The Cell Cycle of the Budding Yeast *Sterigmatomyces halophilus*: Oscillations in the Amounts and Activities of the Terminal Components of the Respiratory Chain

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Cells harvested from exponentially growing cultures of the budding yeast *Sterigmatomyces halophilus* were fractionated according to their age in the cell cycle by isopycnic-zonal centrifugation in Urografin gradients. Activities of five enzymes were assayed in cell-free extracts, prepared using a French pressure cell. The activities of catalase and three enzymes of the mitochondrial inner membrane (NADH dehydrogenase, succinate dehydrogenase and cytochrome *c* oxidase) doubled during the cell cycle but showed complex oscillatory patterns. Acid phosphatase activity increased continuously during the cell cycle. Fourth-order finite difference analysis of low-temperature difference spectra showed that the levels of three *b*-type cytochromes increased continuously during the cycle. In contrast, amounts of cytochromes *c*₁, *c* and *aa*₃ oscillated over one cycle, with cytochromes *c* and *aa*₃ rising to two maxima per cycle in phase with cytochrome *c* oxidase activity. Liganding of redox-active metals, and discontinuous synthesis and proteolysis of apocytochromes are discussed as possible mechanisms for cell cycle-dependent fluctuations in the composition and function of the respiratory chain.

INTRODUCTION

In the cell cycles of many eukaryotic micro-organisms, the activities of certain enzymes increase discontinuously (for a review, see Lloyd *et al.*, 1982*a*). It is not clear, however, whether these changes in activities represent actual changes in amounts of enzyme proteins or modulation of their activities. Only a few studies have been made of changes in enzyme amounts during the cell cycle. Because of technical difficulties in quantitatively purifying individual enzymes during the cell cycle, immunological assays have been used. For example, the amount of catalase protein oscillates with a periodicity of about 1 h so as to give seven maxima per cell cycle in *Acanthamoeba castellanii* (Edwards *et al.*, 1981). An alternative approach has been to examine the rate of synthesis of individual proteins, revealed by two dimensional electrophoresis, using a dual-label procedure involving long-term and pulse labels. Using this technique, Elliott & McLaughlin (1978) examined the behaviour of 111 proteins during the cell cycle of *Saccharomyces cerevisiae* and concluded that they all showed a continuous, exponentially increasing, rate of synthesis. However, these authors considered three classes of proteins to be less well resolved by this method – the very acidic, the very basic, and those of low solubility, such as the membrane-associated proteins of mitochondria. Fortunately, certain proteins in the last class, namely the cytochromes, are directly measurable by spectroscopic examination of intact cells.

The direct determination of cytochrome content has the distinct advantage that no extraction of proteins is necessary and that, in some cases, the spectrally determined quantities may be compared with measurements of functional activity (Poole & Lloyd, 1974; Poole *et al.*, 1974;

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Cottrell *et al.*, 1975). The paucity of similar measurements may be due, in part, to (1) the relative insensitivity of the technique (and the consequent necessity for large numbers of cells of one particular age) and (2) the extensive overlap of individual cytochrome absorptions, even at low temperatures, making accurate quantification difficult (Salmon & Poole, 1980*a*; von Jagow *et al.*, 1973).

In the present study, the activities of three mitochondrial enzymes and the amounts of spectroscopically detectable cytochromes were examined during the cell cycle of the budding yeast *Sterigmatomyces halophilus*. Additionally, the activities of two marker enzymes (Lloyd & Cartledge, 1974) for other cellular membraneous systems (catalase for peroxisomes and acid phosphatase for lysosomes) were measured. Sufficient material for such studies was provided by large-scale zonal fractionation of exponentially growing cultures into different density and size classes (Salmon & Poole, 1983). Fourth-order finite difference analysis of the complex cytochrome spectra was used to resolve and quantify the individual components, some of which accumulate continuously, whilst cytochrome *c* and its immediate electron acceptor, cytochrome *aa*₃, oscillate in phase with cytochrome *c* oxidase activity.

Parts of this work have been presented previously in abstract form (Salmon & Poole, 1980*b*).

