MITOCHONDRIAL EFFECTS OF COPPER-LIMITED GROWTH OF *TORULOPSIS UTILIS*

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1. Introduction

The dependence of cytochrome oxidase activity of yeast on the concentration of copper in the growth medium was shown by Elvehjem [1] in 1931. Giorgio et al. [2] showed that the growth of *Saccharomyces cerevisiae* was diminished if the medium contained less than 0.15 μM copper. Wohlrab and Jacobs [3] demonstrated that growth of *S. cerevisiae* in a medium containing between 0.04 μM and 0.08 μM copper led to a decrease of approx. 70% in the cell concentration of cytochrome aa_3. All of the above studies were performed in batch culture. In view of certain advantages of continuous culture over batch culture in the study of the biochemical effects of various forms of growth limitation [4, 5], I have carried out a study of copper-limited growth of *Torulopsis utilis* in a chemostat. Studies with other growth-limiting conditions have been described elsewhere [5, 6].

2. Materials and methods

T. utilis (N.C.Y.C. 193) was grown in a chemostat at a dilution rate of 0.25 hr^{-1} as described previously [5], using a medium similar to that used for glycerol-limited growth [5] except that copper was omitted and in certain experiments the medium was subjected to the copper extraction procedure of Giorgio et al. [2] using zinc dibenzyldithiocarbamate (see Results). All other methods were as described by Light and Garland [5]. "Copper-recovered cells" were obtained by harvesting and washing cells from a copper-limited culture and then incubating the cells at 30° with aeration for 16 hr when suspended at a conc. of 4–5 mg dry wt per ml in 40 μM $CuSO_4$.

3. Results

Fig. 1 shows the relationship between the yield of cells and the cytochrome aa_3 content of whole cells in continuous culture at presumably various but otherwise undetermined entering concentrations of copper. The reasons for considering that various entering concentrations of copper were achieved were as follows: Firstly, the major source of copper in the medium is from contamination of the other metal salts added. Thus it could be anticipated that halving the concentration of these salts from that normally used (see [5]) would halve the entering copper concentration, given that a copper extraction procedure [2] was not used. Fig. 1 demonstrates that, although media that was not extracted for copper supported glycerol-limited growth, halving the concentration of added salts caused a three-fold reduction in the concentration of cytochrome aa_3 but not of cell yield. Secondly, copper extraction from the growth medium resulted in copper-limited growth, as judged by the fall of cell yield and the appearance of considerable concentrations of unused glycerol with the culture. Thirdly, addition of copper to this copper extracted medium caused an increase in cell yield and a decrease in the concentration of unused glycerol in the culture. Under these conditions, variations of cell yield would represent variations in the entering copper concentration. The minimal cell content of cytochrome aa_3 under copper-limited growth conditions is about 5 nmole/g dry wt which is a 10-fold reduction from the normal with glycerol-limited copper-supplemented cells.

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Table 1

Respiratory properties of glycerol-limited, copper-limited and copper-recovered cells and mitochondria.

	Glycerol-limited		Copper-limited		Copper-recovered (without cycloheximide)		Copper-recovered (with cycloheximide)
	Cells	Mitochondria	Cells	Mitochondria	Cells	Mitochondria	Cells
Concentration of cytochromes (nmole/g)							
aa$_3$	48.3	–	7.9	–	33.5	–	5.5
b	29.0	–	46.5	–	43.5	–	31.3
cc$_1$	57.4	–	78.2	–	89.9	–	34.6
Oxygen uptake rate (ng atom/mg/min)							
Endogenous substrate	140	70	55	25	62	29	33
Pyruvate with malate (state 3)	–	175	–	84	–	152	–
TMPD/Asc (state 3)	–	350	–	86	–	247	–
P:O ratios (polarographic assay)							
Pyruvate with malate	–	3.0	–	2.45	–	2.42	–
NADH	–	1.65	–	1.5	–	1.3	–
L-Glycerol-3-phosphate	–	1.8	–	1.7	–	1.8	–
Respiratory inhibition by Rotenone (%)							
Endogenous substrate	85	–	0	–	30	–	0
Pyruvate with malate	–	95	–	10	–	75	–
NADH	–	0	–	0	–	10	–

Cytochrome concentrations and respiration rates are referred either to g dry wt for cells or mg protein for mitochondria. For experimental conditions, see text and [5].

