Effects of Glucose Repression and Anaerobiosis on the Activities and Subcellular Distribution of Tricarboxylic Acid Cycle and Associated Enzymes in *Saccharomyces carlsbergensis*

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Cell-free extracts were prepared from sphaeroplasts of aerobically and anaerobically grown, glucose-derepressed and glucose-repressed *Saccharomyces carlsbergensis*. The activities of the enzymes of the tricarboxylic acid cycle and of related enzymes were measured, together with their distributions after differential centrifugation. Glucose repression lowered activities of enzymes of the tricarboxylic acid cycle by 60 to 89% under aerobic conditions. Activities were still further decreased under anaerobic glucose-derepressed conditions. 2-Oxoglutarate decarboxylase activity was not detected after anaerobic growth; malate synthase and isocitrate lyase activities were not detected in organisms grown either aerobically or anaerobically. Glucose had little effect on the activities of tricarboxylic acid cycle enzymes under anaerobic conditions, with the exception of citrate synthase whose activity increased under anaerobic glucose-repressed conditions. Differential centrifugation of cell-free extracts showed different distribution patterns of the enzymes under the four growth conditions. The distribution patterns and enzyme activities reflected the differential effects of glucose repression and/or anaerobiosis.

INTRODUCTION

The cytochrome contents and activities of electron transport chain and associated enzymes of mitochondria from *Saccharomyces* species are altered substantially by the presence of a repressing carbon source (e.g. glucose) and/or anaerobic conditions during growth (Schatz, 1965; Criddle & Schatz, 1969; Cartledge & Lloyd, 1972*a, b, c*). These authors also showed that differential or zonal centrifugation of cell-free extracts from either glucose-repressed or anaerobically grown cells revealed different distribution patterns of cytochromes and enzymes, when compared with those of cells grown aerobically with derepressing amounts of glucose.

Despite the wealth of information available about the electron transport chain components, little has been published concerning activities and distributions of enzymes of the tricarboxylic acid cycle under these conditions. Polakis & Bartley (1965) showed lower activities of all enzymes of the tricarboxylic acid cycle present under supposedly repressed conditions [0.9% (w/v) glucose] when compared with derepressed conditions [0.9% (w/v) galactose], using aerobically grown *Saccharomyces cerevisiae*. Similar results were found for various enzymes of the tricarboxylic acid cycle by Duntze *et al.* (1969) and Perlman & Mahler (1974), although again low glucose concentrations were used to achieve repression. Vary *et al.* (1969) found lowered activities of fumarase and malate and succinate dehydrogenases in anaerobically grown *S. cerevisiae*.

In this paper, we present results for the activities and subcellular distributions of the

tricarboxylic acid cycle and associated enzymes from cell-free extracts of *S. carlsbergensis*, after growth under the following conditions: (i) aerobic derepressed, (ii) aerobic repressed, (iii) anaerobic derepressed and (iv) anaerobic repressed.

METHODS

Maintenance and growth. *Saccharomyces carlsbergensis* (NCYC 74S) was maintained on potato dextrose agar slopes. The complex medium for aerobically grown cultures was that of Duell *et al.* (1964). Batch cultures (4 l) were grown in a 5 l fermenter (L.H. Engineering Co., Stoke Poges, Bucks.) at 30 °C, with forced aeration (4 l air min⁻¹, stirring rate 300 rev. min⁻¹). The complex medium and method of growth of anaerobic cultures were as described by Cartledge & Lloyd (1972*c*) except that the batch cultures (1.5 l) were grown in 2 l Quickfit growth vessels (Gallenkamp) in an anaerobic cabinet.

Glucose was added at 0.8 and 10% (w/v) for derepressed and repressed conditions, respectively. Starter cultures were grown in 100 ml medium in 250 ml conical flasks shaken for 22 h at 30 °C in a rotary orbital incubator (Gallenkamp).

Organisms were counted in an Improved Neubauer haemocytometer (Hawksley, Lancing, Sussex) after suitable dilution and mixing to separate clumps. Batch cultures were harvested in late-exponential or early-stationary phase when the cell count was between 6.0×10^7 and 9.0×10^7 organisms ml⁻¹.

Harvesting, preparation and disruption of sphaeroplasts. Sphaeroplasts were formed as described by Cartledge & Lloyd (1972*a*) for aerobic cultures and Cartledge & Lloyd (1972*c*) for anaerobic cultures. Partially digested cells, intact sphaeroplasts and nuclei were removed by centrifugation (2000 g, 5 min) in an 8 × 50 ml rotor of an MSE 18 centrifuge, and the supernatant fraction (cell-free extract) was retained.

