Involvement of mitochondria in the assimilatory metabolism of anaerobic *Saccharomyces cerevisiae* cultures

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The possible physiological role of mitochondria in anaerobically grown *Saccharomyces cerevisiae* was investigated via enzyme localization and inhibitor studies. Almost all of the activity of citrate synthase (EC 4.1.3.7) was recovered in the mitochondrial fraction after differential centrifugation of spheroplast lysates. The enzyme exhibited a high degree of latency which was demonstrated by sonication of the mitochondrial fractions. Since citrate synthase is an important enzyme in anabolic reactions, a consequence of this localization is the requirement for transport of metabolites across the mitochondrial membranes. Such transport is likely to require energy which, as a result of anaerobiosis, cannot be supplied by respiration. It was therefore investigated whether ATP translocation into the mitochondria by an ADP/ATP translocase might be involved in anaerobic mitochondrial energy metabolism. It was shown that addition of the ADP/ATP translocase inhibitor bongkreikic acid to anaerobic cultures indeed inhibited growth, although only partially. It is concluded that mitochondria of *S. cerevisiae* fulfil a vital role in anaerobic sugar metabolism.

Keywords: *Saccharomyces cerevisiae*, (pro)-mitochondria, ADP/ATP-translocator, anaerobic continuous culture, citrate synthase

INTRODUCTION

The existence of mitochondria in anaerobically grown yeast cells has been disputed for a long period in the literature. Early reports (Wallace & Linnane, 1964; Linnane, 1965; Chapman & Bartley, 1968) claim that such cells are completely devoid of mitochondria and that adaptation to aerobic conditions involves *de novo* synthesis of these organelles. These reports were based on electron microscopic studies. Later it was shown that inadequate staining or fractionation procedures had led to these conclusions (Damsky *et al.*, 1969; Cartledge *et al.*, 1972; Cartledge & Lloyd, 1972, 1973; Jenkins *et al.*, 1984). Since the membranous structures found under anaerobic conditions were quite different from the well-known aerobic mitochondria, the term 'pro-mitochondria' was proposed to emphasize the relationship to aerobic organ-

elles, as well as the differences between these organelles and fully functional mitochondria.

In all reports on the disappearance of mitochondria under anaerobic conditions, a discussion of the physiological consequences was restricted to the respiratory system, i.e. cytochromes and oxidases and the F₁-ATPase (Groot *et al.*, 1971). However, it is well-established that most enzymes of the citric acid cycle as well as some enzymes for sterol biosynthesis (Shimizu *et al.*, 1973) and amino acid synthesis (Ryan & Kohlhaw, 1974; Jauniaux *et al.*, 1978) are localized inside the mitochondria and hence disappearance of the organelle could be lethal during growth under anaerobic conditions. Evidence for the indispensability of mitochondria is the fact that yeast proteins mediating protein import into mitochondria are essential for cell viability (Jensen & Yaffe, 1988; Baker & Schatz, 1991; Stuart *et al.*, 1994).

Studies on the localization of mitochondrial enzymes under anaerobic conditions are complicated due to the very fragile structure of these mitochondria (Criddle & Schatz, 1969; Damsky *et al.*, 1969). Hence, in this study,

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great attention has been given to the isolation of intact mitochondria. Citrate synthase activity was taken as a marker enzyme in view of its key role in the TCA-cycle and since earlier reports showed that its activity under anaerobic conditions was not found in a particulate fraction (Wales *et al.*, 1980). Under anaerobic conditions, all ATP is produced in the cytoplasm during glycolysis. If pro-mitochondria also fulfil a role in biosynthesis of cell material during anaerobic growth, energy must be supplied to the organelle both for driving transport processes across its membranes and for the energy-demanding reactions within (Groot *et al.*, 1971). Import of ATP into mitochondria may occur via an ADP/ATP translocator in exchange for ADP. The possible physiological function of yeast mitochondria under strictly anaerobic conditions was therefore also studied by testing the sensitivity of anaerobic cells to bongkreikic acid, a well-known inhibitor of the ADP/ATP translocator.

METHODS

Micro-organism. *Saccharomyces cerevisiae* CBS 8066 was obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and maintained on malt agar slopes.

