Regulation of Mitochondrial Biogenesis

OCCURRENCE OF NON-FUNCTIONING COMPONENTS OF THE MITOCHONDRIAL RESPIRATORY CHAIN IN *SACCHAROMYCES CEREVISIAE* GROWN IN THE PRESENCE OF PROTEINASE INHIBITORS: EVIDENCE FOR PROTEOLYTIC CONTROL OVER ASSEMBLY OF THE RESPIRATORY CHAIN

Alexander V. GALKIN, Tamara V. TSOI and Valentin N. LUZIKOV

Department of Subcellular Biogenesis, Belozersky Laboratory of Molecular Biology and Bio-organic Chemistry, Lomonosov State University, Moscow 117234, U.S.S.R.

(Received 4 September 1979)

Yeast was grown in glucose- or galactose-containing media without or with proteinase inhibitors, phenylmethanesulphonyl fluoride and pepstatin. Culture growth was practically not affected by these compounds. Yeast growth on glucose in the presence of either phenylmethanesulphonyl fluoride or pepstatin entails accumulation of cytochromes *c*, *c*₁, *b* and *aa*₃ to a 25–30% excess above the control by the stationary phase, while cell respiration is unaffected. During growth on galactose the maximal cytochrome content (per unit weight of biomass) is reached in the mid-exponential phase and then decreases by 30–40% towards the stationary phase, while cell respiration remains constant. Addition of phenylmethanesulphonyl fluoride or pepstatin in the mid-exponential phase blocks the decrease in cytochrome levels and has no effect on cell respiration. Mitochondrial populations isolated from stationary-phase control and phenylmethanesulphonyl fluoride-grown cells of glucose cultures display identical succinate oxidase and partial-respiratory-chain activities, despite the differences in cytochrome contents. However, the activities of individual respiratory complexes measured after maximal activation are nearly proportional to the amounts of corresponding components. The same situation holds true for mitochondrial populations from mid-exponential-phase, stationary-phase control and stationary-phase inhibitor-grown cells of galactose cultures. The findings suggest that the 'surplus' respiratory-chain components do not participate in electron flow because of the lack of interaction with adjacent carriers.

Ever-increasing attention of researchers has in recent years been focused on the possible role of proteolytic enzymes in the biogenesis of mitochondria. Proteinases are believed to control the quantity of mitochondrial material in the cell (Gear *et al.*, 1974) and the rates and synchrony of the turnover of mitochondrial proteins (Rajwade *et al.*, 1975). According to Wheeldon *et al.* (1974), a considerable portion of nascent polypeptides in isolated rat liver mitochondria is quickly broken down by an endogenous proteinase. Rapid degradation of mitochondrial translation products *in vitro* (Kalnov *et al.*, 1978) and *in vivo* (Bakalkin *et al.*, 1978) has been demonstrated in this laboratory with *Saccharomyces cerevisiae*. It should be borne in mind that the mentioned proteins are thought to play an organizing role in the assembly of

the catalytic complexes of the mitochondrial inner membrane (Tzagoloff, 1971; Ebner *et al.*, 1973; Werner *et al.*, 1974). Hence proteinases may regulate mitochondrial assembly and differentiation.

To assess the regulatory role of proteinases in mitochondriogenesis, it seems promising to study how the yeast respiratory system develops when intracellular proteolytic enzymes are somehow suppressed. One of the possible means for this is the use of proteinase inhibitors. It has been shown that phenylmethanesulphonyl fluoride and pepstatin inhibit the degradation of the total yeast-cell protein (Bakalkin *et al.*, 1976) and inactivate yeast proteinases B and A *in vivo* (Kalnov *et al.*, 1979a); phenylmethanesulphonyl fluoride has also been demonstrated to be effective in blocking the proteolysis of mitochondrial translation products *in*

vitro (Kalnov *et al.*, 1978, 1979b) and *in vivo* (Kalnov *et al.*, 1979a; Luzikov, 1979) and in suppressing the breakdown of the generally labelled yeast mitochondrial protein (Ryrie & Gallagher, 1979).

Along this line, the present paper reports the results of work on the effects of phenylmethanesulphonyl fluoride and pepstatin on the contents of mitochondrial respiratory-chain components and various respiratory-chain activities during yeast culture growth in glucose and galactose media.

Materials and Methods

Chemicals

Sorbitol, mannitol, Trizma base, EDTA, bovine serum albumin, cytochrome *c*, sodium ascorbate, sodium succinate, malonic acid, Lubrol WX, antimycin A and phenylmethanesulphonyl fluoride were from Sigma Chemical Co., St. Louis, MO, U.S.A.; yeast extract was from Serva, Heidelberg, German Federal Republic; *NNN'*-tetramethyl-*p*-phenylene diamine and maleic acid were from E. Merck, Darmstadt, German Federal Republic; NADH and carbonyl cyanide *m*-chlorophenylhydrazone were from Calbiochem, Lucerne, Switzerland; ubiquinone-2 was from Ferak, Berlin, German Democratic Republic. Pepstatin was a generous gift from Professor H. Umezawa (Microbial Chemistry Research Foundation, Tokyo, Japan).

Growth of *Saccharomyces cerevisiae* and introduction of proteinase inhibitors

Saccharomyces cerevisiae wild-type diploid was grown batchwise in aerobic conditions as described previously (Luzikov *et al.*, 1971, 1976). The semi-synthetic growth medium contained 2.5 g of glucose or 20 g of galactose, 1 g of KH_2PO_4 , 1 g of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2 g of yeast extract per litre.