Fractionation of cell-free extracts by differential centrifugation. All centrifugation procedures were carried out in the 8 × 50 ml rotor of an MSE 18 centrifuge at 4 °C. Cell-free extract (10 to 20 ml) was centrifuged (10000 g, 10 min) and the supernatant was retained. The pellet was resuspended in 5 ml of disruption buffer (0.25 M-sucrose, 2 mM-MgCl₂ and 10 mM-Tris/HCl, pH 7.4), and the above procedure was repeated. The supernatants were combined, and the pellet (P1) was resuspended to a known volume in disruption buffer. The combined supernatants were centrifuged (20000 g, 20 min), the resulting supernatant was retained and the pellet was washed in disruption buffer as before. The pellet (P2) was resuspended in disruption buffer to a known volume, and the volume of the final supernatant (FS) was measured.

Enzyme assays. Spectrophotometric assays were carried out at 30 °C. The following enzymes were assayed by previously published methods: citrate synthase (EC 4.1.3.7) (Srere *et al.*, 1963); aconitase (EC 4.2.1.3) (Fansler & Lowenstein, 1969); alcohol dehydrogenase (EC 1.1.1.1) (Kerstens & De Ley, 1966); L-lactate dehydrogenase (EC 1.1.2.3) (Symons & Burcoyne, 1966); malate synthase (EC 4.1.3.2) and isocitrate lyase (EC 4.1.3.1) (Dixon & Kornberg, 1959); fumarate reductase (Massey & Singer, 1957). Malate dehydrogenase (EC 1.1.1.37) was assayed as described by Kitto (1969) except that 0.1 M-potassium phosphate buffer, pH 7.8, was used. Fumarase (EC 4.2.1.2) was assayed by the method of Racker (1950), except that 0.03 M-potassium phosphate buffer, pH 7.8, was used. NADP-linked isocitrate dehydrogenase (EC 1.1.1.42) was assayed in a reaction mixture containing 1.8 mM-DL-isocitrate, 131 μM-NADP, 600 μM-manganese chloride and 0.03 M-potassium phosphate buffer, pH 7.2. Succinate dehydrogenase (EC 1.3.99.1) was assayed in a reaction mixture containing 13.3 mM-sodium succinate, 8.61 μM-dichlorophenolindophenol, 2.2 mM-phenazine methosulphate, 33.3 mM-potassium cyanide and 0.1 M-potassium phosphate buffer, pH 7.5. 2-Oxoglutarate decarboxylase, part of the 2-oxoglutarate dehydrogenase complex, was assayed by the reduction of ferricyanide, which, in turn, was measured as Prussian Blue by the colorimetric method of Reed & Willms (1966). The reaction mixture contained 35.7 mM-2-oxoglutarate, 143 μM-thiamin pyrophosphate, 214 μM-magnesium sulphate, 17.8 mM-potassium ferricyanide and 0.11 M-potassium phosphate buffer, pH 7.0. The decarboxylase was assayed in preference to the dehydrogenase complex as the decarboxylase assay was found to be more reliable.

Preliminary experiments were done with cell-free extracts to ensure that the rates of enzyme reactions measured were proportional to the amount of extract protein used, were not limited by substrate concentration and were assayed at optimum pH. Where necessary, appropriate dilutions of cell-free extracts and fractions were used to obtain a suitable enzyme rate, and the validity of the assays was confirmed by the satisfactory enzyme recovery values finally obtained.

The results presented are averages of at least three experiments.

Protein was measured by the Lowry method, with bovine serum albumin as a standard.

Chemicals. Potassium ferrocyanide, potassium ferricyanide, ethanol, sodium dithionite and glucose were from BDH; other substrates were from Sigma.

Table 1. *Specific activities of the tricarboxylic acid cycle and related enzymes in cell-free extracts of Saccharomyces carlsbergensis grown under four different conditions*

Figures in parentheses show the specific activities expressed as a percentage of the corresponding activity for growth under aerobic, glucose-derepressed conditions.