Media. Mineral medium, supplemented with vitamins and trace elements, was prepared according to Verduyn *et al.* (1992). Glucose was added as sole source of carbon and energy, to a concentration of 30 g l⁻¹ unless stated otherwise. Ergosterol and Tween 80, supplements required for anaerobic growth of *S. cerevisiae* (Andreassen & Stier, 1953, 1954) were dissolved in pure ethanol and sterilized by heating the solution for 10 min at 100 °C. These components were added to the medium to a concentration of 6 mg l⁻¹ and 660 mg l⁻¹, respectively. To prevent foaming, 50 µl silicone antifoam l⁻¹ was added to the reservoir medium.

Growth conditions. The yeast was grown in continuous cultures, using a 2 litre laboratory fermenter of the type described by Harder *et al.* (1974), with a 1 litre working volume. The pH was controlled at 5.0 by automatic titration of 1 M KOH. The stirrer speed was maintained at 1000 r.p.m. and the cells were grown at 30 °C at a dilution rate of 0.1 h⁻¹ unless stated otherwise.

To maintain anaerobic conditions, the fermenter was flushed with 1 litre min⁻¹ pure nitrogen gas, containing less than 5 p.p.m. oxygen (obtained from Air Products). To minimize diffusion of oxygen, Norprene tubing (Cole-Parmer Instruments) was used. Since dissolved oxygen in the growth medium will significantly contribute to the overall influx of traces of oxygen, the medium was stripped of oxygen using a second fermenter in which it was vigorously flushed with the nitrogen. From this fermenter the medium was pumped directly into the fermentation vessel. The outlet gas passed a cooled condenser (4 °C) and was subsequently led through a glycerol trap to prevent back diffusion of oxygen into the fermenter.

The dissolved-oxygen tension was measured continuously with an autoclavable polarographic (Clark type) oxygen electrode, type Ingold 322 756702/74247, connected to an Ingold O₂ amplifier type 170. As a result of the precautions mentioned above, the actual dissolved oxygen tension in the anaerobic cultures was below 0.005 % air saturation.

For comparative purposes, cells were also grown aerobically in chemostat cultures. All parameters were kept the same, but the nitrogen gas was replaced with air and the glucose concentration in the reservoir was reduced to 5 g l⁻¹.

Analytical methods. Dry weight was determined by filtration of culture samples over weighed polysulfone filters (pore size 0.45 µm, Gelman Sciences). The filter was washed with demineralized water, dried in a microwave (Sharp) for 20 min at medium power and re-weighed.

Cultures were routinely analysed for metabolites. Organic acids were determined by HPLC on a HPX-87H column (300 × 7.8 mm, Bio-Rad) at 30 °C. The column was eluted with 0.005 M H₂SO₄ at a flow rate of 0.6 ml min⁻¹. The detector was a Waters 441 UV-meter at 210 nm, which was coupled to a Waters 741 data module. Glycerol and ethanol were also measured using this column by the use of a refraction index detector which was coupled in line with the UV detector.

Isolation of mitochondria from anaerobic cells. For the isolation of mitochondria, the procedure of Bruinenberg *et al.* (1985) was adopted. To prevent adaptation to aerobic conditions, all buffers and reagents were made anaerobic by flushing with argon and all incubations were carried out under an atmosphere of this gas. When the entrance of oxygen could not be avoided the samples were kept at 0 °C to prevent adaptation to aerobic conditions. Zymolyase [1000 U (g cells)⁻¹] was used for spheroplasting. During incubation small samples were taken and diluted 200-fold in water. The osmotic shock caused lysis of the spheroplasts, as determined by measuring OD₆₆₀ of the solution. Incubation was stopped when the OD was 10 % of the starting value. A crude mitochondrial fraction was obtained from the spheroplast lysates via differential centrifugation as described by Bruinenberg *et al.* (1985). The mitochondrial fractions were stabilized by the addition of 1 mg bovine serum albumin (BSA) ml⁻¹. The fractions P1 and P2 were obtained after centrifugation in an SS34 rotor of a Sorvall RC5B at 10000 r.p.m. at 10 min and 20000 r.p.m. at 20 min, respectively.

When appropriate, the fractions were disrupted by ultrasonic treatment at 4 °C by the use of an MSE sonicator. Full power treatment (30 s, unless indicated otherwise, at 150 W) was alternated with equal periods of cooling in ice-water.

Respiration measurements. Respiration measurements on cells and mitochondria were performed with a polarographic oxygen electrode (Clark type) in a stirred vessel at 30 °C (Biological Oxygen Monitor). Cells were diluted in mineral medium without glucose. After determination of the endogenous respiration glucose was added to a final concentration of 20 mM. The oxygen uptake rate in the presence of glucose was taken as maximum respiratory capacity of the cells. Activity is expressed as µmol O₂ min⁻¹ (g cell protein)⁻¹ and based on a protein content of whole cells of 47 % (Verduyn *et al.*, 1990).

