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Promitochondria of Anaerobically Grown Yeast, V. Energy Transfer in the Absence of an Electron Transfer Chain

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ABSTRACT **Promitochondria of anaerobically grown *Saccharomyces cerevisiae* lack cytochromes aa_3, b, c_1, and c, as well as ubiquinone, yet catalyze a P_i-ATP exchange reaction that is sensitive to uncouplers, rutamycin, and atractyloside. The promitochondrial P_i-ATP exchange reaction is abolished by the cytoplasmic "petite" mutation, as well as by growth of the cells in the presence of erythromycin, which indicates a role of mitochondrial protein synthesis in the assembly of the energy transfer system. These observations demonstrate that mitochondrial energy transfer can occur in the absence of a respiratory chain.**

Anaerobically grown *Saccharomyces cerevisiae* cells contain incomplete mitochondria that lack a respiratory chain (1, 2). These "promitochondria" are precursors of the functional mitochondria that are formed upon adaptation of the cells to oxygen (3). Since promitochondria retain an oligomycin-sensitive mitochondrial ATPase (F_1) (compare ref. 1), it appeared possible that they still exhibit a mitochondrial energy transfer system. Such a system should reveal itself by a P_i–ATP exchange reaction that is sensitive to the usual inhibitors and uncouplers of oxidative phosphorylation.

We now report that isolated promitochondria from wild type yeast indeed catalyze such a P_i–ATP exchange reaction. This result sheds new light on the mechanism of oxidative phosphorylation and on the function of promitochondria within the anaerobic yeast cell.

MATERIALS AND METHODS

Yeast strains

The following strains of *S. cerevisiae* were used: (*a*) DT-XII (P_ρ^+ diploid) and the corresponding cytoplasmic "petite" mutant DT-XIIa (compare ref. 4); (*b*) D 273-10B (αP_ρ^+) (compare ref. 1); (*c*) D 587-4B ([ERY] αP_ρ^+, his 1) and the corresponding extrachromosomally inherited erythromycin-resistant strain D 587-4B-1 ([ery] αP_ρ^+, his 1). The cells were grown anaerobically to the early stationary phase on 0.3% glucose–Tween 80–ergosterol (1). Protoplasts and promitochondria were prepared in a manner to be described elsewhere, except that the preparation of the protoplasts was carried out in the presence of 40 μg of cycloheximide/ml in order to prevent adaptation of the cells to oxygen (5). Mitochondria were prepared from protoplasts of the aerobically grown (9) yeast cells.

Abbreviation: ANS, 8-amino-naphthalene-1-sulfonate.

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P_i-ATP exchange reaction

Mitochondria or promitochondria (0.2–1.0 mg of protein) were incubated for 5 min at 30°C in 0.5 ml of medium containing 10 mM ATP, 25 mM sodium [^{32}P]phosphate, pH 7.0 (10–20 cpm/nmol), 0.5 mg/ml bovine serum albumin, 0.2 mM NaCN, and 300 mM sucrose. The reaction was terminated by the addition of 0.5 ml of 10% trichloroacetic acid, and the incorporation of ^{32}P into ATP was determined as described before (6). The P_i–ATP exchange reaction in both mitochondria and promitochondria has a pH optimum at pH 7.0. The rate of the reaction is linear with time up to 25 min and with respect to protein concentration up to 4 mg/ml. Rutamycin, valinomycin, and the uncoupler 1799 (see ref. 7) were added as methanolic solutions.

ANS fluorescence

Changes in the fluorescence of 8-amino-naphthalene-1-sulfonate (ANS) were measured on an Eppendorf fluorimeter with the wavelength combination of 313 *plus* 366 nm as exciting light; the emitted light was measured after passing it through a 400–3000 nm filter. The reaction medium (final volume 3 ml) contained 330 mM sucrose, 25 mM sodium phosphate buffer (pH 7.0), 30 μM ANS, and 1.7 mg of promitochondrial protein.

