Haem Synthesis during Mitochondrogenesis in Yeast

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(Received 19 August 1970)

The activities of δ -aminolaevulate synthetase and δ -aminolaevulate dehydratase have been assayed in *Saccharomyces cerevisiae* during glucose repression and de-repression. δ -Aminolaevulate dehydratase increased concomitantly with the increase in oxygen uptake during the de-repression phase caused by the depletion of glucose in the medium. δ -Aminolaevulate synthetase showed an oscillatory behaviour and a spurt in its activity always preceded the increase in oxygen uptake. The activity of both the enzymes was lowered if the cells were incubated with glucose or cycloheximide, but not with chloramphenicol.

The presence of glucose in the growth medium of baker's yeast (*Saccharomyces cerevisiae*) brings about an inhibition in the formation of mitochondria, a phenomenon commonly referred to as 'glucose repression' (Polakis & Bartley, 1965; Yotsuyanagi, 1962; Jayaraman, 1969). Jayaraman, Cotman, Mahler & Sharp (1966) observed that the activities of various mitochondrial enzymes and cytochromes remained very low when the yeast cells were exposed to glucose. On depletion of glucose in the medium, there was an increase in the activities of several mitochondrial enzymes and cytochromes concomitant with the formation of structurally and functionally integral mitochondria. The various enzyme activities and cytochromes appeared in the mitochondrial particles at different intervals of time, indicating that they are not laid down concurrently *en bloc* and that there may be considerable autonomy in their synthesis. These experiments did not distinguish between low enzyme activity seen under conditions of glucose repression and low enzyme protein. However, Mahler, Mackler, Slonimski & Grandchamp (1964) and Kraml & Mahler (1967), using respiratory deficient mutants of yeast, showed by immunochemical methods that although succinate dehydrogenase and cytochrome oxidase activities were not detectable in the mutants they contained cross-reacting materials to these enzyme complexes. In petite mutants of *S. cerevisiae* Tuppy & Birkmayer (1969) showed that the non-availability of cytohaemin may be responsible for the deficiency of cytochrome oxidase. Thus it appeared possible that haem synthesis may be a limiting factor under conditions of glucose repression,

accounting for the low haem protein concentrations. In the present investigation, the contents of two key haem biosynthetic enzymes, namely δ -aminolaevulate synthetase and δ -aminolaevulate dehydratase, have been studied in *S. cerevisiae* under the following conditions: (a) repressed phase brought about by the presence of glucose in the medium and characterized by an exponential growth of the cell population and minimal mitochondrial content; (b) de-repressed phase brought about by exhaustion of glucose in the medium and characterized by an exponential increase in mitochondrial content and a stationary growth phase; (c) in the presence of added glucose at the beginning of the de-repressed phase, which increases growth but blocks the increase in mitochondrial content; (d) in the presence of chloramphenicol and cycloheximide, which inhibit mitochondrial and cytoplasmic protein synthesis respectively.

MATERIALS AND METHODS

Chemicals. δ -Aminolaevulinic acid hydrochloride was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Chloromycetin and cycloheximide were obtained as gift samples from Parke, Davis and Co. Ltd., Bombay, India, and Upjohn Co., Kalamazoo, Mich., U.S.A., respectively.

Organism. *S. cerevisiae* 3095 was obtained from the National Collection of Industrial Micro-organisms, National Chemical Laboratory, Poona, India. The stock culture was maintained on agar slants containing 3% of malt extract and 1% of glucose.

Procedures for growth. The experimental procedure consisted of two phases. Yeast was inoculated from a small inoculum into 1-litre flasks each containing 250 ml of medium A: sucrose (1%); yeast extract (Difco or Oxoid)

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(0.2%); KH_2PO_4 (0.1%); MgSO_4 (0.05%); NaCl (0.05%); $(\text{NH}_4)_2\text{SO}_4$ (0.1%). The cells were allowed to grow for 12 h at 25°C with shaking. The cells were then harvested by centrifugation and were found to be near the end of the exponential phase of growth with a low respiratory capacity. The yield of cells obtained was about 4 g wet wt./flask. The cell pellet was washed once with sterile water and centrifuged again. The sediment was suspended in sterile water to a concentration of 5 g wet wt. of cells/5 ml of suspension. This suspension was added to 1-litre flasks containing 250 ml of medium B: glucose (1.0%); yeast extract (0.5%); KH_2PO_4 (0.9%); MgSO_4 (0.05%); CaCl_2 (0.04%); $(\text{NH}_4)_2\text{SO}_4$ (0.06%). Each flask received 1 g wet wt. of cells. The time of addition of the cell suspension to medium B was taken as zero time. The flasks were then shaken at 30°C and samples were removed at different time-intervals. This procedure of transfer to a repression medium was found essential for two reasons: (a) the characteristic increase in yeast respiration was marked in this medium and (b) the timing of events was highly reproducible.