Phenylmethanesulphonyl fluoride was introduced into the cell suspension in portions of 50 $\mu\text{mol/litre}$ at intervals of 90–120 min, beginning from 6–7 h of growth with the glucose-grown culture (cumulative 0.25 mM by the end of the experiment); pepstatin was added twice (at 6 and 9 h of growth) in amounts of 0.5 mg/litre, yielding a final concentration of about 1.5 μM . With galactose-grown cultures, the inhibitors were administered in a similar manner beginning from the mid-exponential phase (8.5 h of growth) to cumulative concentrations of 0.2 mM-phenylmethanesulphonyl fluoride and 1.5 μM -pepstatin.

Low-temperature spectrophotometry

The difference (reduced minus oxidized) spectra of intact cells were recorded at 77 K (Estabrook & Mackler, 1957) in a DW-2a UV/VIS Aminco

spectrophotometer equipped with a low-temperature attachment. Yeast cells harvested after various periods of growth were washed three times with ice-cold water and suspended in 0.55 M-mannitol/50 mM-potassium phosphate buffer, pH 7.4. Oxidation and reduction were performed by adding H_2O_2 and $\text{Na}_2\text{S}_2\text{O}_4$ respectively (Poole *et al.*, 1974). Anaerobic reduction of cytochromes was achieved by incubating cell suspensions for at least 5 min with 20 mM-ethanol or 0.25% glucose (O_2 was exhausted from the medium within 2 min of incubation). Suspensions were frozen in 2 mm-thick cuvettes and scanned from 400 to 650 nm at a 1 nm bandwidth.

Isolation of membrane fractions

Yeast cells were homogenized by the procedure of Tzagoloff (1969) in a medium containing 0.6 M-sorbitol, 1 mM-EDTA, 0.25% bovine serum albumin, 10 mM-potassium phosphate and 20 mM-Tris adjusted to pH 6.7 (at 4°C) with maleic acid. Debris was sedimented at 800 g and washed by the same medium. Supernatants were combined and centrifuged at 70 000 g for 30 min. Pellets were suspended in the isolation medium and either used immediately or stored in small portions at -20°C or in liquid N_2 .

Under carefully controlled conditions the percentage of disrupted cells was similar for all samples and varied within not more than 5% of the mean between independent experiments.

Recovery of mitochondria was estimated by determining the quantity of cytochromes and inner-membrane marker activities in cell homogenates, 70 000 g pellets and supernatants. The membrane fractions quantitatively contained cytochromes *b* and *aa*₃ of the homogenates. Only minor amounts of cytochrome *c* and no NADH oxidase, succinate oxidase or cytochrome oxidase activities were detectable in 70 000 g supernatants. Furthermore cytochrome oxidase activity in the washed 800 g pellets (which can be taken as an index of the portion of mitochondria co-sedimented with the cell debris and nuclei) was somewhat variable but never amounted to more than 4% of the corresponding activity in the membrane fractions or whole homogenates. Therefore no corrections were made for this portion in further calculations. Thus the 70 000 g pellets were assumed to contain all the mitochondrial structures of yeast cells.

Measurements of cytochrome contents and enzyme activities

Cytochrome contents were calculated from the difference (reduced minus oxidized) spectra of membrane fractions recorded at room temperature in the presence of 1 μM -carbonyl cyanide *m*-chlorophenylhydrazone in a DW-2a UV/VIS Aminco spectrophotometer. Wavelength pairs and absorption coefficients ($\text{mM}^{-1} \cdot \text{cm}^{-1}$) were: cyto-

Fig. 1. Effect of proteinase inhibitors on aerobic growth of *S. cerevisiae* in glucose-containing medium

○, Control suspension (no additions); ●, in the presence of phenylmethanesulphonyl fluoride; △, in the presence of pepstatin. For details see the text. (a) Growth in culture: curve 1, concentration of cell suspension; curve 2, respiration on 0.25% glucose; curve 3, maximally stimulated respiration on 1% (v/v) ethanol with 50 μ M-carbonyl cyanide *m*-chlorophenylhydrazine. (b)–(e) Cytochrome content of the intact cells. Data are presented in the form of absorbance of each cytochrome in the difference (reduced by dithionite minus oxidized) spectra recorded as described in the Materials and Methods section with cell suspensions containing 15 mg dry wt./ml. No attempt has been made to express the cytochrome contents in molarities because no reliable absorption coefficients are available for these experimental conditions. (b) Cytochrome *c*

chromes *c* + *c*₁ (551–540 nm), 20; cytochrome *b* (562–575 nm), 19.1; cytochrome *aa*₃ (605–630 nm), 24 (Lee *et al.*, 1965). Enzyme activities were assayed in Aminco or Hitachi spectrophotometers in 50 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA. All water-insoluble reagents were dissolved in methanol. Cytochrome *c* was reduced by titration with ascorbate, and the absorption coefficient was taken to be 18.5 mM⁻¹·cm⁻¹ at 550 nm. Ubiquinone-2 was reduced with NaBH₄ as described by Lester *et al.* (1959), and the absorption coefficient was 12.5 mM⁻¹·cm⁻¹ at 275 nm. The O₂ concentration during oxidase measurements was practically constant in the range 0.24–0.25 mM and exceeded by far the *K*_m for cytochrome oxidase (less than 0.05 μ M). Succinate was used at 20 mM, which also was practically saturating. To activate succinate dehydrogenase before relevant assays, membrane preparations were preincubated with 20 mM-succinate. Other conditions are indicated in the Figure legends.