Determination of ubiquinone

Yeast (pro)mitochondria were extracted as described by Kröger and Klingenberg (8). Difference spectra (volume 1 ml), before and after reduction with borohydride, were taken with a Cary spectrophotometer, equipped with a 0–0.1 slide-wire.

Miscellaneous procedures

Published procedures were employed for measuring protein (1), cell growth (9), cyanide-sensitive NADH oxidase (1), and ATPase (10). Cytochrome oxidase was measured according to Wharton and Tzagoloff (11). A correction was made for any changes in the optical density in the presence of 1 mM NaCN.

RESULTS

Properties of the promitochondrial P_i–ATP exchange reaction

Promitochondria catalyze a P_i–ATP exchange reaction at a rate of about 25% of that of mitochondria (Table 1). Since the promitochondrial exchange reaction is completely in-

hibited by the uncoupler 1799 as well as by rutamycin, it obviously represents a part of a functional energy transfer system (12). The exchange reaction is also fully sensitive to atractyloside as well as to valinomycin *plus* K^+. This indicates that promitochondria possess an adenine nucleotide translocator as well as a K^+ "pump" (compare ref. 13) and the same sidedness as mitochondria from aerobic yeast and from mammalian sources. Although CN^- inhibits incorporation of $^{32}P_i$ into ATP in aerobic mitochondria by about 25%, it has no effect on the exchange reaction in promitochondria. This inhibition probably reflects synthesis of ATP coupled to oxidation of endogenous substrate (6). This synthesis is obviously absent in the promitochondria, in which cytochrome *c* oxidase activity is only 0.6% of the mitochondrial control and cyanide-sensitive NADH oxidase is not detectable. Closely similar results were obtained with mitochondria and promitochondria prepared from the haploid yeast strain D 273-10B.

Involvement of ubiquinone in the P_i–ATP exchange reaction

It has been implied that electron carriers like ubiquinone may play an important role in the P_i–ATP exchange reaction by acting as an electron shuttle (Compare refs. 12, 14). Although it has already been reported that ubiquinone is absent from anaerobically grown yeast cells (15), we decided to extend this finding to isolated promitochondria. In Fig. 1 the reduced *minus* oxidized difference spectrum of an extract of mitochondria and promitochondria is shown. The difference spectrum of the extract from aerobic yeast mitochondria is typical of ubiquinone with an isosbestic point at 290 nm and a minimum at 272 nm (16). Our mitochondrial preparations contained about 4.5 nmol of ubiquinone/mg protein, in agreement with earlier established values (17). In contrast, extracts from promitochondria did not contain detectable ubiquinone. This observation excludes a requirement for ubiquinone in the P_i–ATP exchange reaction and makes the involvement of a phosphorylated ubiquinone compound in ATP synthesis unlikely.