Enzyme	Enzyme specific activity [nmol min ⁻¹ (mg protein) ⁻¹]			
	Aerobic, glucose-derepressed	Aerobic, glucose-repressed	Anaerobic, glucose-derepressed	Anaerobic, glucose-repressed
Citrate synthase	128	13 (11)	7 (5)	44 (34)
Aconitase	221	49 (22)	19 (9)	1 (0.3)
NADP-linked isocitrate dehydrogenase	94	35 (37)	10 (11)	14 (15)
2-Oxoglutarate decarboxylase	27	4 (16)	< 0.3 (0)	< 0.3 (0)
Succinate dehydrogenase	44	6 (14)	1 (2)	1 (3)
Fumarase	510	98 (19)	2 (0.3)	3 (0.5)
Malate dehydrogenase	4120	1630 (40)	175 (4)	155 (3)
Alcohol dehydrogenase	1380	1680 (122)	93 (7)	1040 (76)
L-Lactate dehydrogenase	148	23 (15)	5 (3)	3 (2)
Fumarate reductase	73	125 (170)	12 (16)	8 (11)
Isocitrate lyase	ND	ND	ND	ND
Malate synthase	ND	ND	ND	ND

ND, Not detectable.

RESULTS

Characterization of fractions

Previous studies of cytochrome *c* oxidase distribution (Cartledge & Lloyd, 1972*a*) indicated that mitochondria from sphaeroplasts of *Saccharomyces carlsbergensis* are completely sedimented in fractions P1 and P2. Cytochrome *c* oxidase, however, is undetectable in cell-free extracts of anaerobically grown organisms (Cartledge & Lloyd, 1972*c*) and in this case the presence of mitochondria-like organelles only in P1 and P2 fractions is indicated by the complete recovery of oligomycin-sensitive ATPase in these fractions.

Enzyme activities in cell-free extracts

The highest activities of enzymes of the tricarboxylic acid cycle occurred in the cell-free extracts of aerobically grown, glucose-derepressed organisms (Table 1). These activities were 60 to 89% lower in extracts of aerobically grown, glucose-repressed organisms and, except for citrate synthase, the activities were even lower in extracts after anaerobic growth. Citrate synthase behaved differently in that its activity in extracts of anaerobically grown, glucose-repressed organisms was higher than in extracts of either anaerobically grown, glucose-derepressed or aerobically grown, glucose-repressed organisms. 2-Oxoglutarate decarboxylase activity was not detected in extracts obtained from organisms grown under either of the anaerobic conditions. L-Lactate dehydrogenase activities followed the same pattern as the majority of enzymes of the tricarboxylic acid cycle, i.e. those other than citrate synthase.

Alcohol dehydrogenase was more active in extracts from aerobically grown, glucose-repressed organisms than in extracts from aerobically grown, glucose-derepressed organisms. The lowest activity occurred in the anaerobically grown, glucose-derepressed extracts. Fumarate reductase behaved similarly to alcohol dehydrogenase under aerobic conditions.

Malate synthase and isocitrate lyase activities could not be detected in extracts of organisms grown under any of the four growth conditions.

Table 2. *Percentage distributions and recoveries of the tricarboxylic acid cycle and related enzymes from fractions of cell-free extracts of Saccharomyces carlsbergensis grown under four different conditions*

Distributions are expressed as percentages of the total recovered activity.

Fraction	...	Aerobically grown										Anaerobically grown									
		Glucose-derepressed					Glucose-repressed					Glucose-derepressed					Glucose-repressed				
		Distribution (%)			Re-covery (%)	Distribution (%)			Re-covery (%)	Distribution (%)			Re-covery (%)	Distribution (%)			Re-covery (%)				
		P1	P2	FS		P1	P2	FS		P1	P2	FS		P1	P2	FS					
Protein		33	6	61	99	40	12	48	96	21	15	64	103	18	5	77	97				
Citrate synthase		88	6	6	101	56	15	28	85	12	6	82	85	8	0	92	93				
Aconitase		96	4	0	79	89	11	0	86	28	6	66	117	70	0	30	90				
NADP-linked isocitrate dehydrogenase		44	15	41	79	48	10	42	110	47	5	48	98	70	8	22	81				
2-Oxoglutarate decarboxylase		82	4	14	59	47	4	49	114	ND	ND	ND	—	ND	ND	ND	—				
Succinate dehydrogenase		98	2	0	79	50	5	5	90	50	9	41	114	36	2	62	102				
Fumarase		95	4	1	73	91	7	2	78	—	—	—	—	—	—	—	—				
Malate dehydrogenase		57	6	37	68	48	8	44	60	53	20	27	85	89	8	3	89				
Alcohol dehydrogenase		21	2	77	93	25	19	56	86	7	2	91	90	9	1	90	92				
L-Lactate dehydrogenase		88	4	8	69	75	11	14	69	24	36	40	74	58	8	34	100				
Fumarate reductase		14	5	81	92	5	3	92	92	—	—	—	—	48	7	45	94				

ND, Not detectable; —, not determined.