Cell respiration was measured with a Clark oxygen electrode and a Radiometer PO-4 polarograph at 30°C in 50 mM-potassium phosphate buffer, pH 7.4.

Determination of protein

Protein was determined with Folin–Ciocalteu phenol reagent (Sigma Chemical Co.) as proposed by Albro (1975), with bovine serum albumin as standard.

Presentation of data

The amounts of cytochromes and protein and activities of the membrane fractions were determined with respect to unit weight of cells from which the fractions were isolated (see under 'Isolation of membrane fractions'). Thus the 'activity of a membrane fraction' is defined as the total activity displayed by mitochondrial structures initially contained in the amount of cells equivalent to 1 g dry wt. The same applies to the cytochrome contents and protein.

Results

Whole-cell studies on the development of the respiratory system and the effects of proteinase inhibitors

During culture growth in glucose-containing medium the cell respiration increases until the late exponential phase (Fig. 1a). Throughout the examined period of culture growth respiration is completely inhibited by cyanide (1 mM) or antimycin (0.2 μ M); significant and constant stimulation

(548–533 nm); (c) cytochrome *c*₁ (553–533 nm); (d) cytochrome *b* (559–572 nm); (e) cytochrome *aa*₃ (603–630 nm).

by an uncoupler is also observed at all times of growth (cf. curves 2 and 3 in Fig. 1a). The cell contents of cytochromes *c*, *c*₁, *b* and *aa*₃ increase roughly in parallel with the respiratory activity (Figs. 1b–1e).

The data in Fig. 1 also demonstrate culture growth and the development of the respiratory system in the presence of phenylmethanesulphonyl fluoride or pepstatin. Here it must be mentioned that the assays of proteolytic activities in cell homogenates by the method of Lenney (1975) reveal a pronounced (more than 90%) inhibition of proteinases B and A on the addition of 50 μ mol of phenylmethanesulphonyl fluoride and 1 mg of pepstatin respectively per litre of suspension either in the early exponential phase or in the late exponential phase of culture growth (Kalnov *et al.*, 1979a). Under conditions described in the Materials and Methods section phenylmethanesulphonyl fluoride only transiently inhibits the growth rate by less than 15%, and the culture completely recovers within 2 h after the first addition (Fig. 1a, curve 1). Pepstatin produces a similar growth pattern. By the early stationary phase identical cell numbers (Fig. 1a) and biomass yields (0.88 g dry wt. of cells/litre of suspension) were obtained with control, phenylmethanesulphonyl fluoride-grown and pepstatin-grown cultures.

The cell respiration, its sensitivity to cyanide or antimycin and stimulation by the uncoupler are not affected by proteolytic inhibitors (Fig. 1a, curves 2 and 3). At the same time it is apparent that cytochromes *c*, *c*₁, *b* and *aa*₃ gradually accumulate above the corresponding control values, producing a 25–30% elevation by the early stationary phase in the presence of phenylmethanesulphonyl fluoride and only slightly less with pepstatin (Figs. 1b–1e).

When yeast cells are grown in a galactose containing medium, the constant cell respiration rate is already reached by the mid-exponential phase. Complete inhibition by 1 mM-cyanide or 0.2 μ M-antimycin and an equal extent of stimulation by carbonyl cyanide *m*-chlorophenylhydrazine was observed for all samples (Fig. 2a, curves 2 and 3). The cell contents of cytochromes *c*, *c*₁, *b* and *aa*₃ increase up to the mid-exponential phase, but then, most peculiarly, gradually decrease 1.7–2-fold by the early stationary phase (Figs. 2b–2e).

If phenylmethanesulphonyl fluoride or pepstatin is introduced into the cell suspension at the time of growth corresponding to the peak cytochrome content (8.5 h; Fig. 2), the pattern of cytochrome behaviour is dramatically altered. The fall in the amount of cytochrome *c* is significantly attenuated (Fig. 2b), and the decrease in cytochromes *c*₁, *b* and *aa*₃ is completely blocked (Figs. 2c–2e). The effect of pepstatin is only slightly less pronounced than that of phenylmethanesulphonyl fluoride. At the

Fig. 2. Effect of proteinase inhibitors on yeast aerobic growth in galactose-containing medium

○, Control suspension; ●, in the presence of phenylmethanesulphonyl fluoride; △, in the presence of pepstatin. All conditions were as described for Fig. 1 except that respiration in (a), curve 2, was measured on 2% (w/v) galactose and spectra for (b)–(e) were recorded with 22 mg dry wt./ml cell suspensions.

same time no changes are observed in cell respiration either in the absence or in the presence of the uncoupler (Fig. 2a, curves 2 and 3) and in its sensitivity to 1 mM-cyanide or 0.2 μ M-antimycin. Similarly to glucose-grown cultures, addition of proteolytic inhibitors only transiently affects

culture growth rate; cell numbers (Fig. 2*a*) and biomass yields by the early stationary phase (1.35 g dry wt./litre of suspension) were identical for control and inhibitor-grown batches. Again, proteinases A and B are markedly inhibited by pepstatin and phenylmethanesulphonyl fluoride, as judged by the proteolytic assays in cell homogenates (A. V. Galkin, unpublished work).

Combined addition of both phenylmethanesulphonyl fluoride and pepstatin produces results (not shown) indistinguishable with respect to culture growth, respiration or cytochrome contents from those obtained with phenylmethanesulphonyl fluoride alone.

