADH-cytochrome *c* oxidoreductase and cytochrome *c* oxidase) remain low until glucose is exhausted from the medium (Poole & Lloyd, 1973). The activities of these enzymes in organisms harvested from cultures grown with glycerol to the mid-exponential phase of growth indicate that at this stage the cells are catabolite-de-repressed; this is confirmed by the observation that the cellular content of

Fig. 3. Changes in cell population, total cell protein, protein in cell-free extracts and amounts of enzymes during growth of a glycerol-containing, synchronously dividing culture of *S. pombe*

(a) shows the synchrony index (F_1) of the first doubling in cell numbers (●) and total (□) and released (○) protein of cell suspensions used to prepare cell-free extracts. The vertical line indicates the midpoint of doubling in cell numbers. (b) shows amounts of cytochrome *c* oxidase (●), catalase (○), acid *p*-nitrophenylphosphatase (▲), NADH-cytochrome *c* oxidoreductase (■) and succinate dehydrogenase (▽). Amounts of enzymes are expressed as units/ml of culture.

Fig. 4. Changes in cellular content of cytochromes during the growth of a glycerol-containing synchronously dividing culture of *S. pombe*

F_1 denotes the synchrony index of the first doubling in cell numbers (a). (b) shows amounts of cytochromes $a+a_3$ (●), c_{548} (○), b_{554} (▲) and b_{560} (▽). Amounts of cytochromes are expressed as nmol/100 ml of culture.

cytochromes, particularly that of cytochromes $a+a_3$, is greatly increased by growth with the non-fermentable substrate.

Whereas the respiration rate of synchronous cul-

tures growing in the presence of 1% glucose shows an oscillatory pattern (Poole *et al.*, 1973), the O_2 -uptake rates of the catabolite-de-repressed cultures studied here show a double step per cell-cycle; the

Scheme 1. Cell-cycle maps of timings of midpoints of increases in O_2 uptake and enzyme amounts during the cell-cycle of glycerol-grown *S. pombe*

In (a) and (b) the abscissa represents (as a linear scale extending from 0 to 1.0) the cell-cycle taken as the period between the midpoints of the first and second doublings in cell numbers in synchronous cultures. ∇ denote the midpoints of increase of O_2 uptake (a) or cellular content of cytochromes $a + a_3$ (b) observed in the period before the first doubling in numbers (i) or in the following complete cell-cycle (ii). In (c), the abscissa represents the cell-cycle (assumed to be of length 5 h in synchronously dividing cultures) where 0 and 1.0 are defined by the midpoint of doubling in cell numbers. ∇ denote the midpoints of increase or maxima in enzyme amounts. In all cases, arrows with crossbars are mean values with s.d.

relative timings of the two maxima seen in glucose-containing cultures are similar to those of the abrupt increases in cultures containing glycerol (Scheme 1a). These observations confirm the previous suggestion (Poole *et al.*, 1973) that the feedback control mechanism involved in the generation of respiratory oscillations does not act at the level of terminal respiration itself, but is rather a consequence of the interaction of respiratory processes with those of glycolytic reactions involved in glucose utilization (i.e. a Pasteur effect). The oscillatory behaviour of respiration in synchronized cultures of *Saccharomyces cerevisiae* growing with glucose as carbon source was not evident in maltose-grown cultures (Dharmalingam & Jayaraman, 1973), and it appears likely that differences in growth substrates can account for the discrepancies between the various reports of respiratory oscillations (Nosoh & Takamiya, 1962; Wiemken *et al.*, 1970) and stepwise increases (Scopes & Williamson, 1964; Osumi & Sando, 1969; Marchant, 1971; Greksák & Hanicová, 1973) in the respiration of synchronous yeast cultures.

The peak patterns of expression of catalase, succinate dehydrogenase, NADH-cytochrome *c* oxido-

reductase and acid *p*-nitrophenylphosphatase in glycerol-grown synchronous cultures of *S. pombe* are similar to those observed previously in catabolite-repressed cells (Poole & Lloyd, 1973). Thus it appears that the control mechanisms involved in the expression of these enzymes at set times in the cell-cycle proceed independently of catabolite repression itself. Several general models explaining periodic enzyme synthesis during the cell-cycle have been suggested (Mitchison, 1971; Halvorson *et al.*, 1971) including one based on oscillatory repression. However, the mode of expression of both cytochrome *c* oxidase activity and of spectrophotometrically detectable cytochromes $a + a_3$ is strikingly different in cells growing under conditions of catabolite repression from that in those growing with glycerol as carbon source; the complex peak pattern seen in cells growing with glucose is replaced by a pattern of two steps per cell-cycle. The mechanisms by which newly synthesized enzymes are subsequently modified with loss of activity to give peak patterns of expression have not been investigated. It is evident that in glucose-grown *S. pombe* loss of cytochrome *c* oxidase activity is accompanied by loss of haemoprotein (Poole *et al.*, 1974);

that this process does not occur in the case of growth with glycerol points to an involvement of catabolite repression.

The relative timings of the midpoints in increases of respiration rate, cytochromes $a+a_3$ and cytochrome c oxidase and of maxima in amounts of other enzymes in glycerol-grown *S. pombe* are summarized in Scheme 1.

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