METHODS

Maintenance, growth and harvesting of the organism. *Sterigmatomyces halophilus* CBS 4609 was maintained on a solid, complex medium and grown in a defined liquid medium with glucose as the growth-limiting substrate as described previously (Salmon & Poole, 1980*a*). Exponentially growing cultures were harvested by passing the culture through an MSE continuous action rotor and resuspending the cells in a small volume of 3% (w/v) NaCl (Salmon & Poole, 1983).

Culture fractionation. Fractionation of the cell population into various size classes, representing successive stages in the cell cycle, was by isopycnic-zonal centrifugation through Urografin gradients (Salmon & Poole, 1983). Fractions were collected, and the cells harvested and washed as described previously (Salmon & Poole, 1983) and resuspended in 50 mM-Tris/H₂SO₄ buffer (pH 7.2).

Preparation of cell-free extracts. Cells, resuspended in a small volume (5 to 10 ml) of 50 mM-Tris/H₂SO₄ buffer (pH 7.2) were broken by two slow passages through a cold (4 °C) French pressure cell (Milner *et al.*, 1950) at 138 MPa (20000 lbf in⁻²). Unbroken cells and cell wall debris were removed by acceleration to 10000 r.p.m. in the 16 × 15 ml rotor (8500 g, *r*_{av}, 7.6 cm) of an MSE 18 centrifuge, followed by immediate deceleration. The supernatant, which was termed the cell-free extract, was carefully decanted to avoid disturbance of the pellet.

Cytochrome spectra and their numerical analyses. Difference spectra at 77 K and their fourth-order finite difference analyses were obtained exactly as described by Salmon & Poole (1980*a*). Suspensions of cells in 50 mM-Tris/H₂SO₄ buffer (pH 7.2) were reduced with a few grains of NaBH₄ or oxidized with 4.5 mM-H₂O₂ before freezing the samples by immersion in liquid nitrogen. To improve signal-to-noise ratios, six successive scans were summed for each spectrum. No baseline corrections were made.

The intensities of the cytochrome α -bands in reduced minus oxidized difference spectra of intact *S. halophilus* were directly proportional to cell concentration up to 1.6×10^{10} cells ml⁻¹. Above these concentrations, the intensities decreased due to the high non-specific absorption and light scattering of the suspensions. Cell concentrations were, therefore, adjusted to about 1×10^{10} cells ml⁻¹ for quantifying cytochromes.

To enable the measured amounts of cytochromes to be tested against a null hypothesis (see below), the reproducibility of the analysis for each absorption maximum was estimated by preparing 10 replicate reduced minus oxidized difference spectra of cells from an exponentially growing culture. For cytochrome *aa*₃, the intensity of the peak at 600 nm was measured and the coefficient of variation calculated (s.d./mean). In the region 530 to 570 nm, the fourth-order finite difference analysis for each spectrum was computed. The mean positions of the revealed absorption maxima and the coefficients of variation in the intensity of each peak were then calculated. The mean peak positions (nm) with their coefficients of variation in intensity (in parentheses) were: 543.5 (0.17); 547.2 (0.10); 551.1 (0.21); 554.5 (0.70); 556.6 (0.27); 559.8 (0.10); 563.1 (0.26); 600 (0.10). The coefficients of variation in the positions of the absorption maxima were all about 5×10^{-4} .

Enzyme assays. Preliminary experiments were performed, using a cell-free extract of cells from an exponentially growing culture, to determine the pH optima of the enzymes and to measure the range over which the rates of enzyme reactions were linear with both time and amount of extract used. The spectrophotometric assays were carried out at room temperature (about 20 °C) with a Pye Unicam SP1700 spectrophotometer; acid phosphatase was determined at 25 °C.