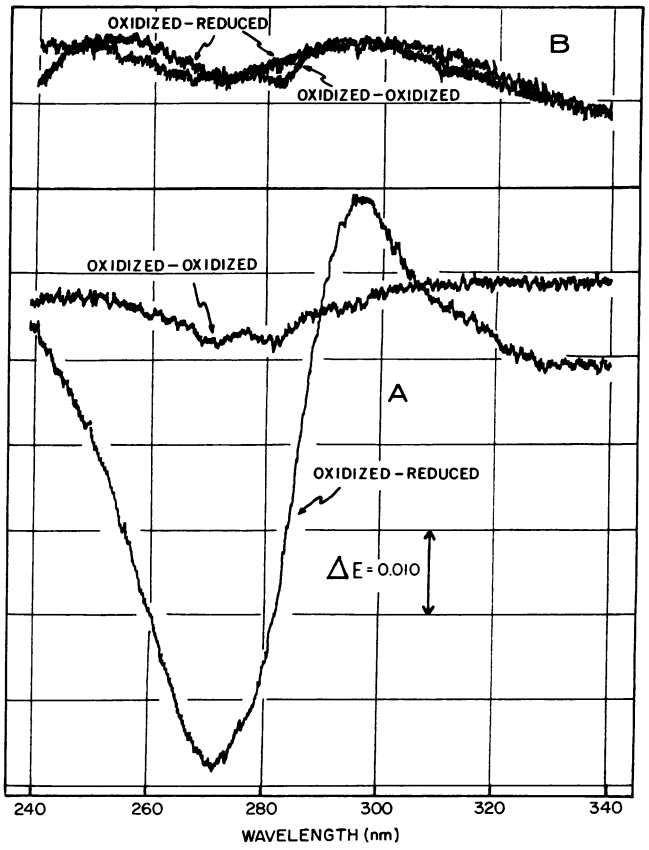

FIG. 1. Reduced *minus* oxidized spectra of extracts of mitochondria (*A*) and promitochondria (*B*) from strain DT-XII. After extraction of 2.7 mg of mitochondrial protein or 3.9 mg of promitochondrial protein, the contents of the extract were taken into absolute ethanol (3 ml) and oxidized with $FeCl_3$ (see *Methods*)

TABLE 1. *The P_i–ATP exchange reaction in promitochondria and mitochondria*

Omission or additions	Promitochondria (nmol ATP/min per mg)	Mitochondria (nmol ATP/min per mg)
None	32.4	141
Rutamycin (50 μg/ml)	0.2	0
1799 (20 μM)	0.1	0
Atractyloside (0.5 mM)	0.2	7
Valinomycin (0.2 μg/ml) + KCl (5 mM)	1.8	23
−NaCN	32.2	175
Cytochrome *c* oxidase (ΔE_{550}/min per mg)	0.018	2.77
NADH oxidase (nmol/min per mg)	0.0	389

This experiment was carried out with mitochondria and promitochondria prepared from the wild-type strain DT-XII. The P_i–ATP exchange activity was determined using 0.8 mg of mitochondria/ml and 0.5 mg of promitochondria/ml.

Energy-linked changes of ANS fluorescence

Another sensitive indicator for the presence of a mitochondrial energy transfer system is the energy-linked change in the fluorescence of ANS that is bound to mitochondrial membranes (18, 19). This method also has the advantage of revealing the sidedness of the membrane studied. Fig. 2*A* shows that addition of ATP to promitochondria causes a quenching of ANS fluorescence. Subsequent addition of uncoupler restores the fluorescence to a level that is reached in a control experiment (Fig. 2*B*) in which uncoupler is added before the ATP. These experiments show that ATP "energizes" the promitochondrial membrane and that the sidedness of this membrane is the same as that of intact mitochondria from aerobic yeast and from mammalian sources.

Proton permeability and swelling

It is now well established that the mitochondrial inner membrane is largely impermeable to protons (20). The same holds true also for yeast promitochondria, provided that the pH of the suspending medium is below 6 (results to be published). After an acid-pulse, only slow equilibration of H^+ occurs; addition of uncoupler has little effect (Fig. 3). Fast proton

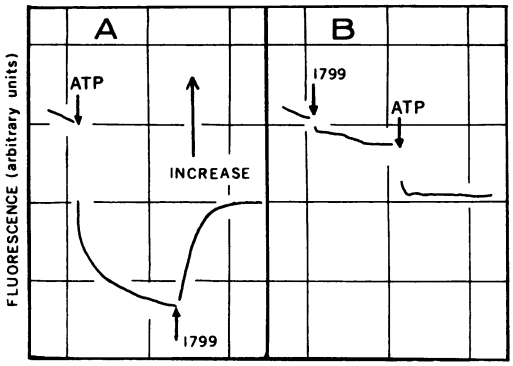

FIG. 2. Effect of ATP and the uncoupler 1799 on the fluorescence of ANS in the presence of promitochondria (strain DT-XII). At the arrows ATP and 1799 were added at a final concentration of 2 mM and 10 μM, respectively.

equilibration is observed only after the membrane is made permeable to K^+ by the addition of valinomycin (21). This observation shows that promitochondria are closed vesicles, impermeable to both H^+ and K^+. The low K^+ permeability of promitochondria is also reflected by the fact that they swell only slowly in isotonic potassium acetate unless valinomycin is added.