Addition of compounds. Chloramphenicol was added at a concentration of 4 mg/ml of medium. Because of its limited solubility at the concentration used it was usually added as a solid to medium B at zero time and dissolved by heating. After cooling, the medium was inoculated with the yeast. The behaviour of yeast cells, from the parameters measured, was not affected in the repression phase. Cycloheximide was dissolved in a small volume of sterile water and was added at a concentration of 100 µg/ml of medium after 3 h growth of the cells in medium B. Glucose was added as a 40% (w/v) sterile solution at a concentration of 1 g/g wet wt. of cells after 3 h growth of the cells in medium B.

Measurement of growth. Growth was followed turbidimetrically. A 5 ml portion of the sample was taken from the flasks just before harvesting and turbidity was measured in a Klett colorimeter with a red filter. After the cells were harvested the wet weight of the pellet was also determined.

Oxygen uptake of cells. A 5 ml portion of the yeast culture was quickly centrifuged (2–3 min) in a clinical centrifuge. The supernatant was decanted and the sediment was suspended in water to make a final volume of 1 ml. A 0.1 ml sample of this suspension was added to 1.9 ml of potassium phosphate buffer, pH 7.4 (0.1 M), containing 0.5% of glucose and the oxygen uptake was measured by using a Gilson Oxygraph at 30°C. The results are expressed as nmol of oxygen taken up/g of cells.

Extraction of yeast cells. At the required time-intervals the individual flasks were removed from the shaker and immediately put in an ice bath. After taking out portions for measurements of turbidity and oxygen uptake, a 40 ml portion was pipetted out into weighed centrifuge tubes and quickly centrifuged. The sedimented pellet was weighed to determine the wet weight. To the sediment was added an equal weight of carborundum (100 mesh)-Celite (1:1, w/w) and thoroughly mixed. The almost dry powder-like mixture was transferred to a cold mortar and ground well for 1 min. For δ -aminolaevulate dehydratase extraction the cell paste was extracted with cold 0.05 M-glycine-NaOH buffer, pH 9.2 (5 ml of buffer/g wet wt. of cells). The supernatant was decanted off the settled carborundum and then centrifuged at 4000 rev./min for

10 min in a Sorvall refrigerated centrifuge. The supernatant was carefully decanted, centrifuged again at the same speed and the final supernatant was used for the assay. For δ -aminolaevulate synthetase, the extraction buffer used was 0.25 M-sucrose–20 mM-tris (pH 7.4)–5 mM-EDTA. Both the extraction buffers yielded a protein content in the extract ranging from 50 to 60 mg/g wet wt. of the cells.

Assay of δ -aminolaevulate dehydratase. The optimum conditions of the assay were essentially those described by Clara de Barreiro (1967) for this enzyme in *S. cerevisiae*. The incubation mixture contained, in a total volume of 2 ml: 120 µmol of glycine-NaOH buffer, pH 9.6, 10 µmol of δ -aminolaevulinic acid and 8–10 mg of protein as the yeast-cell extract. The incubation was carried out for 1 h at 55°C. The reaction was stopped with 1 ml of trichloroacetic acid-mercury reagent and the porphobilinogen formed was determined with modified Ehrlich reagent (Granick & Mauzerall, 1958). The yeast-cell extract did not contain any direct-reacting Ehrlich-positive material.

Assay of δ -aminolaevulate synthetase. The incubation mixture contained, in a total volume of 2 ml: 120 µmol of tris-HCl buffer, pH 7.4, 200 µmol of glycine, 20 µmol of sodium succinate, 20 µmol of ATP, 0.3 µmol of pyridoxal phosphate, 0.2 µmol of coenzyme A, 10 µmol of MgCl_2 , 8 µmol of EDTA, about 1 mg of succinyl-CoA synthetase [succinate-CoA ligase (ADP), EC 6.2.1.5] prepared from *Rhodospseudomonas spheroides*, and 8–10 mg of protein as yeast-cell extract. The incubation was carried out with shaking for 1 h at 37°C. The reaction was stopped with 0.5 ml of 12.5% (w/v) trichloroacetic acid and the solution centrifuged. δ -Aminolaevulate present in the supernatant was determined by using Ehrlich reagent, after fractionation of its pyrrole condensation product with acetylacetone with columns (1 cm × 2.5 cm) of Dowex-1 (acetate form) by the method described by Hayashi, Yoda & Kikuchi (1969). The yeast cell extract did contain δ -aminolaevulate at all stages of growth and a heat-denatured blank was run for each preparation.