The efficiency of breakage of cells in the French pressure cell was variable. To make allowances for this in the calculation of enzyme activities per cell, total protein was measured in samples prior to disruption, and again in the

cell-free extract. The percentage recovery of protein was calculated and the measured activities corrected to 100% release of protein (Poole & Lloyd, 1973). Within individual experiments, the protein recoveries varied little; in three selected examples of cell cycle fractionations the mean values were 50.1%, 37.1% and 28.5% and the respective coefficients of variation were 0.11, 0.11 and 0.19. No reproducible oscillations in protein recovery occurred that could account for the observed fluctuations in enzyme activities (see Results). In a control experiment, the mean protein recovery on 12 replicate samples from an exponential culture was 39% (coefficient of variation 0.15). Enzyme assays were performed on extracts prepared from cell suspensions at several concentrations and the activities per cell calculated. The coefficient of variation of these results represents the maximum variation inherent in the determination of enzyme activity.

The assay for succinate dehydrogenase (succinate:2,6-dichlorophenolindophenol oxidoreductase; EC 1.3.99.1) was based on that of Arrigoni & Singer (1962). The assay mixture contained, in a final volume of 3 ml: 6.7 mM-sodium succinate, 2.2 mM-phenazine methosulphate, 82 μ M-2,6-dichlorophenolindophenol, 3.27 mM-KCN, 100 mM-Tris/HCl buffer pH 7.5, and 20 to 100 μ l of cell-free extract. The order of addition of reagents was extract, succinate (which were incubated together for 5 to 15 min), mixture (buffer, cyanide, dichlorophenolindophenol) and (to start) phenazine methosulphate. Prior incubation of the enzyme with substrate activates the enzyme and prevents KCN deactivating it (Singer *et al.*, 1973). Measured rates were independent of the incubation times used. The rate of dye reduction was measured at 600 nm against that in control reaction mixtures lacking succinate. The coefficient of variation for this assay was 0.12 with nine samples analysed.

Cytochrome *c* oxidase (ferrocycytochrome *c*:oxygen oxidoreductase; EC 1.9.3.1) activity was measured by following the oxidation of cytochrome *c* at 550 nm (Smith, 1955). The assay mixture contained, in a final volume of 1 ml: 8 μ M-cytochrome *c* (reduced prior to use with NaBH_4), 20 mM-potassium phosphate buffer pH 6.5, and 5 to 100 μ l of cell-free extract. The rate of oxidation was measured against controls lacking the cell-free extract. The coefficient of variation for this assay was 0.08 with 10 samples analysed.

Estimation of NADH dehydrogenase (NADH:cytochrome *c* oxidoreductase; EC 1.6.99.3) activity was based on the method of Mahler (1955). The assay mixture contained, in a final volume of 1 ml: 175 μ M-NADH, 21.5 μ M-cytochrome *c*, 10 mM-KCN, 16 mM-potassium phosphate buffer pH 7.0, and 20 to 100 μ l of the cell-free extract. The reduction of cytochrome *c* was followed at 550 nm against controls lacking NADH. The coefficient of variation for this assay was 0.12 with 10 samples analysed.

Catalase (H_2O_2 : H_2O_2 oxidoreductase; EC 1.11.1.6) activity was assayed by the method of Lück (1963). The assay mixture contained, in a final volume of 3 ml: 13.3 mM- H_2O_2 , 20 mM-potassium phosphate buffer pH 7.5, and 100 to 200 μ l of cell-free extract. The rate of loss of H_2O_2 was measured at 240 nm against controls lacking the cell-free extract. The coefficient of variation for this assay was 0.14 with 6 samples analysed.

Acid phosphatase [*o*-phosphoric-monoester phosphohydrolase (acid optimum); EC 3.1.3.2] activity was determined by the method of Torriani (1960). The assay mixture contained, in a final volume of 1 ml: 5 mM-*p*-nitrophenyl phosphate, 0.02% (w/v) Triton X-100, 100 mM-sodium acetate buffer pH 5.0, and 100 to 200 μ l of the cell-free extract. The reaction was stopped by the addition of 2 ml 1 M-NaOH, and the colour that developed at 420 nm determined against a control lacking the cell-free extract. The coefficient of variation for this assay was 0.12 with 6 samples analysed.

All assays were completed within 10 h of the preparation of the cell-free extracts. Activities are expressed as μ mol of substrate transformed min^{-1} per cell or as μ mol of product formed min^{-1} per cell.