Experiments on anaerobic reduction of cytochromes in glucose- and galactose-grown cells demonstrated that, irrespective of the growth phase and the presence of proteinase inhibitors, cytochromes can be reduced by incubation of the anaerobic suspensions with physiological substrates (glucose or galactose, and ethanol). In all cases cytochromes *c*, *c*₁ and *aa*₃ are reduced quantitatively, and cytochrome *b* is reduced by about 75% of the extent obtained with dithionite. Addition of ethanol and cyanide in aerobic conditions produces practically the same reduction; antimycin blocks the reduction of cytochromes *c*, *c*₁ and *aa*₃. No peak shifts or appearance of new absorption bands could be observed in the spectral region examined (400–650 nm).

Cytochrome contents and enzyme activities of mitochondrial populations isolated from glucose-grown cells

Table 1 summarizes the characteristics of membrane fractions obtained from early-stationary-phase cells of the control and phenylmethanesulphonyl fluoride-grown cultures. These fractions contain the entire mitochondrial populations of the corresponding cells, as judged by the determination of cytochromes and mitochondrial inner-membrane marker activities in cell homogenates, 70000g pellets and supernatants. As can be seen, preparations from inhibitor-grown cells contain 25–30% more cytochromes *c* + *c*₁, *b* and *aa*₃ than the control ones. In all cases cytochromes *c* + *c*₁ and *aa*₃ can be quantitatively reduced in anaerobic conditions by succinate, NADH, or ascorbate plus tetramethyl-*p*-phenylenediamine; succinate-reducible cytochrome *b* always constitutes about 60% of the dithionite-reducible one (Table 1). Succinate-reducible flavoproteins (not shown) were found to be 20–30% increased in mitochondrial preparations from phenylmethanesulphonyl fluoride-grown cells.

It is also apparent from Table 1 that, despite the differences in cytochrome contents, both preparations display the same succinate oxidase and

Table 1. *Characteristics of membrane fractions isolated from glucose-grown cells*

Source of preparation	Oxidase activities (μg-atoms of O/min per g dry wt. of cells)					
	Succinate oxidase			Ascorbate oxidase		
	+5 μM-cytochrome <i>c</i>			+20 μM-cytochrome <i>c</i>		
Stationary-phase control cells	Cytochrome <i>c</i> + <i>c</i> ₁	Succinate	Dithionite	Cytochrome <i>aa</i> ₃		
Stationary-phase phenylmethanesulphonyl fluoride-grown cells	41.7	34.4	57.8	14.4	46.0	380
	52.0	42.8	72.0	18.5	46.5	388

Membrane fractions containing the entire mitochondrial populations were isolated as described in the Materials and Methods section from cells grown in a glucose medium to the early stationary phase in control conditions and in the presence of phenylmethanesulphonyl fluoride. Succinate oxidase and ascorbate oxidase activities were assayed polarographically at 30°C with 20 mM-succinate and 20 mM-ascorbate plus 1 mM-tetramethyl-*p*-phenylenediamine. For other details see the Materials and Methods section.

ascorbate oxidase activities, even at saturating substrate concentrations either in the absence or in the presence of exogenous cytochrome *c*. Further, ubiquinol oxidase activity is identical in the membrane fractions from control and phenylmethane-sulphonyl fluoride-grown cells at all substrate concentrations (Fig. 3), and addition of exogenous cytochrome *c* enhances the activities but to the same

extent. Succinate–cytochrome *c* reductase segments (Fig. 4) in the compared preparations also display similar rates of cytochrome *c* reduction, irrespective of its concentration.

In all preparations succinate oxidase, ubiquinol oxidase and succinate–cytochrome *c* reductase were not less than 98% inhibited by antimycin (0.2 μ M). Cyanide (0.5 mM) suppressed ascorbate oxidase by 95% or more, and succinate oxidase and ubiquinol oxidase were inhibited completely.

The data in Table 2 allow one to compare the activities of the three constituent individual com-

Fig. 3. Ubiquinol oxidase activities of membrane fractions isolated from glucose-grown cells

Yeast cells were grown till the early stationary phase in the absence (○) or in the presence of phenylmethanesulphonyl fluoride (●). The assay medium contained 1 mM-malonate; the temperature was 25°C. The reaction was started by the addition of ubiquinol-2 in a minimal volume of methanol. The initial rate is expressed as μ mol of ubiquinol oxidized/min per g dry wt. of cells (see the Materials and Methods section).

Fig. 4. Succinate–cytochrome *c* reductase activities of membrane fractions from glucose-grown cells

○, Control; ●, grown with phenylmethanesulphonyl fluoride. Activities were assayed at 30°C; the reaction was started by the addition of cytochrome *c*. The initial rate is expressed as μ mol of cytochrome *c* reduced/min per g dry wt. of cells in the presence of 20 mM-succinate and 1 mM-cyanide.

Table 2. Activation of the constituent complexes of mitochondrial respiratory chain

Membrane fractions were obtained from stationary-phase cells grown in a glucose medium in control conditions and in the presence of phenylmethanesulphonyl fluoride. Activation was achieved by freezing–thawing of the membrane preparations in the isolation medium in the presence of 0.05% Lubrol WX; the detergent was also present in the assay medium for activated samples (concentrations in parentheses). The activities presented below pertain to the following conditions: succinate–ubiquinone reductase, 20 mM-succinate and 5 μ M-ubiquinone-2 (0.01% Lubrol); ubiquinol–cytochrome *c* reductase, 15 μ M-ubiquinol-2 and 11 μ M-cytochrome *c* (0.03% Lubrol); cytochrome oxidase, 5 μ M-ferrocycytochrome *c* and approx. 0.24 mM dissolved O₂ (0.06% Lubrol). Activities are given per g dry wt. of cells.