Oxygen consumption of mitochondria was measured in 25 mM potassium phosphate buffer (pH 7.0) containing 5 mM MgCl₂, 0.65 M sorbitol and 0.17 mM ADP using 0.25 mM NADH as substrate. To verify whether the oxygen consumption by whole cells and isolated mitochondria was mediated by cytochrome oxidase, activity was tested by adding cyanide to the solution to a final concentration of 1 mM.

Enzyme assays. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed according to Bruinenberg *et al.* (1983). The activity of NADH dehydrogenase (EC 1.6.99.3) was determined as described by Bruinenberg *et al.* (1985). Citrate synthase was assayed in a 100 mM Tris/HCl buffer (pH 8.0) containing 0.10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 0.05 mM acetyl CoA. After addition of the sample, the reaction was started with oxaloacetate (final concentration 0.25 mM). The appearance of coenzyme A was followed spectrophotometrically at 412 nm ($\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$). Protein determinations in mitochondrial fractions were disturbed by the addition of BSA and therefore not performed.

Fig. 1. Electron micrographs of subcellular fractions from anaerobically grown, glucose-limited *S. cerevisiae* cells (bars, 1 µm). Cells were grown at a dilution rate of 0.10 h⁻¹. (a) P1-fraction, mainly containing pro-mitochondria. (b) P2-fraction, mainly containing membranes and vesicles.

Chemicals. Tween 80 was obtained from Merck. Zymolyase (100000T) was purchased from the Kirin brewery, Japan. Antifoam was obtained from BDH. Bongkreikic acid was a gift of Professor J. A. Duine from our department.

RESULTS

Isolation of mitochondria

S. cerevisiae cells were grown under strictly anaerobic conditions and glucose limitation at a dilution rate of 0.1 h⁻¹. The procedure for the isolation of pro-mitochondria was based on the method of Bruinenberg *et al.* (1985) and van Urk *et al.* (1989), developed for the isolation of mitochondria from aerobically grown cells of *Candida utilis* and *S. cerevisiae*, respectively. The essentials of these procedures are enzymic degradation of the cell wall with Zymolyase in hypertonic medium, followed by dialysis of the spheroplast suspension to lower its osmotic value gently. Spheroplasts are then mechanically disrupted by a few strokes in a Potter–Elvehjem homogenizer. Mitochondrial fractions are subsequently isolated by differential centrifugation. These fractions were named P1, P2 and S according to earlier experiments (Wales *et al.*,

1980; Bruinenberg *et al.*, 1985; van Urk *et al.*, 1989). The P1 fraction contains mostly mitochondria, the P2 fraction contains the remaining membranes and the S fraction is the final supernatant or soluble fraction.

In the method of van Urk *et al.* (1989), pretreatment of the aerobically grown cells with DTT and EDTA was necessary to obtain spheroplasts at a satisfactory rate. DTT, however, interfered with our measurements of citrate synthase activity due to remaining residues after washing. These traces of DTT reacted with the DTNB used in the citrate synthase assay. Further checking showed that pretreatment of cells with DTT, EDTA or the combination of the two did not enhance the rate of spheroplasting of anaerobically grown *S. cerevisiae* with Zymolyase and hence this pretreatment was omitted from the procedure.

When pro-mitochondria of anaerobically grown cells are to be studied, special care has to be taken to prevent adaptation of the cells to aerobic surroundings during the isolation procedures, which were therefore carried out under an atmosphere of argon. Purging argon or nitrogen through the liquid was avoided since high shear forces

resulted in premature lysis of the spheroplasts (results not shown).

When anaerobiosis could not be maintained (e.g. during centrifugation steps), cells were cooled down to 0 °C under argon gas prior to further handling.

Since it has been shown that premature lysis of spheroplasts will lead to damaged mitochondria (Bruinenberg, 1985), we optimized the concentration of the osmotic stabilizer sorbitol. Measurements of the activity of (cytoplasmic) glucose-6-phosphate dehydrogenase released in the supernatant of the incubation mixture showed that 2 M sorbitol was optimal with respect to spheroplast stability and the time required to reduce the sorbitol concentration to 0.65 M by dialysis. The optimum concentration of Zymolyase was found to be 2000 U in 20 ml cell suspension (100–120 mg cells ml⁻¹).