Protein assay. Protein (typically 0.5 to 1 mg ml^{-1}) was determined in cell-free extracts by Lowry's method, with BSA as standard. However, Tris buffer interferes in this assay by both decreasing chromophore development and by contributing blank colour (Rej & Richards, 1974). This interference was overcome by incorporating Tris at the same concentration (1.33 mM) in all the standards as well as samples (Rej & Richards, 1974).

The total protein content of whole cells was determined as described by Salmon & Poole (1983). Tris interference was not as marked in this assay, but corrections were made if the final concentration of Tris exceeded 0.15 mM. Total protein estimates were carried out in quadruplicate.

Cell counting and sizing. These were performed using a Coulter Counter model Z_{B1} fitted with a 50 μ m aperture probe and Channelyzer C1000 as described by Salmon & Poole (1983).

Chemicals. Cytochrome *c* (type III, from horse heart), NADH (Grade III, from yeast), 2,6-dichlorophenolindophenol and *p*-nitrophenyl phosphate were obtained from Sigma. Phenazine methosulphate was from Koch-Light, Urografin 370 was from Schering Chemicals, Burgess Hill RH15 9NE, U.K. and BSA was from Armour Pharmaceuticals, Eastbourne, U.K. General reagents were of analytical grade wherever possible.

RESULTS

Enzyme activities during the cell cycle

Using isopycnic sedimentation in Urografin gradients, cells from exponentially growing cultures of *S. halophilus* were separated into homogeneous size classes, representing successive

Fig. 1. Changes in enzyme activities in cell-free extracts prepared at successive stages of the cell cycle of *S. halophilus*. The enzymes assayed were (a) NADH dehydrogenase, (b) succinate dehydrogenase, (c) cytochrome *c* oxidase, (d) catalase and (e) acid phosphatase. The central bold line of each family of three smooth curves through the sets of experimental data represents an exponential rate of increase in activity that doubles during the cell cycle (i.e. between mean cell volumes of 9.5 and $19 \mu\text{m}^3$). This line is the null hypothesis. The curves above and below this line indicate the extent of two standard deviations from this theoretical curve. The chance of any point lying outside this range is 1 in 20, if the null hypothesis is valid. Enzyme activities are expressed as either $\text{amol substrate consumed min}^{-1}$ per cell or as $\text{amol product produced min}^{-1}$ per cell.

stages in the cell cycle (Salmon & Poole, 1983). Extracts of these fractions were prepared and assayed for several enzyme activities. Three mitochondrial enzymes, NADH dehydrogenase, succinate dehydrogenase and cytochrome *c* oxidase showed an overall doubling in activity on a per cell basis (Fig. 1*a, b, c*). However, all these enzymes showed a discontinuous pattern of activity, which was significantly different (95% level) from an exponential rate of increase (the null hypothesis), as can be judged from the disparity of the experimental data from the theoretical curve. In each case, several experimental points lay outside two standard deviations from the hypothesis (Fig. 1*a, b, c*).

Two maxima in activity were observed for cytochrome *c* oxidase (Fig. 1*c*); the first occurred in fractions containing cells with a mean cell volume of about $12.5 \mu\text{m}^3$, and the second in cells of about $15.5 \mu\text{m}^3$. Note that the latter is indicated by *two* datum points and that a maximum at this cycle stage was consistently observed in seven experiments (see Fig. 4*c*). In contrast, both NADH dehydrogenase (Fig. 1*a*) and succinate dehydrogenase (Fig. 1*b*) displayed a single oscillation per cycle, though with the maxima in activities occurring in cells of different volumes. For NADH dehydrogenase, the maximum was in fractions containing cells with a mean volume of $13.5 \mu\text{m}^3$, and for succinate dehydrogenase in cells of about $11.5 \mu\text{m}^3$.