Participation of mitochondrial protein synthesis in the assembly of the energy transfer system

The demonstration of an energy transfer system in promitochondria allows one to answer the question whether the formation of this system is dependent on promitochondrial protein synthesis. Results obtained with respiring cells would necessarily be ambiguous since any inhibition of mitochondrial protein synthesis would cause the loss of most mitochondrial cytochromes (22), which could indirectly affect the energy transfer system.

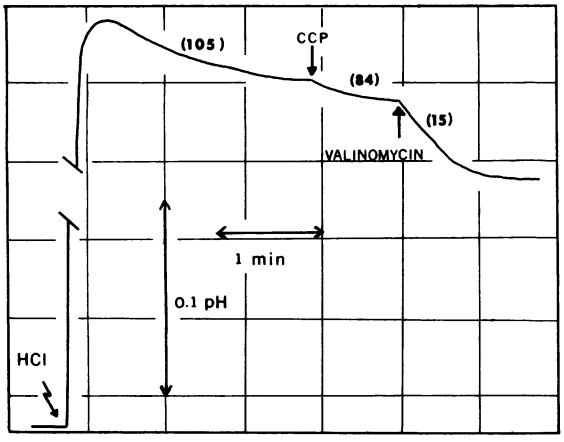

FIG. 3. Proton impermeability of promitochondria of strain DT-XII. A suspension containing 5 mg of promitochondrial protein in 400 mM mannitol, 150 mM KCl, and 3.3 mM glycylglycine (pH 6.5) was incubated for 10 min. The final volume was 2.5 ml, the final pH 5.9. At the first arrow, 825 nmol of HCl was added and the change of the pH was monitored. During the passive proton equilibration the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCP) (4 μM final concentration) and valinomycin (1 μg/ml) were added at the arrows. The figures between parentheses are the half-times in seconds for the passive equilibration of protons (see ref. 21).

TABLE 2. *Effect of growth in the presence of erythromycin on the promitochondrial P_i–ATP exchange reaction*

Expt. no.	Strain used	Erythromycin during growth	nmol ATP/min per mg
1	DT-XII	−	32.4
2		+	0.8
3	D 587-4B	−	10.8
4		+	1.2
5	D 587-4B-1	−	12.5
6		+	14.0

All strains were grown anaerobically. When erythromycin was present, the concentration in the growth medium was 2 mg/ml.

If promitochondrial protein synthesis is blocked *in vivo* by growing strains DT-XII or D 587-4B in the presence of erythromycin, the isolated promitochondria are no longer able to catalyze a P_i–ATP exchange reaction (Table 2, experiments 1–4). It might be argued that this effect of erythromycin is nonspecific since experiments with intact yeast cells require rather high external erythromycin concentrations. In order to exclude this possibility, a control experiment was carried out with an erythromycin-resistant extrachromosomal mutant of strain D 587-4B in which the specific target site of erythromycin, or some closely related site, had been altered. As can be seen from Table 2 (experiments 5 and 6) the promitochondrial exchange reaction in this mutant is no longer affected by growth of the cells in erythromycin. A nonspecific effect of erythromycin can thus be excluded.

It has been shown that the extrachromosomal "petite" mutation irreversibly inactivates promitochondrial protein synthesis (9, 23). If our results with erythromycin-repressed wild type yeast cells are correct, then promitochondria isolated from anaerobically grown "petite" mutants should lack an energy transfer system. The results listed in Table 3 confirm this prediction. The slow rate of P_i–ATP exchange that is still found in the mutant promitochondria is insensitive to rutamycin, uncouplers, or atractyloside. This residual exchange probably reflects contamination of the "petite" promitochondria with glycolytic enzymes (24), since the P_i–ATP exchange reaction is inhibited by iodoacetate and stimulated by the addition of 3-phosphoglycerate and glyceraldehyde 3-phosphate.

