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Ca^{2+} METABOLISM IN YEAST CELLS AND MITOCHONDRIA

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SUMMARY

1. Ca^{2+} in the culture medium stimulates only sligthly the growth and respiration of *Saccharomyces cerevisiae*.

2. Neither energy-linked Ca^{2+} transport nor high-affinity Ca^{2+} binding occur in mitochondria isolated from *Saccharomyces cerevisiae* or from *Torulopsis utilis*.

3. Metabolism-independent, low-affinity binding of Ca^{2+} does, however, occur in mitochondria isolated from both yeasts. The concentration of these sites varies between 40 and 50 nmoles per mg of mitochondrial protein. Their affinity for Ca^{2+} is rather low (K_m = 10–20 μM).

4. Mitochondria of *Saccharomyces cerevisiae* contain about 10 nmoles of endogenous Ca^{2+} per mg of mitochondrial protein, which is bound or sequestered in a very stable manner.

5. The respiration of mitochondria from *Saccharomyces cerevisiae* is stimulated by valinomycin in the presence of KCl, suggesting that energy-linked transport of K^+ occurs.

6. The results show that energy-linked Ca^{2+} transport is not a universal attribute of intact mitochondria from all species. Since the yeast cells lack high-affinity Ca^{2+} binding capacity, they may lack a specific Ca^{2+} carrier. The basic energy-dependent cation pump may however be present in these mitochondria, since they can transport K^+ in the presence of valinomycin.

INTRODUCTION

It is now well known that mitochondria isolated from a variety of animal cell types can accumulate Ca^{2+} from the medium in a respiration-dependent process[1]. In addition, such mitochondria may also bind Ca^{2+} in the absence of metabolic activity[2,3]. However, little information is available on Ca^{2+} movements in mitochondria of lower cells such as yeasts. In fact it is not even certain that Ca^{2+} is an essential mineral for yeast. OLSON AND JOHNSON[4] could demonstrate no Ca^{2+} requirement for growth of *Saccharomyces cerevisiae*, but they did not find it possible to prepare a medium completely free of Ca^{2+}. On the other hand, LOMANDER[5] has shown that

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supplementation of a basal growth medium with Ca^{2+} causes increases both in cell yield and in specific respiratory activity of *Saccharomyces pastorianus*.

Mitochondria with good phosphorylating activity can now be readily isolated from yeast cells[6–9]. This paper reports a study of Ca^{2+} accumulation and binding by mitochondria isolated from *Saccharomyces cerevisiae*, a species which lacks the capacity for energy-coupling at Site 1 of the respiratory chain, and from *Torulopsis utilis*, a yeast in which all three energy-coupling sites are functional[7].

The results show that no energy-dependent Ca^{2+} accumulation of the type observed in liver mitochondria takes place in mitochondria from either yeast. Moreover, neither yeast shows high-affinity, respiration-independent Ca^{2+} binding; they therefore appear to lack a Ca^{2+} carrier in the membrane. Both types of yeast, however, can bind some Ca^{2+} in a low-affinity, metabolism-independent process.

MATERIALS AND METHODS

Yeast strains

Two types of yeast were employed: *Saccharomyces cerevisiae*, Strain D-261, and a strain of *Torulopsis utilis* (*Candida utilis*) obtained from Dr. T. Ohnishi.

Growth of yeast

For routine fermentor growth, the natural medium of BECK *et al.*[8] was employed. For studies of the effects of Ca^{2+} nutrition, the completely synthetic medium of OLSON AND JOHNSON[4] was used, except that in most experiments the primary carbon source was 0.1 % glucose + 3 % ethanol, rather than 1 % glucose. This medium contains 0.25 % asparagine and 0.1 % sodium citrate. In small-scale experiments cells were grown at 30° in 2-l flasks containing 200 ml of medium, on a New Brunswick Model G-10 gyrotary shaker for 30–48 h. For large-scale production, cells were grown 21 h in 10-l batches in a New Brunswick fermentor as described previously[9].

Determination of cell respiration

Cell respiration (Q_{O_2}) was determined as described by BECK *et al.*[8]. The substrate was 0.1 M glucose.

Preparation of mitochondria

Mitochondria were prepared from *Saccharomyces cerevisiae* by the method of BECK *et al.*[8], and from *Torulopsis utilis* by the method of BALCAVAGE AND MATTOON[10]. They were either used immediately, or kept frozen under liquid N_2 until needed[11].

Chemical and biochemical measurements on mitochondria

Endogenous Ca^{2+} was measured by atomic absorption spectrophotometry on extracts obtained after removal of mitochondrial proteins by heating at 100° for 10 min. The determinations were carried out in the presence of lanthanum to eliminate the interference by phosphates.

Oxidative phosphorylation was determined as previously described[10]. Ca^{2+} uptake was measured isotopically following rapid filtration of the medium through millipore membranes. The medium was that used for oxidative phosphorylation and consisted of 0.01 M sodium phosphate (pH 6.7) in 0.6 M mannitol. The respiratory

substrate was added at 17 mM. When indicated, P_i was omitted. Uncoupling by valinomycin was studied in the same medium, containing KCl.