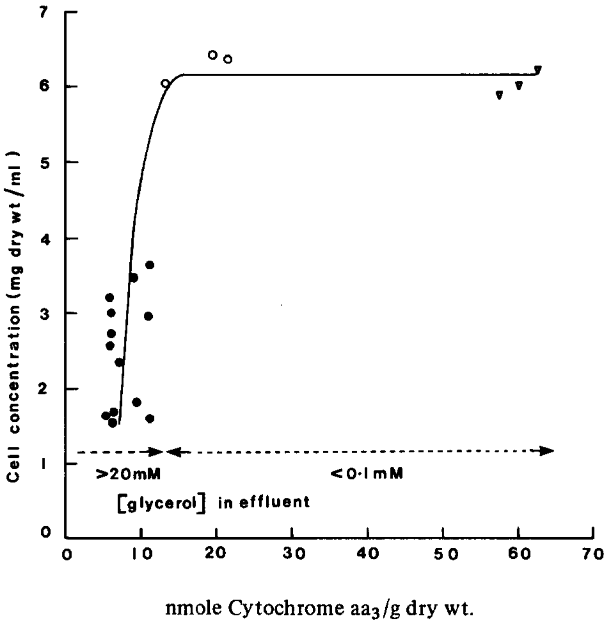

Fig. 1. The cytochrome aa_3 concentration and yield of *T. utilis* cells grown continuously at varying entering concentrations of copper and a constant entering concentration of glycerol. Three levels of copper concentration were achieved. Experimental points (▽) correspond to the glycerol medium of Light and Garland [5] without added copper; points (○) correspond to the same medium but with the concentration of all added metal salts halved; points (●) correspond to different batches of the latter medium that had been subjected to a copper extraction procedure [2]. The transition between copper-limited and glycerol-limited growth occurred in the region of the sharp inflexion in the graph as is shown by the concentration of glycerol in the effluent from the chemostat.

Table 1 compares some respiratory properties of whole cells and isolated mitochondria from glycerol- or copper-limited growth. Also shown in table 1 are the effects of aerobic incubation of copper-limited cells in the presence of 40 μM $CuSO_4$ in the absence of cell growth. The following conclusions may be drawn: a) Copper limitation specifically decreased the cell concentration of cytochrome aa_3 without decreasing that of cytochrome b or cc_1. b) The respiration rates of both whole cells and mitochondria were markedly diminished by copper limitation of growth. This suggests that cytochrome oxidase had become rate limiting for respiration. c) Rotenone sensitivity was absent from copper-limited cells and their mitochondria. d) The copper-recovery procedure caused a 5-fold increase in the cell concentration of cytochrome aa_3, an increase in the mitochondrial respiration rate, and a return of rotenone sensitivity. e) These effects of the recovery procedure did not occur if cycloheximide (0.1 mg/ml) was added to the recovery incubation medium. f) Polarographic assays of P/O ratios using a variety of substrates demonstrated the presence of all 3 energy conservation sites associated with the respiratory chain. In all instances respiration was completely inhibited by cyanide.

4. Conclusions

The effects of copper-limited growth on the cytochrome aa_3 content of *T. utilis* were as expected from the earlier work of others [1–3]. An unexpected finding was that copper limitation caused rotenone insensitivity at cell growth rates where nitrogen-, magnesium-, phosphate- or glycerol-limitation do not [5]. Iron- and sulphate-limited growth also cause rotenone insensitivity [5, 6], and both analytical [7] and spectroscopic [8] evidence points to this effect being due to alterations of the mitochondrial content of iron–sulphur protein. Speculatively, rotenone insensitivity might arise not only from the absence of a specific iron–sulphur protein but also from altered packing of respiratory enzymes in the mitochondrial membrane as a consequence of drastic changes in their relative concentrations. In this context it may be noted that the detergent-solubilized NADH dehydrogenase (i.e. NADH ferricyanide reductase) of *T. utilis* mitochondria co-purifies with cytochrome aa_3 [9].

The copper-recovery procedure is of interest in that it apparently provides a non-growing cell system in which spectroscopically identified cytochrome aa_3 is restored in a cycloheximide-sensitive fashion. This transition is less complex than the widely studied anaerobic to aerobic transition [10], and may well be an excellent system in which to study the synthesis and location of a specific new protein into a pre-existing membrane. The effects of cycloheximide are consistent with the independent conclusions of Birkmayer [11] and Subik et al. [12] that cytoplasmic protein synthesis is involved in the synthesis of cytochrome aa_3.

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References

[1] C.A. Elvehjem, J. Biol. Chem. 90 (1931) 111.

[2] A.J. Giorgio, G.E. Cartwright and M.M. Wintrobe, J. Bacteriol. 86 (1963) 1037.

[3] H. Wohlrab and E.E. Jacobs, Biochem. Biophys. Res. Commun. 28 (1967) 1003.

[4] P. Ann Light, Fifth International Symposium on Continuous Culture, Oxford, July 1971, Society for Chemical Industry Monograph, in press.

[5] P. Ann Light and P.B. Garland, Biochem. J. 124 (1971) 123.

[6] B.A. Haddock and P.B. Garland, Biochem. J. 124 (1971) 155.

[7] R.A. Clegg and P.B. Garland, Biochem. J. 124 (1971) 135.

[8] C.I. Ragan and P.B. Garland, Biochem. J. 124 (1971) 171.

[9] S.O.C. Tottmar and C.I. Ragan, Biochem. J. 124 (1971) 853.

[10] W.L. Chen and F.G. Charalampous, J. Biol. Chem. 244 (1969) 2767.

[11] G.D. Birkmayer, European J. Biochem. 21 (1971) 258.

[12] J. Subik, S. Kuzale, J. Kolarov, L. Kovac and M. Lachowicz, Biochim. Biophys. Acta 205 (1970) 513.