Preparation	Succinate–ubiquinone reductase (μ mol of ubiquinone reduced/min per g)		Ubiquinol–cytochrome <i>c</i> reductase (μ mol of cytochrome <i>c</i> reduced/min per g)		Cytochrome oxidase (μ mol of cytochrome <i>c</i> oxidized/min per g)	
	Basal	Activated	Basal	Activated	Basal	Activated
Control	40.4	46.5	41	77	18.4	42.0
Phenylmethanesulphonyl fluoride-grown	43.1	55.5	40.3	98	18.6	51.3

plexes of the succinate oxidase chain (succinate-ubiquinone reductase, ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase) in the control and inhibitor-grown mitochondrial populations. When these are assayed in the intact membrane ('basal' activities), little or no difference is observed. However, if the membrane is disrupted by freezing-thawing in the presence of a non-ionic detergent Lubrol WX, the relation of the respective activities between the two preparations is markedly altered. Figs. 5-7 demonstrate the potential catalytic capacity of the individual electron-transfer complexes in the solubilized mitochondrial preparations from control and phenylmethanesulphonyl fluoride-grown cells.

The double-reciprocal plots for succinate-ubiquinone reductase (Fig. 5) show that the activity of the inhibitor-grown population exceeds the control at all ubiquinone-2 concentrations, displaying an identical apparent K_m . The two preparations appear to be similarly sensitive to malonate (results not shown).

Cytochrome oxidase activity is also higher in the phenylmethanesulphonyl fluoride-grown preparation (Fig. 6), in accordance with a higher cytochrome *aa₃* content (Table 1). Again, the slope of the Eadie-Hofstee plot (the negative of the

Fig. 5. Succinate-ubiquinone reductase activities of mitochondrial preparations solubilized by freezing-thawing in the presence of Lubrol WX

Membrane fractions were isolated from stationary-phase cells grown in the glucose medium in the absence (control, ○) and in the presence (●) of phenylmethanesulphonyl fluoride. Activities were assayed at 25°C in a medium containing 0.01% Lubrol WX, 20mM-succinate, 0.4 μM-antimycin and 1 mM-KCN; the reaction was started by the addition of ubiquinone-2. *v* is expressed as μmol of ubiquinone reduced/min per g dry wt. of cells. Straight lines are least-square fittings.

apparent K_m) is similar to the control one over a substantial range of cytochrome *c* concentrations. However, a moderate but distinct deviation from linearity is observed at higher substrate concen-

Fig. 6. Cytochrome oxidase activities of glucose-grown solubilized membrane preparations

Preparations were from stationary-phase control cells (○) and stationary-phase phenylmethanesulphonyl fluoride-grown cells (●). Membranes were solubilized by freezing-thawing in the presence of 0.05% Lubrol WX. The assay medium contained 0.06% Lubrol, 0.4 μM-antimycin and 0.24 mM dissolved O_2 ; the temperature was 30°C. *v* is expressed as μmol of ferrocytochrome *c* oxidized/min per g dry wt. of cells, [S] as μM-ferrocytochrome *c*.

Fig. 7. Ubiquinol-cytochrome *c* reductase activities of solubilized glucose-grown preparations

Membrane fractions from control (○ and △) and phenylmethanesulphonyl fluoride-grown (● and ▲) stationary-phase cells were solubilized by freezing-thawing in the presence of 0.05% Lubrol WX. The reaction was carried out at 25°C in a medium containing 0.03% Lubrol, 1 mM-malonate and 1 mM-KCN, with 1.2 μM-cytochrome *c* (○ and ●) or 11.7 μM-cytochrome *c* (△ and ▲). The reaction was started by addition of ubiquinol. The initial rate is expressed as μmol of cytochrome *c* reduced/min per g dry wt. of cells.

trations and the relative elevation in activity reaches almost 30%.

With ubiquinol–cytochrome *c* reductase (Fig. 7) both substrates were variable. At low concentrations of either substrate (e.g. cytochrome *c*) the other one fails to produce any differences in activity between the two preparations (lowest curve). At higher cytochrome *c* concentrations the surplus in activity of the inhibitor-grown population becomes more and more pronounced with increasing ubiquinone concentration, approximating the excess in cytochrome contents (cf. Table 1).

Cytochrome contents and respiratory enzyme activities in mitochondrial populations from galactose-grown cells

Membrane preparations containing the entire mitochondrial populations (see preceding subsection) were isolated from mid-exponential-phase control cells possessing the highest cytochrome content, as judged by whole-cell determinations (Fig. 2), from stationary-phase control cells and from cells grown from mid-exponential to stationary phase in the presence of both phenylmethanesulphonyl fluoride and pepstatin. As shown in Table 3, membrane fractions of the stationary-phase control cells contain 30–40% less cytochromes than those of the exponential-phase or the stationary-phase inhibitor-grown cells. Cytochromes of all preparations can be reduced by anaerobic incubation with respiratory-chain substrates, as with the membrane fractions of glucose-grown cells. A similar

Fig. 8. Ubiquinol oxidase activities of mitochondrial populations from galactose-grown cells

Preparations were from exponential-phase cells (Δ), stationary-phase control cells (○) and stationary-phase inhibitor-grown cells (●). The reaction was carried out at 30°C in the presence of 1mM-malonate and 4μM-cytochrome *c*. Other details were as given in Fig. 3 legend.