H^+ movements were followed on a Beckman Expandomatic pH meter, connected to a strip-chart recorder. Calibrations of the system were made by addition of known amounts of either NaOH or HCl. The oxidation–reduction state of cytochrome *b* was recorded with a dual wavelength spectrophotometer.

High- and low-affinity Ca^{2+} binding was studied at 0°, as described by REYNAFARJE AND LEHNINGER[3] for rat-liver mitochondria.

Chemicals

All chemicals used were reagent grade. Valinomycin was the kind gift of Dr. F. Bygrave; $^{45}Ca^{2+}$ was purchased from ICN Corp., U.S.A.

RESULTS

Effect of Ca^{2+} on growth and respiration

Although calcium salts are frequently used to supplement media used for growing yeast, it is uncertain whether the organism in fact requires Ca^{2+} (refs. 4, 5). The possibility was considered that Ca^{2+} might be required only for mitochondrial function. Since *Saccharomyces cerevisiae* is a facultative anaerobe, it requires functional mitochondria only when utilizing nonfermentable carbon sources during aerobic growth. Consequently, the relative growth-stimulating effects of added Ca^{2+} were determined for yeast utilizing glucose, which is fermentable, and ethanol, which is nonfermentable. Table I shows that in flask experiments, supplementation of the

TABLE I

EFFECTS OF Ca^{2+} SUPPLEMENTATION ON *S. cerevisiae* GROWTH ON FERMENTABLE AND NONFERMENTABLE SUBSTRATES

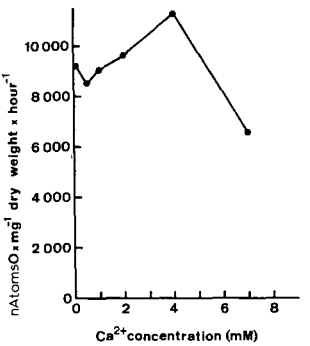
Cells were grown 30 h in 200 ml of synthetic medium in 2-l erlenmeyer flasks incubated at 30° on a rotary shaker. Supplemented medium contained 1.0 mM $CaCl_2$. Total yield is given.

Carbon source	*Cell dry weight (g)*	
	Control	*+ Ca^{2+}*
1% Glucose	0.72	0.81
2% Ethanol	0.41	0.50

synthetic medium with 1.0 mM Ca^{2+} gives small increases in yield with both types of substrate. Although the salts used in preparing the medium were of reagent grade, they do contain small amounts of this cation as impurities. Nevertheless, the experiment does suggest that even when the cell is entirely dependent upon a respiratory substrate and functional mitochondria for its ATP synthesis, Ca^{2+} is not a critical nutrient. Fig. 1 shows that addition of Ca^{2+} to the synthetic ethanol medium caused a small, but significant increase in the respiratory capacity (Q_{O_2}) of *Saccharomyces cerevisiae* grown in flask culture. This suggests that Ca^{2+} supplementation, though not essential, is beneficial for mitochondrial function in yeast. The apparent drop in Q_{O_2} seen at the highest Ca^{2+} concentration is probably an artifact

arising from the presence of insoluble calcium salts which increase the apparent dry weight. It must be concluded either that Ca^{2+} is not required by *Saccharomyces cerevisiae* or that the amounts of Ca^{2+} already present in the reagent grade inorganic salts and in the very small amounts of calcium pantothenate used in preparing the medium are able to support a high level of mitochondrial activity.

When cells are grown aerobically in 10-l batches in a fermentor, no appreciable difference in yield between Ca^{2+}-supplemented and unsupplemented medium was obtained. In view of the small effects seen in the flask experiments, aeration efficiency may be an important variable in regulating Ca^{2+} metabolism in yeast. As will be shown below, even cells grown in an unsupplemented medium contained significant amounts of Ca^{2+}. Consequently yeast seems to be highly efficient in "scavenging" even traces of Ca^{2+} from the medium. It should be noted that in the fermentor experiments the sodium salt of pantothenic acid was used to reduce still further the endogenous Ca^{2+} content. These experiments do not give strong support to the view that Ca^{2+} is required for mitochondrial function; however, the possibility remains that trace amounts of Ca^{2+} might suffice to maintain respiratory activity.



