A MUTANT OF *SACCHAROMYCES CEREVISIAE* LACKING α-KETOGLUTARATE DEHYDROGENASE ACTIVITY

J. ŠUBÍK, J. KOLAROV and T.M. LACHOWICZ
Department of Microbial Genetics, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wróclaw, Poland

Received 2 August 1972

1. Introduction

A number of yeast mutants with defects in the structure and function of mitochondria has been described. The inability to grow on nonfermentable substrates is the common phenotypic property of these mutants. Respiration-deficient mutants, cytoplasmic or nuclear, lacking one or more cytochromes [1–5] as well as a mutant with normal respiratory activity but with a defect in oxidative phosphorylation [6–8] belong to this group of yeast mutants.

Mutants with lesion in the tricarboxylic acid cycle represent another category of yeast mutants unable to grow on the nonfermentable carbon sources. Mutants glt_1 lacking the aconitate hydratase activity are capable of respiring with various substrates which are oxidized to the level of acetate but are incapable of degrading two carbon substrates to CO_2 via the tricarboxylic acid cycle. These mutants have, besides a requirement for glutamate, the "petite morphology" [9, 10]. In addition, mutant glt_2 requires lysine and most of its segregants were found to be ρ^- [11].

This paper deals with the biochemical characterization of another mutant of *Saccharomyces cerevisiae* with a defect in the tricarboxylic acid cycle, which lacks the α-ketoglutarate dehydrogenase activity.

Abbrevations: TMPD = tetramethyl-*p*-phenylendiamine; CCCP = carbonylcyanide *m*-chlorophenylhydrazone.

2. Materials and methods

The mutant used, designated *Saccharomyces cerevisiae* S 20 X 20, is an adenine requiring diploid strain, homozygous for the affected nuclear gene, prepared by Lachowicz et al. [12] from wild-type strain D 225-5A by nitrite mutagenesis. For comparison, diploid wild-type strain DTXII was employed.

The methods of culture and of isolation of mitochondria as well as some enzyme determinations and analytical techniques were the same as employed by Kováč and Hrušovská [8]. The α-ketoglutarate was assayed enzymatically [13], after deproteinization of the samples with $HClO_4$ and neutralization with KOH. Enzymes used in the assay were obtained from Boehringer, Mannheim.

The α-ketoglutarate dehydrogenase activity was determined by procedures of Hager and Kornberg [14] and of Garland [15]. NAD^+ reduction was followed spectrophotometrically at 340 nm by a split-beam dual-wavelength spectrophotometer (Hitachi Perkin-Elmer Model 356).

Oxidative decarboxylation of α-ketoglutarate was determined manometrically with ferricyanide as electron acceptor [14].

The source of chemicals used is indicated in parentheses: ATP (Serva), antimycin A (Sigma), coenzyme A (Calbiochem), hexokinase (Sigma, Type III), α-ketoglutaric acid (Sigma), NAD^+ and NADH (WEB Arneimittelwerk Dresden), thiamine pyrophosphate-HCl (Serva), tetramethyl-*p*-phenylendiamine (Serva). Carbonylcyanide-*m*-chlorophenyl hydrazone [16] was synthesized in the laboratory by Dr. M. Greksák.

Table 1
Growth of mutant S 20 × 20 in comparison with the wild-type yeast.

Strain	Number of cells/ml		Growth on			
	Glucose (0.5%)	Ethanol (2%)	glycerol,	lactate,	pyruvate,	acetate
DT XII	256×10^6	340×10^6	+	+	+	+
S 20 × 20	55×10^6	18×10^6	–	–	–	–
S 20 × 20 $\rho-$	40×10^6	–	–	–	–	–

Cells were grown in a semi-synthetic medium containing in 1 l: 1 g KH_2PO_4, 0.7 g $MgSO_4 \cdot 7\ H_2O$, 0.4 g $CaCl_2$, 0.5 g NaCl, 1.2 g $(NH_4)_2SO_4$, 5 mg $FeCl_3$, 5 g peptone, 5 g yeast extract and substrate in concentration as indicated in the table. Number of cells was determined when the cultures reached the stationary phase (after 24–48 hr). Mutant S 20 × 20 $\rho-$ was prepared by acriflavine (10 μg/ml) mutagenesis. – Means no growth.