Succinyl-CoA synthetase was prepared from *R. spheroides* cells by the method described by Hayashi *et al.* (1969). The preparation catalysed the formation of 10–12 µmol of succinyl-CoA/mg of protein per h.

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Growth and oxygen uptake during the glucose repression-de-repression cycle. A number of experiments have been carried out correlating the oxygen uptake of whole cells with the growth pattern of the yeast in 1% glucose medium (medium B). The results of a typical experiment are shown in Fig. 1. The cell population increased about threefold in the first 3.5 h and then entered a stationary phase. The oxygen uptake of the cells remained stationary at a low value (about 7000 nmol/g wet wt.) during the increase in growth phase and then increased almost threefold when the growth phase became stationary. These results are almost identical with those reported by Jayaraman *et al.* (1966) with a different

Fig. 1. Growth, oxygen uptake, δ -aminolaevulate synthetase and δ -aminolaevulate dehydratase activities in *S. cerevisiae* during the glucose repression–de-repression cycle. Experimental details are given in the text. The experiments have been repeated at least four times. Results presented are those obtained in a typical experiment. ●, Growth; ▲, uptake of oxygen; ■, δ -aminolaevulate synthetase; ○, δ -aminolaevulate dehydratase.

strain of *S. cerevisiae*. In the present series of experiments the de-repression phase started at about 3.75 h after the growth of cells in medium B. It may be pointed out that in Fig. 1 values have been given only from the 2 h time-point after transfer of the cells to medium B. It has been found that the lowest value for oxygen uptake is reached 2 h after the transfer of cells to medium B, and then the value remains steady until de-repression starts.

δ -Aminolaevulate dehydratase and δ -aminolaevulate synthetase activities during the glucose repression–de-repression cycle. From Fig. 1 it can be seen that the specific activity of δ -aminolaevulate dehydratase remained steady and at a low activity during the repression phase. Almost simultaneously with the increase in oxygen uptake δ -aminolaevulate dehydratase activity showed a parallel and equivalent increase during the de-repression phase.

In contrast with δ -aminolaevulate dehydratase, δ -aminolaevulate synthetase showed a rather complex behaviour. There was a fall in the δ -aminolaevulate synthetase activity during the early part of the repression phase. Just before the onset of the de-repression phase the enzyme doubled its activity and then once again decreased. Again there was a peak of activity at about 4 h and the enzyme activity continued to fall. Two peaks of enzyme activity have been repeatedly observed,

although there was a quantitative variation from experiment to experiment in the ratio of the two maxima. It is significant to point out that the appearance of the first peak of δ -aminolaevulate synthetase activity always preceded the time of the onset of the de-repression phase. The pattern of results obtained was the same when the two enzyme activities were expressed as units/g of cells.

Effect of antibiotics and glucose on the two enzyme activities. Because of limited solubility chloramphenicol was added at zero time. Cycloheximide and extra glucose were added at 3 h of growth in medium B.

Addition of chloramphenicol at zero time did not affect the growth, oxygen uptake or the two enzyme activities during the repression phase. During the de-repression phase the antibiotic blocked oxygen uptake, which is in agreement with the reports (Huang, Biggs, Clark-Walker & Linnane, 1966; Linnane & Stewart, 1967) that the antibiotic blocks mitochondrogenesis (Tables 1 and 2). From Table 1 it is clear that chloramphenicol did not significantly affect the increase in δ -aminolaevulate dehydratase activity during the de-repression phase, although at 6 h there appeared to be a slight inhibitory effect on the enzyme. Cycloheximide inhibited the increase in oxygen uptake as well as enzyme activity during

Table 1. *Factors affecting δ -aminolaevulate dehydratase activity in Saccharomyces cerevisiae during the repression-de-repression cycle*

Yeast cells were grown in medium A and then transferred to medium B. Chloramphenicol was added at zero time. Cycloheximide and glucose were added at 3 h after growth in medium B. The experimental details are given in the Materials and Methods section. The experiments have been repeated three times and the results presented are a set of typical values.