Electron microscopic examination of the fractions obtained after differential centrifugation revealed that the P1-fraction mainly consisted of relatively intact mitochondria. Micrographs of the P2-fraction showed only irregular membranous structures and some vesicles (Fig. 1).

Respiration of mitochondria of anaerobically grown cells

The possible, undesirable, aerobic adaptation during handling of anaerobically grown cells would be reflected in the ability of the cells to consume oxygen. To validate the isolation procedure with respect to this adaptation, cells were incubated under conditions equivalent to those in the isolation procedure, i.e. under argon or kept at 0 °C, and were subsequently checked for the maximal respiratory capacity. Zymolyase and sorbitol were not added in these control experiments. Cells incubated this way for at least 4 h did not acquire a higher respiratory activity. This remained constant at a low value of 10 µmol min⁻¹ (g cell protein)⁻¹. Approximately 50 % of this activity was insensitive towards cyanide, whereas the respiration of aerobically grown cells was blocked completely by this inhibitor (Table 1). No respiration of

Table 1. Maximum respiratory capacity of anaerobically and aerobically grown cells of *S. cerevisiae*, and of mitochondrial preparations of these cultures, measured in the presence and absence of 1 mM KCN

Growth conditions	$Q_{O_2}^{max}$ [µmol O ₂ min ⁻¹ (g protein) ⁻¹]			
	Cells		Mitochondria*	
	-CN	+CN	-CN	+CN
Aerobic	150	0	360†	NT
Anaerobic	10	5	ND	ND

ND, Not detectable; NT, not tested.

* In mitochondrial fractions respiration was measured with NADH instead of glucose as a substrate, in the presence of ADP.

† Value obtained from van Urk *et al.* (1989).

Table 2. Subcellular localization of citrate synthase and glucose-6-phosphate dehydrogenase in anaerobically grown *S. cerevisiae*

Distributions are expressed as percentage of recovered activity. This recovery was based upon the total activity in the cell free extract (fraction T), being 3 and 20 µmol min⁻¹ for citrate synthase and glucose-6-phosphate dehydrogenase, respectively. Data ± SD are based on four independent isolations of mitochondria. P1, mitochondrial pellet fraction; P2, rest of particulate material.

Fraction	Citrate synthase	Glucose-6-phosphate dehydrogenase
P1	79.0 ± 3.3	0.8 ± 0.6
P2	10.3 ± 4.1	1.0 ± 1.0
Total particulate	89.2 ± 7.3	1.7 ± 1.2
Soluble	10.8 ± 7.3	98.3 ± 1.2
Recovery	87.3 ± 15.6	88.4 ± 8.4

Fig. 2. The release of citrate synthase activity following ultrasonic treatment of the mitochondria isolated from anaerobically grown *S. cerevisiae* cells. Results are presented as percentage of the maximal activity after sonication.

NADH was found with the mitochondria of anaerobically grown cells, indicating that no adaptation of cells to aerobic conditions had occurred during the isolation procedure.

Subcellular localization of citrate synthase

Glucose-6-phosphate dehydrogenase is a well-known cytosolic marker enzyme. As can be concluded from the distribution of the activity of this enzyme over the fractions examined (Table 2), the mitochondrial fractions isolated were hardly contaminated with cytosolic enzymes.

Citrate synthase in the mitochondrial fraction P1 exhibited latency. Only after sonication was the maximum activity reached. This is demonstrated in Fig. 2, in which the activity of the enzyme in the P1-fraction is depicted as a

Fig. 3. Washout curve of an anaerobic continuous culture of *S. cerevisiae* [dilution rate = 0.2 h^{-1} , S_R (concentration of growth-limiting nutrient in the reservoir) = $27.5 \text{ g glucose l}^{-1}$] after addition of bongkreikic acid ($5 \mu\text{M}$) as a specific inhibitor of the ADP/ATP-carrier of the mitochondrial membrane at $t = 0 \text{ h}$ (●). Dashed curve refers to washout kinetics when $\mu = 0$. The solid line drawn through the data points is the result of a curve fit procedure assuming that the organism is washed out according to $x = x_0 \cdot e^{(\mu-D)t}$ where x = biomass, μ = specific growth rate and D = dilution rate. The data show that anaerobic growth in the presence of bongkreikic acid still proceeds with $\mu = 0.1 \text{ h}^{-1}$ for at least five generations.

function of the sonication time. The sonication procedure hardly damaged the enzyme, since prolonged exposure time did not significantly reduce the activity. The citrate synthase activity in the P1-fraction increased approximately fivefold after the sonication procedure, whereas the small amount of enzyme in the P2-fraction did not exhibit significant latency (less than 10 %).