Table 2
Respiratory activity of the mutant cells.

Substrate	Q_{O_2} (μl O_2/mg/hr)
Endogenous	2.1
25 mM Glucose	29.3
25 mM Glucose + 10 μM CCCP	48.1
25 mM Ethanol	31.2
25 mM Ethanol + 10 μM CCCP	67.2

Polarographic vessel contained in 2 ml: 50 mM potassium glutarate, 10 mM potassium phosphate, 100 mM KCl and mutant cells (1.5 mg dry weight) grown 24 hr in semi-synthetic medium with 0.5% glucose. Final pH 4.3. Temperature 30°.

Table 3
The extent of oxidation of various substrates by mutant S 20 × 20.

Substrate	Oxygen uptake (moles per mole substrate)		
	Theoretical		Observed
	For complete oxidation	For oxidation to the level of acetate	
Glucose	6.0	2.0	1.08
Ethanol	3.0	1.0	0.97
Acetaldehyde	2.5	0.5	0.34

Stoichiometry of oxygen uptake was determined by the standard direct Warburg method. The main compartment of the Warburg flasks contained in 2.0 ml: 0.2 M citrate phosphate, 6.28 mg (dry weight) of mutant cells and substrate, final pH 4.3. The central well contained 0.2 ml 2 M KOH and a piece of fluted paper. The measurements commenced after 7 min of thermal equilibration at 30° by addition of substrate from the side arm. Amounts of substrate are indicated in parentheses: glucose (2 resp. 4 μmoles), ethanol (5 resp. 10 μmoles) and acetaldehyde (5 resp. 10 μmoles).

3. Results and discussion

Mutants S 20 × 20 was grown under aerobic conditions with low growth yields on solid and liquid media with glucose or ethanol as substrate. No growth was observed when glycerol, D- or L-lactates, pyruvate or acetate were used as carbon source (table 1). The mutant could not utilize either the carbon or the energy sources present in yeast extract and peptone. Contrary to the oxidative phosphorylation deficient mutant [17], the superimposition of cytoplasmic mutation to respiratory deficiency over the nuclear mutation in mutant S 20 × 20 did not result in the arrestation of cell division.

Mutant colonies formed on agar plates with a glucose medium did not stain with tetrazolium. However, the mutant cells possessed all the cytochromes. Relative ratios of cytochromes of the wild-type and mutant cells (DT XII/S 20 × 20) were: 2.86, 1.72 and 2.62 for cytochromes *a*, *b* and *c*, respectively. The respiratory activity of mutant cells was lower than that of wild-type cells and, as indicated in table 2, this activity was *in vivo* under control of phosphorylation reactions. Contrary to the low rate of glucose oxidation, the rate of glucose fermentation of the

Table 4
Oxidation rates and phosphorylation efficiencies of mitochondria isolated from mutant S 20 × 20.

Substrate	Oxidation rate (ng atoms O/min/ mg protein)	P/O
None	20.8	*
10 mM α-Ketoglutarate	18.0	*
1 mM NADH	80.3	1.53
5 mM Citrate	66.3	1.47
5 mM Pyruvate + 1 mM Malate	61.2	1.69
5 mM Ethanol	49.0	1.63
10 mM α-Glycerol-phosphate	40.0	1.64
20 mM Succinate	48.0	1.53
10 mM D (−) Lactate	72.0	*
10 mM L (+) Lactate	41.2	*
7.5 mM Ascorbate + 0.1 mM TMPD	110.0	0.71

* not determined.