Table 3. Characteristics of membrane fractions isolated from galactose-grown cells

Pooled mitochondrial populations were isolated as described in the Materials and Methods section from cells indicated in the Table. Succinate oxidase and ascorbate oxidase activities were measured polarographically at 30°C with 20 mM-succinate and 20 mM-ascorbate plus 1 mM-tetramethyl- <i>p</i> -phenylenediamine as respective substrates. Concentrations of exogenous cytochrome <i>c</i> given below produced maximal stimulation of the activities.	Source of preparation	Cytochrome content (nmol/g dry wt. of cells)					Protein content (mg/g dry wt. of cells)	Oxidase activities (μg-atoms of O/min per g dry wt. of cells)			
		Cytochromes <i>c</i> + <i>c</i> ₁	Cytochrome <i>b</i> , reduced by			Cytochrome <i>aa</i> ₃		Succinate oxidase			
			Succinate	Dithionite				+5 μM-cytochrome <i>c</i>	Ascorbate oxidase + 20 μM-cytochrome <i>c</i>		
		Mid-exponential-phase control cells	48.7	34.4	59.3	13.0		182	26.2	38.3	278
		Stationary-phase control cells	26.8	24.0	39.1	8.6		109	27.1	38.9	261
		Stationary-phase inhibitor-grown cells	43.7	33.1	57.3	12.4		164	26.8	37.8	263

relation was obtained for the contents of succinate-reducible flavoproteins.

Nevertheless all three preparations display similar succinate oxidase, ascorbate oxidase (Table 3) and ubiquinol oxidase (Fig. 8) activities, regardless of substrate concentrations and the presence of exogenous cytochrome *c*. As to the succinate-cytochrome *c* reductase (Fig. 9), the exponential preparation, most strikingly, shows activity only half that of the stationary-phase control, and this difference cannot be overcome by increasing cytochrome *c* concentration. The activity of the inhibitor-grown preparation only approximates the control one at high substrate concentrations. These rather surprising observations are not due to the presence of 'inside-out' membranes because succinate-ferri-cyanide reductase activity of all samples is completely inhibited by antimycin. When succinate-cytochrome *c* reductase activity is assayed in the presence of various concentrations of Lubrol WX, differential effects are seen, as exemplified in Fig. 9 inset. The activity of the stationary-phase control preparation is either unaffected by the detergent or even slightly inhibited, especially at high cytochrome *c* concentrations. At the same time, activation is pronounced in the exponential-phase preparation and also clearly detectable in the inhibitor-grown

one. The above findings suggest that in exponential-phase and inhibitor-grown mitochondrial populations, unlike the stationary-phase control one, a portion of the electron-donor sites for cytochrome *c*

Fig. 10. Succinate-ubiquinone reductase activities of solubilized galactose-grown preparations

Membranes were disrupted by freezing-thawing in the presence of Lubrol WX. Initial velocities of ubiquinone-2 reduction ($\mu\text{mol}/\text{min}$ per g dry wt. of cells) were determined at 30°C in the presence of 20 mM-succinate, 0.4 μM -antimycin, 1 mM-KCN and 0.01% Lubrol. Preparations were from: Δ , exponential-phase cells; O, stationary-phase control cells; $\bullet$, stationary-phase inhibitor-grown cells.

Fig. 9. Succinate-cytochrome *c* reductase activities of galactose-grown preparations

Membrane fractions were isolated from exponential-phase (Δ), stationary-phase control (O) and stationary-phase inhibitor-grown ($\bullet$) cells. The assay medium contained 20 mM-succinate and 1 mM-KCN; the temperature was 30°C . The initial rate is expressed as μmol of cytochrome *c* reduced/min per g dry wt. of cells. For the inset activities with 4.5 μM -cytochrome *c* were measured after pre-incubation of the membranes with the indicated concentrations of Lubrol WX.

Fig. 11. Ubiquinol-cytochrome *c* reductase activities of solubilized galactose-grown preparations

Preparations were from exponential-phase cells (Δ), stationary-phase control cells (O) and stationary-phase inhibitor-grown cells ($\bullet$). The assay medium contained 0.03% Lubrol WX, 1 mM-malonate, 1 mM-cyanide and 18 μM -cytochrome *c*. Activities are expressed as μmol of cytochrome *c* reduced/min per g dry wt. of cells.

Fig. 12. Eadie-Hofstee plots for cytochrome oxidase activities of solubilized galactose-grown preparations. v is expressed as μmol of ferrocytochrome c oxidized/min per g dry wt. of cells, $[S]$ as μM -ferrocytochrome c . Preparations were from exponential-phase cells (Δ), stationary-phase control cells ($\circ$) and stationary-phase inhibitor-grown cells ($\bullet$). Assay conditions were as given in Fig. 6 legend.

is inaccessible for the exogenous acceptor, but that labilization of the membrane by the detergent renders them reactive.

The potential catalytic capacity of the individual respiratory-chain complexes was estimated in preparations solubilized by freezing-thawing in the presence of Lubrol (see the preceding subsection). When activated maximally, succinate-ubiquinone reductase (Fig. 10), ubiquinol-cytochrome c reductase (measured at saturating cytochrome c concentration; Fig. 11) and cytochrome oxidase (Fig. 12) activities of the exponential-phase and inhibitor-grown preparations exceed those of the stationary-phase control, and the relation of activities is nearly proportional to the contents of corresponding components (Table 3). Similarly to the fractions from glucose-grown cells, all activities are suppressed by appropriate respiratory inhibitors.