Catalase (a marker enzyme for peroxisomes) and acid phosphatase (a marker enzyme for lysosomes) (Lloyd & Cartledge, 1974) also showed an overall doubling in activity on a per cell basis with respect to cell volume (Fig. 1*d, e*). Catalase showed an oscillatory pattern of activity

Fig. 2. Representative difference spectra and their respective fourth-order finite difference analyses. Each of the spectra on the left of the Figure is the sum of six replicate reduced (NaBH_4) minus oxidized (H_2O_2) difference spectra, recorded at 77 K and a scan speed of 0.2 nm s^{-1} using a bandwidth of 1.0 nm. Cell concentrations in each case were about $10^{10} \text{ cells ml}^{-1}$ but the spectra shown are scaled to a per cell basis. The mean cell volume in each gradient fraction shown is in the centre of the Figure. The fourth-order finite difference analysis (right) of each spectrum was calculated using differencing intervals of 1.8, 1.4, 1.2 and 1.0 nm.

with a single peak occurring in cells with a mean cell volume of about $15.5 \mu\text{m}^3$ (Fig. 1*d*). Acid phosphatase, unlike the other enzymes, showed a continuous pattern of activity, which was consistent with an exponential rate of increase (Fig. 1*e*).

Cellular content of cytochromes during the cell cycle

The cytochrome content in cells of different size classes, obtained from zonal isopycnic separations, was determined in low-temperature difference (NaBH_4 -reduced minus H_2O_2 -oxidized) spectra. The contributions of the individual components to the composite α -band of *b*- and *c*-type cytochromes were estimated from the bands revealed by fourth-order finite difference analysis of these spectra. This is illustrated in Fig. 2, which shows, for four representative cell cycle fractions, the spectra and their numerical analyses. The positions of the absorption maxima at about 544, 547, 551, 555, 557, 560 and 564 nm in the numerical analyses (Fig. 2) correspond closely to those observed previously for mitochondrial particles prepared from this organism (Salmon & Poole, 1980*a*). On the basis of differential reduction and by comparison with the results of other workers, Salmon & Poole (1980*a*) assigned the maxima at 548 and 554 nm to the α_1 - and α_2 -bands, respectively, of cytochrome *c*, that at 551 nm to cytochrome *c*₁ and those at 554, 557, 560 and 563 nm to *b*-type cytochromes. The unfavourable signal-to-noise ratios in the α -band region of cytochromes *aa*₃ precluded the numerical analysis of this absorption.

The variations across the gradient in the intensities of bands absorbing at about 547, 551, 557, 560 and 563 to 564 nm in the fourth-order finite difference spectra, and 600 nm in the difference

Fig. 3. Cellular content of cytochromes during the cell cycle of *S. halophilus*. Low temperature (77 K) reduced minus oxidized difference spectra were obtained of different size classes of cells, after isopycnic fractionation of an exponentially growing culture. Spectral conditions and the computation of fourth-order finite difference analyses were as described in Fig. 2. (a) Cytochrome *c*, (b) cytochrome *c*₁, and (c, d, e) three *b*-type cytochromes were estimated from the intensities of maxima in such analyses at 547, 551, 557, 560 and 563 nm, respectively. The amount of cytochrome *aa*₃ (f) was determined from the height of the 600 nm absorbance in unanalysed difference spectra. The families of three curves for each cytochrome amounts are expressed in arbitrary units per cell.

spectra, are shown in Fig. 3. The *b*-type cytochromes, represented by the absorptions at about 557, 560 and 564 nm (Fig. 3c, d, e respectively) all showed continuous patterns of accumulation, which were compatible with the null hypothesis (of exponential rate of increase during the cell cycle). However, the amounts of *c*-type cytochromes, measured from the absorptions at 547 nm (the α_1 -band of cytochrome *c*) and at 551 nm (the α -band of cytochrome *c*₁), and of the *a*-type cytochromes measured from the intensity of the absorption at 600 nm (composite α -band of cytochromes *a* and *a*₃) did not give satisfactory fits to the null hypothesis, showing oscillatory patterns. Cytochrome *c* (Fig. 3a) showed two maxima in amount during the cell cycle; the first occurred in cells with a mean volume of about 12.5 μm^3 , and the second in cells of about 16.5 μm^3 . Cytochrome *aa*₃ also showed two maxima in amount per cycle, which occurred in cells of similar sizes (12.5 μm^3 and 15.5 μm^3 , Fig. 3f). Cytochrome *c*₁ showed a single broad peak with its maximum in cells with a mean volume of about 13.5 μm^3 (Fig. 3b). All these cytochromes showed an overall doubling in amount on a per cell basis during the cell cycle (Fig. 3).