Time after transfer (h)	Additions	Growth (g wet wt./flask)	Uptake of O ₂ (μ mol/g of cells)	Dehydratase activity (nmol of porphobilinogen formed/mg of protein)
3	None	3.0	7.2	0.80
	Chloramphenicol	2.9	7.0	0.80
4½	None	3.9	9.2	1.52
	Chloramphenicol	3.4	10.1	1.52
	Cycloheximide	3.3	8.0	0.52
	Glucose	4.8	10.2	0.72
6	None	3.9	22.0	2.00
	Chloramphenicol	3.5	8.8	1.60
	Cycloheximide	3.2	7.8	0.52
	Glucose	5.8	6.8	0.44

Table 2. *Factors affecting δ -aminolaevulate synthetase activity in Saccharomyces cerevisiae during the repression-de-repression cycle*

Experimental details are as given under Table 1.

Time after transfer (h)	Additions	Growth (g wet wt./flask)	Uptake of O ₂ (nmol/g of cells)	Synthetase activity (nmol of δ -aminolaevulate formed/mg of protein)
3	None	3.0	5.8	0.80
	Chloramphenicol	3.0	5.5	0.75
3½	None	3.5	7.0	0.80
	Chloramphenicol	3.2	6.6	0.75
	Cycloheximide	3.6	6.4	0.63
	Glucose	4.0	7.4	0.68
4	None	4.1	6.5	1.00
	Chloramphenicol	3.6	5.4	1.00
	Cycloheximide	4.1	5.4	0.60
	Glucose	5.2	6.5	0.40

the de-repression phase. Addition of glucose blocked increases in oxygen uptake and δ -aminolaevulate dehydratase activity but enhanced growth. A similar picture was obtained with δ -aminolaevulate synthetase (Table 2). At the time-periods studied the increase in δ -aminolaevulate synthetase activity was not striking; however, it can be seen that glucose and cycloheximide caused a decrease in the activities of the enzyme present at 3 h. Chloramphenicol had no effect on this enzyme.

DISCUSSION

The results of the present investigation indicate that δ -aminolaevulate dehydratase, a cytoplasmic enzyme, increased during the de-repression phase in

S. cerevisiae concomitantly with the increased oxygen uptake and formation of mitochondria. The increase in enzyme activity is blocked by cycloheximide but not by chloramphenicol, indicating its probable extramitochondrial site of synthesis. The enzyme is subject to glucose repression. Thus conditions that affect mitochondrial synthesis seem to affect δ -aminolaevulate dehydratase activity in a parallel fashion, even though the enzyme is not mitochondrial in origin or location. It appears possible that this enzyme may have a regulatory function in providing a mitochondrial component, namely haem, the availability of which can determine the formation of haem proteins. δ -Aminolaevulate dehydratase has been shown to have a regulatory function in haem synthesis in organisms such as *Propionibacterium shermanii* (Menon &

Shemin, 1967), *Neurospora crassa* (Muthukrishnan, Padmanaban & Sarma, 1969) and *Euglena gracilis* (Ebbon & Tait, 1969). δ -Aminolaevulate synthetase in the present experiments shows an oscillatory behaviour, and an increase in enzyme activity always precedes the beginning of the de-repression phase. It is possible that a spurt in its activity may provide enough δ -aminolaevulate to trigger the process of haem synthesis, and its oscillatory behaviour may be due to its susceptibility to feedback inhibition or repression by haem or to both. δ -Aminolaevulate synthetase shows an oscillatory behaviour in animal systems (Tschudy, Waxman & Collins, 1967) and is regulated by the end product haem (Burnham & Lascelles, 1963; Granick, 1966). This can possibly account for the decreasing trend in the activity of δ -aminolaevulate synthetase at late stages of de-repression in *S. cerevisiae*. Porra & Irving (1970) have shown that δ -aminolaevulate synthetase activity is present in cell-free extracts of *Torulopsis utilis* grown under nitrogen but not in extracts from aerobically grown cells. The latter cells contain as much as 30 times the haem content of the former on a protein basis. Despite its complex behaviour in *S. cerevisiae*, the enzyme is subject to glucose repression and its activity is decreased by cycloheximide but not by chloramphenicol.

The results presented in this paper, taken together with the observations of other workers quoted in the introductory paragraph, strongly suggest a regulatory role for the two enzymes in mitochondrogenesis in yeast.

Thanks are due to Dr T. Ramasarma for keen interest in the work and to Miss V. Varalakshmi and Mr S. Suryaprakash for expert technical assistance.

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