TABLE 3. *Lack of P_i–ATP exchange in promitochondria from a cytoplasmic "petite" mutant*

Expt. no.	Additions	nmol ATP/min per mg
1	None	2.7
	Atractyloside (0.2 mM)	2.6
2	None	1.5
	Rutamycin (50 μg/ml)	1.5
	1799 (20 μM)	1.5

The experiments were carried out with the promitochondria from anaerobically grown DT-XIIa. In Expt. 1, 0.82 mg/ml promitochondrial protein was used; in Expt. 2, 1.90 mg/ml.

DISCUSSION

Our results leave little doubt that the P_i–ATP exchange activity of promitochondria reflects the presence of a typical mitochondrial energy transfer system. Spectral and enzymatic analyses show that promitochondria contain neither ubiquinone nor the cytochromes aa_3, b, c_1, and c. Moreover, promitochondria from strain DT-XII (the strain used in most of the experiments) exhibited little, if any, electron spin resonance signal at g = 1.94 at liquid nitrogen temperature upon reduction with dithionite. We conclude that these respiratory carriers are not obligate participants in the P_i–ATP exchange reaction. The P_i–ATP exchange reaction in promitochondria thus seems to be the first instance of a mitochondrial energy transfer system that is dissociated from a respiratory chain.

It is possible, of course, that the promitochondrial P_i–ATP exchange proceeds via unidentified respiratory carriers or via traces of ubiquinone that are below the limit of detection. It might also be argued that the residual dehydrogenases, as well as the low amounts of a pigment resembling cytochrome b_5 (compare ref. 1), may participate in promitochondrial energy transfer. While these possibilities cannot be ruled out entirely, they seem rather unlikely. Firstly, our methods would have detected ubiquinone levels 30–40 times lower than those present in aerobic yeast mitochondria. Secondly, present information about aerobic *S. cerevisiae* mitochondria indicates that neither NADH nor succinate dehydrogenase is involved in energy conservation (25). Finally, the properties of the cytochrome b_5-like pigment suggest that it is either present in the outer promitochondrial membrane or in "microsomal" particles that contaminate the promitochondrial fraction (1).

The observations reported here show that the ATP- and water-forming reactions of oxidative phosphorylation are not obligatorily coupled to redox reactions in the respiratory chain (compare 12). Previous indirect evidence for this view was based mainly on the fact that the mitochondrial exchange reactions were not affected by respiratory inhibitors or by reduction of the respiratory chain (6, 26). It was found, moreover, that removal of cytochrome *c* (27, 28) or cytochrome oxidase (29) from mitochondrial membranes did not abolish the exchange reactions. The present results show clearly that a mitochondrial energy transfer system can function even if virtually *all* respiratory carriers are absent.

One of the most intriguing results emerging from this study is the concept of mitochondrial function in the absence of oxygen. Our findings raise the possibility that promitochondria can utilize glycolytically-generated ATP for energy-linked processes such as ion transport (compare Table 1). Thus, yeast promitochondria may be not merely inert mitochondrial precursors, but organelles in their own right which fulfill distinct physiological functions.

NOTE ADDED IN PROOF

In collaboration with Dr. P. D. Boyer it was found that promitochondria also catalyze an $[^{18}O]H_2O$–P_i exchange reaction that is inhibited by rutamycin and the uncoupler 1799. The rate of this reaction is approximately 20% of that catalyzed by aerobic mitochondria. This observation further supports the conclusion (12) that the water-forming reactions of oxidative phosphorylation are independent of the redox reactions in the respiratory chain.

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