The intensity of the absorption at 544 nm varied exactly in phase with that at 547 nm (results not shown) and displayed a constant stoichiometry of 0.62 ± 0.05 (s.d. in 27 independent

Fig. 4. Cell cycle maps indicating the timings of maxima of the activities of (a) succinate dehydrogenase, (b) NADH dehydrogenase, (c) cytochrome oxidase and (d) catalase, and of amounts of cytochrome (per cell) of (e) cytochrome *c*, (f) cytochrome *c*₁ and (g) cytochrome *aa*₃, during the cell cycle of *S. halophilus*. It was assumed that cell age was directly proportional to mean cell volume over the range 9.5 to 19 μm^3 . The arrows with bar lines indicate the mean and standard deviation of the timing of each maximum.

measurements in cells of different sizes) to the absorption at 547 nm. This is further evidence for these two peaks belonging to the spectrum of a single component. Due to the low intensity of the peak at 554 nm and the consequent difficulty in resolving it (as can be judged from the high coefficient of variation of 0.70 obtained), no attempt was made to measure this.

The positions of the maxima in enzyme activities and in cytochrome amounts found in several measurements are shown in Fig. 4. In an analysis of variance of this data, the timings of the maxima in NADH dehydrogenase, succinate dehydrogenase and catalase activities were significantly different (95% level) from one another. The timings of the first maximum in cytochrome *c* oxidase activity were coincident with the maximum in succinate dehydrogenase, and the second with the maximum in catalase activity.

DISCUSSION

A major problem in interpretation of the changes in levels of cellular constituents that occur during the cell cycle is that all methods of cell cycle analysis give, at best, samples that are only *enriched* in cells of a particular age, size or density and which do not constitute truly homogeneous populations. In the present paper and in Salmon & Poole (1983), the culture fractionation procedure was of high resolution, as judged by the coefficients of variation of sizes in the separated fractions, but each fraction will contain very few, if any, cells whose size is unique to that fraction, and cells of the same size will be found in adjacent fractions. Thus, the difference in enzyme or cytochrome level can only be due to a small fraction of the total cells whose mean size is different. This means that variations in levels in an individual cell through the cycle will be greater than those seen in Figs 1 and 3. In culture fractionations of the kind reported here, the failure of the dispersion of cell ages to obscure oscillatory patterns cannot be attributed to a dispersion or desynchronizing event late in the cycle as suggested by Lloyd *et al.* (1982*b*) to explain high amplitude oscillations in imperfectly synchronized cultures of *Acanthamoeba castellanii*. It is conceivable, however, that (1) synergistic phenomena ('self-

synchronization') operate in the concentrated cell cycle fractions to enhance the oscillations, as occurs in high frequency glycolytic oscillations (Aldridge & Pye, 1976), and (2) that Coulter measurements of volume distributions in the absence of hydrodynamic focusing devices overestimate the skewness of the size distributions (e.g. Roberts, 1980).

Of the five enzyme activities measured in this study, only one, that of acid phosphatase, increased continuously, in parallel with total protein (Salmon & Poole, 1983), during the cell cycle of *S. halophilus*. Although various post-translational controls, such as conformational changes, covalent modifications and interaction with endogenous inhibitors may be invoked to explain cell cycle-dependent changes in activity (for a survey see Lloyd *et al.*, 1982a), spectroscopic measurements of cytochrome levels in intact cells have revealed that periodic synthesis and/or degradation of the holoenzymes is involved. No conclusions may be drawn, however, regarding the cytochrome apoproteins since, in a variety of experimental systems, removal of the metal centre from redox-active metal-containing proteins results in loss of spectral features. For example, copper-limited growth of *Neurospora crassa* (Werner *et al.*, 1974) or *Candida utilis* (Keyhani & Chance, 1971) causes disappearance of spectrally detectable cytochrome *aa*₃. In both organisms, however, cytochrome apoproteins are detectable immunologically and, in *C. utilis*, porphyrin *a* is also present in the apo-oxidase (Keyhani & Keyhani, 1980). Such apocytochromes may be membrane-bound (Hennig & Neupert, 1981). These studies identify diverse levels of control in the biosynthetic pathway of functional cytochromes, in addition to post-transcriptional and post-translational controls over protein synthesis.