Fractionation of cell-free extracts by differential centrifugation

With the exception of NADP-linked isocitrate dehydrogenase (44%) and malate dehydrogenase (57%), over 80% of the recovered activities of the enzymes of the tricarboxylic acid cycle and L-lactate dehydrogenase were found in fraction P1 from the extracts of aerobically grown, glucose-derepressed organisms (Table 2). The alcohol dehydrogenase and fumarate reductase distributions were in complete contrast to the other enzymes, a higher percentage of the recovered activity occurring in the final supernatant.

Results from an extract of aerobically grown, glucose-repressed organisms showed that most of the recovered activity (about 90%) of fumarase, succinate dehydrogenase, aconitase and L-lactate dehydrogenase occurred in fraction P1. Lower percentages of the recovered activity of citrate synthase, 2-oxoglutarate decarboxylase and malate dehydrogenase (56, 47 and 48, respectively) were found in fraction P1 under these conditions. Conversely, a high percentage of the recovered activity of alcohol dehydrogenase and fumarate reductase occurred in fraction FS.

With the exception of malate dehydrogenase (53%), fraction P1 of extracts from anaerobically grown, glucose-derepressed organisms contained less than 50% of the recovered activity of each enzyme. This decrease in recovered enzyme activities in fraction P1 (compared with this fraction from organisms grown under both aerobic conditions) was accompanied by a decrease to 21% of recovered protein. The percentage of recovered activities of several enzymes in fraction P1 was much higher in extracts from anaerobically grown, glucose-repressed organisms (malate dehydrogenase 89%, aconitase and NADP-linked isocitrate dehydrogenase both 70%, and L-lactate dehydrogenase 58%). Citrate synthase and alcohol dehydrogenase activities were almost entirely recovered in fraction FS.

DISCUSSION

The comparative values of specific activities (in parentheses, Table 1) show a distinct trend under the four conditions of growth. There is a decrease in activity of enzymes of the tricarboxylic acid cycle with glucose repression (see also Polakis & Bartley, 1965; Duntze *et al.*, 1969; Perlman & Mahler, 1974) and a further reduction in activities under anaerobic conditions. 2-Oxoglutarate decarboxylase activity was not found under anaerobic conditions. Machado *et al.* (1975) found low activities of 2-oxoglutarate dehydrogenase under 'hypoxic' conditions and suggested that the tricarboxylic acid cycle is modified to provide a branched, non-cyclic pathway, first reported in *Escherichia coli* (Amarasingham & Davis, 1965). Fumarate reductase is present, although with low activity, under anaerobic conditions.

A much higher activity of alcohol dehydrogenase was present under aerobic derepressed conditions, and a higher percentage of the activity was located in fraction P1, compared with organisms grown anaerobically. Ball *et al.* (1975) also found an increase in alcohol dehydrogenase activity during aerobic adaptation (with derepressing amounts of glucose) and suggested that repression of cytoplasmic alcohol dehydrogenase followed by an induction of mitochondrial alcohol dehydrogenase occurred.

The lower recovered activities of some of the enzymes of the tricarboxylic acid cycle in fraction P1 of aerobically grown, glucose-repressed organisms, and their even lower activities under anaerobic derepressed conditions, can perhaps be explained by the matrix enzymes leaching out of the more fragile promitochondria (Criddle & Schatz, 1969; Dansky, 1976) during the preparation of the fractions. However, the fact that integral proteins such as 2-oxoglutarate dehydrogenase and succinate dehydrogenase [which is bound to the mitochondrial membrane (Singer & Nicholson, 1972)] are only partly sedimentable is more difficult to explain.

Chapman & Bartley (1968) have shown that adaptation of yeast to both conditions of glucose repression and anaerobiosis lowers the activities of enzymes of the tricarboxylic acid cycle, but concluded that the glucose concentration is the major effector of this. How-

ever, the main conclusion to be drawn from our results is that glucose repression and anaerobiosis show the same qualitative effects on the enzymes studied, but quantitatively the effects of anaerobiosis are much more marked.

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