Effect of bongkreikic acid on anaerobic cultures

Bongkreikic acid is a well-known specific inhibitor of the mitochondrial ADP/ATP-translocase of mammalian cells (beef heart and rat liver), both in intact mitochondria (Erdelt *et al.*, 1972) and in a reconstituted system (Krämer & Klingenberg, 1979). The same was demonstrated for the yeast mitochondrial translocase by following transport activity in proteoliposomes containing reconstituted, purified translocase of aerobically grown *S. cerevisiae* (haploid strain D 273-10B α) cells with ^{14}C -labelled ATP (Knirsch *et al.*, 1989).

To investigate the *in vivo* role of mitochondrial translocase during anaerobic growth, inhibition of growth was followed by recording the washout kinetics of steady state cultures after the addition of bongkreikic acid. This translocase inhibitor was added at zero time to both the medium reservoir and the culture vessel to a concentration of $5 \mu\text{M}$. In this way the concentration of bongkreikic acid remained constant during the washout experiment. From the washout profile after the addition of the inhibitor it can be calculated that the growth rate decreased by 50 % from 0.2 to 0.1 h^{-1} (Fig. 3). Increasing the concentration of bongkreikic acid from 5 to $50 \mu\text{M}$ did not enhance the inhibitory effect (data not shown).

It has been reported (Šubík, 1972) that addition of bongkreikic acid to growing cells of *S. cerevisiae* under anaerobic conditions induced the formation of respiratory-deficient mutants. Whether a similar phenomenon would occur in our continuous culture experiment was checked by plating the samples from the washout culture on appropriate agar media, but no petite colonies were found.

DISCUSSION

Anabolic functions of mitochondria during anaerobiosis

Citrate synthase is a key enzyme of the TCA-cycle (Lowenstein, 1967; Walsh & Koshland, 1985) catalysing the condensation of oxaloacetate and acetyl CoA to produce citrate. The TCA cycle is important under aerobic conditions, as it generates reduced coenzymes to supply the electron transport chain with substrates. The second role of the cycle is to provide the cell with anabolic precursors, such as 2-oxoglutarate, the precursor for the glutamate family of amino acids (Fig. 4). Thus, although under anaerobic conditions the first role is of no significance, the latter anabolic function is still of vital importance.

The localization of several TCA cycle enzymes under anaerobiosis has been studied for *Saccharomyces carlsbergensis* by Wales *et al.* (1980). They found that citrate synthase was almost entirely recovered in the soluble fraction, whereas with aerobically grown cells only 6 % of

Fig. 4. Vectorial processes in mitochondria during anaerobic growth, showing the coupling of the intra- and extramitochondrial nucleotide pools by the ADP/ATP-translocase in the inner mitochondrial membrane. Note that ATPase, when functional in anaerobic mitochondria, would build up a proton motive force by consumption of ATP, instead of dissipating the proton motive force for synthesis of ATP as occurs in aerobic cells. The proton symport (antiport) symbolizes energy-dependent transport processes across the mitochondrial membrane, for example of the metabolites shown, pyruvate, oxaloacetate (OAA), (iso)-citrate, etc. CS: citrate synthase.

this activity was found in the supernatant. The low recovery of activity in the mitochondrial fraction of anaerobically grown cells was explained by leakage of enzyme from the more fragile (Criddle & Schatz, 1969; Damsky, 1969) pro-mitochondria. Therefore, in this study a very gentle procedure for the isolation of the mitochondria was used.

The results, shown in Table 2 and Fig. 2, clearly show that with this procedure most of the enzyme is particulate, also under anaerobic conditions. Isoenzymes, functioning in different locations in the cell, always complicate localization studies. When, for example, yeast cells are grown on ethanol or acetate, the glyoxylate cycle is active. It has been shown that the enzymes of this route are localized in the peroxisomes. Under these conditions two different genes encoding citrate synthase are active, *CIT1* encoding the mitochondrial enzyme and *CIT2* the peroxisomal protein (Lewin *et al.*, 1990). In cells grown on glucose under anaerobic conditions, however, peroxisome synthesis is repressed (Veenhuis & Harder, 1987) and peroxisomal enzymes are not synthesized (Rogers & Stewart, 1973).

Therefore, it seems unlikely that the particulate localization of citrate synthase is partially or totally due to contamination of the mitochondrial fraction with peroxisomes. Furthermore, since it is well-known that peroxisomes are very fragile, the observed latency of citrate synthase (Fig. 2) is also not in accordance with a peroxisomal localization of the enzyme.