The polarographic vessel contained in 2 ml: 0.6 M mannitol, 20 mM KCl, 10 mM Tris maleate, 10 mM potassium phosphate labelled with ^{32}P (45–60 cpm per nmole P), 1.5 mM $MgCl_2$, 0.5 mM ADP, 25 mM glucose, 0.5 mg of hexokinase, 0.25% bovine serum albumin, mitochondrial protein (2.4 and 2.7 mg) and substrate in concentration as indicated in the table. Final pH 6.4. The reaction was started by the addition of mitochondria and terminated, after oxygen concentration decreased to about 50%, by adding 0.1 ml 100% w/v trichloroacetic acid. Inorganic phosphate was extracted as the phosphomolybdate complex and the radioactivity of the non-extractable residue determined by counting by a thin-window Geiger-Müller tube. The results are means of 2 experiments.

mutant cells was comparable to that of wild-type yeast, resulting in a high R_Q (5.6). The Pasteur effect was operating in mutant cells ($Q^{air}_{CO_2}$ = 166.5; $Q^{N_2}_{CO_2}$ = 216.0).

When the stoichiometry of O_2 uptake was determined with limiting amounts of ethanol, acetaldehyde and glucose, respectively, it has been found that the oxidation of these substrates proceeded within mutant cells only to the level of acetate (table 3).

Mitochondria isolated from mutant cells grown on glucose as carbon source could oxidize the following substrates: NADH, citrate, pyruvate and malate, ethanol, α-glycerol-phosphate, succinate, D (−) and L (+) lactates and ascorbate plus TMPD. However, they were not able to oxidize α-ketoglutarate (table 4).

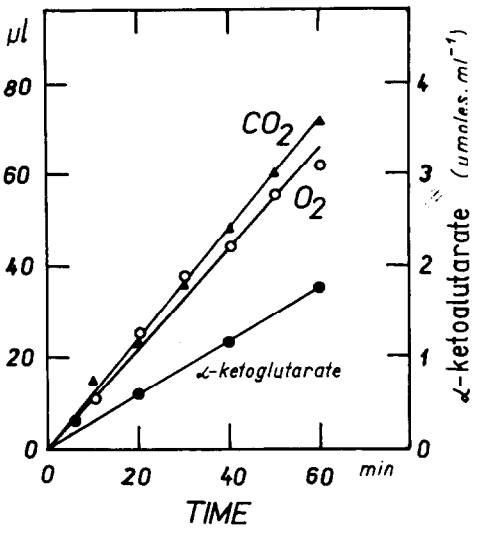

Fig. 1. α-Ketoglutarate accumulation by mutant mitochondria oxidizing citrate. The main compartment of the Warburg flasks contained in 2 ml: 0.6 M mannitol, 20 mM KCl, 1.5 mM EDTA, 10 mM Tris maleate, 10 mM potassium phosphate and mitochondria (1.7 mg protein), final pH 6.4. For the measurement of oxygen uptake the central well contained 0.2 ml 2 M KOH and a piece of fluted paper. The measurements commenced, after 7 min of thermal equilibration at 30°, by addition of 20 mM citrate from the side arm. At intervals indicated in the figure the reaction in Warburg flasks was terminated by adding 0.1 ml 5 N $HClO_4$ and after neutralization by KOH, α-ketoglutarate was determined enzymatically [13].

Phosphorylation efficiency of isolated mutant mitochondria was the same as found previously with the wild-type mitochondria [18].

The lack of α-ketoglutarate dehydrogenase activity was also proved by determination of metabolic quotients of mutant mitochondria with α-ketoglutarate as substrate in a long-term manometric experiment. The rate of oxygen uptake and the rate of CO_2 production was not different from endogenous activities. Essentially the same results were obtained by following the reduction of NAD^+ spectrophotometrically and by manometric determination of the release of CO_2 in assay system containing exogenously supplied thiamine pyrophosphate, NAD^+, cysteine, Mg^{2+} and antimycin A. In the presence of ferricyanide and thiamine pyrophosphate no α-ketoglutarate decarboxylase activity was demonstrated in mutant mitochondria.

The oxidation of citrate by mutant mitochondria

was accompanied by accumulation of α-ketoglutarate (fig. 1).

The fact that the mutant carrying the deficiency in α-ketoglutarate dehydrogenase is a nuclear one indicates that the synthesis of α-ketoglutarate dehydrogenase is coded for by the nuclear DNA. The same seems to hold for the other enzymes of the tricarboxylic acid cycle since these enzymes had been previously demonstrated in mitochondria of the cytoplasmic respiration deficient mutants [19–22].

Acknowledgement

The authors wish to thank Dr. L. Kováč for valuable discussions.

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