Periodic modification and/or degradation of continuously synthesized proteins (Halvorson, 1977) is frequently discounted as an explanation of cell cycle-dependent changes in enzyme levels because estimates of protein turnover rates in exponentially growing cultures are low. However, these measurements have been criticized by Bakalkin *et al.* (1978) for not allowing adequately for post-incorporation and recycling of isotope label: the half-life of the products of mitochondrial translation in yeast was estimated as less than one hour by these authors. Even if the overall protein turnover rate is low, a small number of proteins may still be rapidly degraded. Thurston & Richards (1980) have demonstrated that, in growing cells of *Chlorella fusca*, about 5% of the total protein has a much greater rate of turnover (about 20% h⁻¹) than the overall rate of degradation, which is not more than 2.1% h⁻¹ (Richards & Thurston, 1980).

Gear *et al.* (1974) and Rajwade *et al.* (1975) have suggested that proteases control both the quantity and composition of mitochondria by regulating the rates of turnover of mitochondrial proteins. Several proteolytic systems (e.g. Hare, 1978; Duque-Magalhães & Ferreira, 1980) have been identified in mitochondria. A protease with rapid turnover, responsible for the degradation in cytochrome *c* oxidase of the higher molecular weight subunits that contain the haems and copper atoms (Winter *et al.*, 1980), has been identified in *N. crassa* (Kumar & Padmanaban, 1981). Luzikov *et al.* (1976) and Galkin *et al.* (1979) have demonstrated that the addition of specific inhibitors of protease to the culture medium of *Saccharomyces cerevisiae* prevents the changes in cytochrome content normally observed during growth. The results of the present study suggest that these proteolytic activities could be temporally, as well as spatially, organized.

For the complex composite spectra of mitochondrial cytochromes, the resolving power of fourth-order finite difference analysis was required (for a recent application, see Joergensen, 1981). Such spectra allowed the accurate measurements of the relative band intensities. The only assumptions that were made in this analysis were that the bandwidths and band shapes remained constant throughout the cell cycle (for a discussion of the influence of these parameters on derivative spectra, see Morrey, 1968).

The amounts of *b*-type cytochromes all increased continuously but both the *c*- and *a*-type cytochromes displayed oscillations in amount during the cell cycle (Fig. 3). Cytochrome *c* and cytochrome *aa*₃ oscillated in phase, with two maxima in amounts per cell cycle. The positions of these maxima in the cell cycle coincided with those of cytochrome *c* oxidase activity. Since cytochrome *aa*₃ is the structural counterpart of the measured cytochrome *c* oxidase activity, then at least for this enzyme the variation in activity actually reflected a variation in enzyme amount. In contrast, Cottrell *et al.* (1975) observed a stepwise increase in oxidase activity but a

continuous increase in spectroscopically detectable oxidase in synchronous cultures of *Sacch. cerevisiae* prepared by an induction method. All the cytochromes that showed an oscillatory pattern of synthesis in the present study are of positive redox potential, and the last two components of the respiratory chain (cytochrome *c* and cytochrome *aa*₃) oscillated in phase, perhaps indicating a common regulatory mechanism. However, cytochromes *c* and *c*₁ are both coded for entirely by nuclear genes and cytochrome *aa*₃ in part by mitochondrial genes (for references, see Winter *et al.*, 1980). Further work is required to study the turnover of individual subunits and their association with iron and copper, and to explain the coordinated degradation of cytochromes that are buried in the hydrophobic membrane core (e.g. cytochrome *c* oxidase) or are peripherally associated with the matrix side of the mitochondrial inner membrane.

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