ATP requirements for anabolic functions of mitochondria

The majority of the mitochondrial enzymes are encoded on the nuclear DNA and citrate synthase is no exception in this respect (Schatz & Mason, 1974). Import of these enzymes or precursor proteins requires not only a membrane potential but also hydrolysis of matrix ATP (Eilers *et al.*, 1987; Pfanner *et al.*, 1987; Stuart *et al.*, 1994). The subsequent refolding of imported proteins by mitochondrial chaperones (e.g. hsp60) requires ATP within the mitochondrial matrix (Baker & Schatz, 1991; Stuart *et al.*, 1994). Anabolic reactions also will depend on the supply of energy, whether this is direct as in phosphorylation reactions, or indirect as active transport processes.

Whereas under aerobic conditions ATP is produced within the mitochondria by respiration, under anaerobic conditions the energy requirement for anabolic processes necessarily implies extramitochondrial sources of energy, e.g. import of ATP into the mitochondrion.

The only possible route for mitochondrial uptake of ATP is mediated by the ADP/ATP translocator (see Fig. 4). This carrier is the most abundant protein in the mitochondrial membrane (Klingenberg, 1985) and under respiratory conditions it will export ATP and import ADP at high rates. Under these conditions, the ATP/ADP ratio in the cytosol is considerably higher than in the mitochondrial matrix. Transport therefore occurs against

the chemical gradient of the substrates ATP and ADP. The driving force for this process is the membrane potential due to the fact that the exchange of ATP^{4-} against ADP^{3-} includes charge movement across the membrane. Thus, the direction of the fluxes and the relative contribution of the transport modes carrying ADP and ATP, respectively, depend on the energy state of the membrane (Krämer & Klingenberg, 1980).

Since heterologous exchange (ATP^{4-} , ADP^{3-}) is electrogenic, the mitochondrial import of ATP in anaerobically grown cells creates a membrane potential in the physiological direction, i.e. positive outside (see Fig. 4). On the other hand, once in the matrix, ATP may be hydrolysed by the ATPase, leading to extrusion of protons thereby generating an electrochemical proton gradient. It has to be taken into account, however, that this would also decrease further uptake of ATP by the electrogenic heterologous exchange mode of the ADP/ATP translocator to some extent.

A highly specific and effective inhibitor of the ADP/ATP-translocator is bongkreikic acid. It has been shown that aerobic growth of respiration-deficient mutants of *S. cerevisiae* could be arrested by addition of this drug, indicating the vital importance of mitochondrial import of ATP for anabolic purposes (Šubík *et al.*, 1972; Gbelská *et al.*, 1983). Since anaerobic conditions of wild-type *S. cerevisiae* will lead to a similar mitochondrial energy demand as in the experiments of Šubík *et al.* (1972) and Gbelská *et al.* (1983), it was expected that bongkreikic acid would be as effective in blocking growth under these conditions. Our results show that anaerobic growth was indeed inhibited, but the effect was not complete. Although our results seem contradictory to published studies on the effect of bongkreikic acid on the ADP/ATP-translocator in respiratory-deficient mutants (Šubík *et al.*, 1972; Gbelská *et al.*, 1983), it should be borne in mind that so far no studies have been performed on the effect of this inhibitor on cells that were grown under strict anaerobic conditions. In this respect it is relevant that different genes (*AAC1*, *AAC2* and *AAC3*) encode ADP/ATP-translocator in *S. cerevisiae*. *AAC1* and *AAC2* are expressed under aerobic conditions whereas *AAC3* is specifically expressed under anaerobic conditions (Gawaz *et al.*, 1990; Kolarov *et al.*, 1990). We therefore hypothesize that this third gene product is only partly inhibited by the drug and thus supports anaerobic growth in its presence.

In conclusion, the present study has provided experimental support for the contention that the mitochondria play a vital role in the anabolic metabolism of anaerobically growing cells. The term 'non-functional mitochondria', often used in the literature in the sense of 'non-respiring mitochondria', should therefore cease to be used.

ACKNOWLEDGEMENTS

These investigations were supported by the Foundation for Biological Research (BION), which is subsidized by the Netherlands Organization for Scientific Research (NWO). We

thank Professor Dr J. G. Kuenen and Dr J. T. Pronk for a critical reading of the manuscript.

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Received 7 June 1994; revised 18 July 1994; accepted 22 July 1994.