Discussion

Suitability of the experimental model

The results of the present study indicate that addition of phenylmethanesulphonyl fluoride and pepstatin *in vivo* to either glucose- or galactose-grown cultures only slightly and transiently suppresses yeast growth rate and does not affect growth yields (see the first subsection of the Results section) or cell viability (never less than 97% as judged by staining with Methylene Blue). Ryrie & Gallagher (1979) have also shown that the influence of phenylmethanesulphonyl fluoride on yeast growth rate and yield is negligible.

As follows from a number of reports on the use *in*

vivo of proteinase synthetic inhibitors such as 7-amino-1-chloro-3-tosylamidoheptan-2-one ('tosyl-lysyl chloromethyl ketone') and 1-chloro-4-phenyl-3-tosylamidobutan-2-one ('tosylphenylalanyl chloromethyl ketone') (McIlhinney & Hogan, 1974; Pong *et al.*, 1975; Betz & Weiser, 1976; Ito, 1977), their main side effect is inhibition of protein synthesis. In the present experiments, however, an overall increase in the content of mitochondrial haemoproteins per unit biomass is observed in inhibitor-grown cells as compared with the control (Figs. 1 and 2 and Tables 1 and 3), whereas the biomass yield is unaffected. The results indicate that suppression of synthesis is negligible. This variance from other results (see references cited above) may, firstly, be accounted for by the lower toxicity of phenylmethanesulphonyl fluoride and pepstatin. Secondly, the procedure of inhibitor administration (see the Materials and Methods section) has been specially designed to minimize the possible side effects. Thirdly, the growth media were made ample in yeast extract to support normal protein synthesis from free precursors even under conditions hindering protein catabolism (cf. media compositions and total biomass yields).

Thus phenylmethanesulphonyl fluoride and pepstatin produce no obvious detrimental effects on cell physiology. On the other hand, they are effective *in vivo*, as follows from the work cited in the introduction and the retention of mitochondrial proteins reported in the present paper. Therefore the experimental scheme proposed seems to provide a satisfactory model of mitochondrial development under conditions of selectively blocked proteolysis.

Cytochrome contents versus enzyme activity

From the spectrophotometric data, obtained with whole cells and isolated mitochondrial populations, as well as from the observed increase in mitochondrial membrane protein (e.g. Table 3), it appears that in the presence of proteinase inhibitors the overall quantity of mitochondrial material per unit weight of cells is increased owing to the retention of components that should have normally been eliminated during culture growth. At the same time no surplus cell respiration or mitochondrial respiratory activities related to the same parameter are seen. The similarity of the results for intact cells and isolated fractions shows that the discrepancy between the contents of mitochondrial respiratory-chain components and respiratory activity is not due to differential injury to mitochondrial membranes during isolation.

As follows from the enzymic data, no surplus activity can be obtained with preparations containing 'excess' cytochromes, regardless of the length and position of the measured respiratory-chain segment and concentration of substrates, when

activities are assayed in intact membranes. Nevertheless, when the membrane is disrupted, the potential catalytic capacity of all constituent complexes of the electron-transfer chain is nearly proportional to the contents of corresponding components. This means that the 'excess' entities are essentially normal as such (at least with respect to their spectral and catalytic properties) but improperly aligned within the membrane, so that they cannot react efficiently with adjacent carriers nor with exogenous substitutes of the latter. Though such components can be reduced by respiratory-chain substrates, this only occurs on anaerobic incubation in the presence of excess substrate, and is likely to proceed too slowly to permit participation in mitochondrial oxidation.

Non-functioning respiratory-chain components: natural or artifacts?

According to the data discussed in the preceding subsection, the respiratory-chain components retained in the presence of proteinase inhibitors appear to form essentially normal complexes that, however, prove to be ineffective as members of the mitochondrial electron-transfer system. This can hardly be explained by any side effects of anti-proteolytic agents, such as suppression or distortion of the synthesis and/or assembly process. An intriguing issue arising in connection with the conceivable sequelae of proteinase inhibitors is that several mitochondrial proteins, including some respiratory-chain components, have been reported to be synthesized as larger precursors (see, e.g., Ries *et al.*, 1978; Schatz, 1979; Poyton & McKemie, 1979a,b). An implication of these findings is that mitochondrial assembly involves a proteolytic processing step that might be affected by inhibitor treatment. The situation, however, is still far from being clear. For instance, precursor forms for the three largest ATPase subunits have been immunologically detected in yeast by Maccacchini *et al.* (1979); on the other hand, Ryrie & Gallagher (1979) have directly shown that yeast growth with proteinase inhibitors does not alter the electrophoretic patterns of the ATPase complex, and they suggested that no appreciable modification of its components occurs *in vivo*.

Further, if precursor processing is a crucial step in mitochondrial assembly, as supposed by Schatz (1979), its inhibition should most certainly be expected to result in marked suppression of development of the whole respiratory system, and this is obviously not the case in the present study (e.g. Figs. 1 and 2). Hence one can speculate either that the processing step is optional, which would be rather puzzling [especially for 'polyprotein' precursors such as the one reported by Poyton & McKemie (1979a,b) for cytochrome oxidase], or, more

plausibly, that the processing machinery is unaffected by the proteinase inhibitors used.

Finally, features very similar to that of the mitochondria of inhibitor-grown cells are observed in the preparations from the exponential-phase galactose-grown culture in the absence of proteinase inhibitors. This strongly argues in favour of such aberrations in the respiratory-chain arrangement being indeed naturally occurring. Of course, a possibility that inhibitor treatment, besides protecting the non-functioning entities from elimination, gives rise to some structural alterations cannot be ruled out altogether, since such consequences would not be discerned in the present study inasmuch as they do not affect the spectral or catalytic properties of the elements of the respiratory chain.

Glucose-grown versus galactose-grown cultures

Results obtained with glucose- and galactose-grown cultures seem to complement each other nicely. The experiments with galactose-grown cultures make it likely that in glucose-grown cultures the inactive components are also not induced but simply preserved by proteinase inhibitors. On the other hand, the data on glucose-grown cultures indicate that the observed phenomena are not a unique property of growth on galactose. The fact that without proteinase inhibitors the 'excess' cytochromes can be seen in galactose-grown but not glucose-grown cells may be due to the fact that in the latter case the development of the respiratory system proceeds substantially more slowly (compare Figs. 1 and 2), and the rate of elimination of inactive components is such that they would not accumulate to a detectable extent. During growth on galactose, on the other hand, no appreciable breakdown of cytochromes seems to occur until the mid-exponential phase, since the presence of phenylmethane-sulphonyl fluoride or pepstatin from the onset of growth does not increase the peak of cytochrome contents, although suppressing the subsequent decrease (some data on this can be found in a preliminary report by Luzikov *et al.*, 1976).

References

- Albro, P. W. (1975) *Anal. Biochem.* **64**, 485–493
- Bakalkin, G. Ya., Kalnov, S. L., Zubatov, A. S. & Luzikov, V. N. (1976) *FEBS Lett.* **63**, 218–221
- Bakalkin, G. Ya., Kalnov, S. L., Galkin, A. V., Zubatov, A. S. & Luzikov, V. N. (1978) *Biochem. J.* **170**, 569–576
- Betz, H. & Weiser, U. (1976) *Eur. J. Biochem.* **62**, 65–76
- Ebner, E., Mason, T. L. & Schatz, G. (1973) *J. Biol. Chem.* **248**, 5369–5378
- Estabrook, R. W. & Mackler, B. (1957) *J. Biol. Chem.* **224**, 637–648
- Gear, A. R. L., Albert, A. D. & Bednarek, J. M. (1974) *J. Biol. Chem.* **249**, 6495–6504

- Ito, K. (1977) *J. Bacteriol.* **132**, 1021–1023
- Kalnov, S. L., Serebryakova, N. V., Zubatov, A. S. & Luzikov, V. N. (1978) *Biokhimiya* **43**, 662–667
- Kalnov, S. L., Novikova, L. A., Zubatov, A. S. & Luzikov, V. N. (1979a) *FEBS Lett.* **101**, 355–358
- Kalnov, S. L., Novikova, L. A., Zubatov, A. S. & Luzikov, V. N. (1979b) *Biochem. J.* **182**, 195–202
- Lee, C. P., Estabrook, R. W. & Chance, B. (1965) *Biochim. Biophys. Acta* **99**, 32–45
- Lenney, J. F. (1975) *J. Bacteriol.* **122**, 1269–1273
- Lester, R. L., Hatefi, Y., Widmer, C. & Crane, F. L. (1959) *Biochim. Biophys. Acta* **33**, 169–176
- Luzikov, V. N. (1979) *Symp. FEBS* **53**, 133–142
- Luzikov, V. N., Zubatov, A. S., Rainina, E. I. & Bakeyeva, L. E. (1971) *Biochim. Biophys. Acta* **245**, 321–334
- Luzikov, V. N., Makhlis, T. A. & Galkin, A. V. (1976) *FEBS Lett.* **69**, 108–110
- Maccacchini, M.-L., Rudin, Y., Blobel, G. & Schatz, G. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 343–347
- McIlhinney, A. & Hogan, B. L. M. (1974) *Biochim. Biophys. Acta* **372**, 358–365
- Pong, S.-S., Nuss, D. L. & Koch, G. (1975) *J. Biol. Chem.* **250**, 240–245
- Poole, R. K., Lloyd, D. & Chance, B. (1974) *Biochem. J.* **138**, 201–210
- Poyton, R. O. & McKemmie, E. (1979a) *J. Biol. Chem.* **254**, 6763–6771
- Poyton, R. O. & McKemmie, E. (1979b) *J. Biol. Chem.* **254**, 6772–6780
- Rajwade, M. S., Katyare, S. S., Fatterpaker, P. & Sreenivasan, A. (1975) *Biochem. J.* **152**, 379–387
- Ries, G., Hundt, E. & Kadenbach, B. (1978) *Eur. J. Biochem.* **91**, 179–191
- Ryrie, I. J. & Gallagher, A. (1979) *Biochim. Biophys. Acta* **545**, 1–14
- Schatz, G. (1979) *FEBS Lett.* **103**, 203–211
- Tzagoloff, A. (1969) *J. Biol. Chem.* **244**, 5020–5026
- Tzagoloff, A. (1971) *J. Biol. Chem.* **246**, 3050–3056
- Werner, S., Schwab, A. J. & Neupert, W. (1974) *Eur. J. Biochem.* **49**, 607–617
- Wheeldon, L. W., Dianaux, A.-Ch., Bof, M. & Vignais, P. V. (1974) *Eur. J. Biochem.* **46**, 189–199