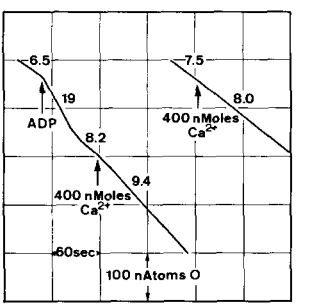


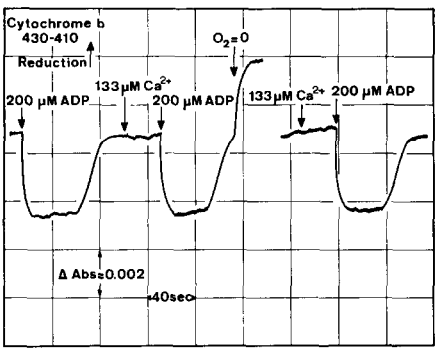
Fig. 1. Effect of Ca^{2+} supplementation on yeast respiratory capacity. Cells were grown in synthetic medium containing 2 % ethanol as primary carbon source. $CaCl_2$ was added to the flasks at the concentrations shown. Cells were grown 48 h in 200 ml of medium in 2-l erlenmeyer flasks incubated at 30° on a rotary shaker. The respiratory rate was determined polarographically, using 0.1 M glucose as substrate.

Fig. 2. Effect of ADP and Ca^{2+} on the respiration of mitochondria from *S. cerevisiae*. Technical details are given in MATERIALS AND METHODS. P_i was present in the medium. Succinate was the respiratory substrate. Mitochondrial concentration, 0.37 mg in a final volume of 3 ml.

The reaction of Ca^{2+} with isolated yeast mitochondria

As shown in Fig. 2, the mitochondria used in this study exhibited good ADP-mediated respiratory control. In a large series of experiments with succinate as the substrate, an average respiratory control ratio of 2.5 was obtained upon addition of ADP. However, in contrast to the behavior of liver and other animal mitochondria, Ca^{2+} failed to stimulate State-4 respiration. Only in some experiments did a very small increase of the respiratory rate occur after addition of Ca^{2+}, but this did not return to the previous State-4 level. Unsuccessful attempts were made to induce respiratory stimulation following the addition of Ca^{2+} by substituting NaCl or KCl for mannitol, by omitting P_i, or by increasing the pH of the incubation medium. The respiratory chain in yeast mitochondria is thus apparently unable to respond to Ca^{2+}. Moreover, the spectrophotometric experiments shown in Fig. 3 lend further

support to this conclusion. Perhaps the most sensitive index of the energy-linked interaction of Ca^{2+} with the membrane of liver and other animal mitochondria is the spectrophotometrically observed transition of cytochrome *b* towards a more oxidized state. Very recently it has indeed been possible to show that the apparent K_m for the response of cytochrome *b* to Ca^{2+} in liver mitochondria is of the order of 1 μM (ref. 12). However, it is clear from the trace shown in Fig. 3 that even higher Ca^{2+} concentrations did not induce a shift in the redox state of yeast cytochrome *b*.



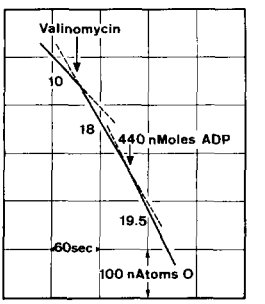


Fig. 3. Effect of ADP and Ca^{2+} on the redox state of cytochrome *b* in *S. cerevisiae* mitochondria. Technical details in MATERIALS AND METHODS. Final volume, 3 ml; temp., 20°. Mitochondrial concentration was 0.12 mg of protein in 3.0 ml.

Fig. 4. Valinomycin-induced uncoupling of respiration in *S. cerevisiae* mitochondria. Conditions as in Fig. 2. Concentrations: KCl, 0.01 M; mitochondria, 0.9 mg of protein; valinomycin, 0.065 μg.

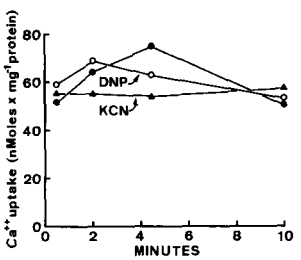
ADP on the other hand induced the normal shift toward a more oxidized steady state. A further indication that Ca^{2+} does not interact with intact yeast mitochondria is shown by their failure to eject H^+ upon addition of Ca^{2+}, whereas addition of Ca^{2+} to liver and other animal mitochondria respiring in State 4 induces a prompt and large ejection of H^+, stoichiometric with the Ca^{2+} uptake[1].

Although no active accumulation of Ca^{2+} occurs in this yeast, it is of considerable interest that addition of valinomycin to yeast mitochondria respiring in the presence of K^+ under State-4 conditions induced a prompt stimulation of respiration, which then responded only minimally to the addition of ADP (Fig. 4). Yeast mitochondria are evidently capable of a respiration-dependent active uptake of K^+.

Low-affinity Ca^{2+} binding by yeast mitochondria. Despite the lack of Ca^{2+}-induced respiratory stimulation, cytochrome *b* redox transitions, or H^+ ejection, yeast mitochondria are capable of respiration-independent binding of Ca^{2+}. The experiment presented in Fig. 5 shows that such binding is very rapid. A saturation level was reached within seconds after the addition of Ca^{2+}; no further increase in the binding took place upon prolonging the incubation to 10 min. This binding is totally independent of either coupled or uncoupled respiration, as shown by the lack of effect of KCN or 2,4-dinitrophenol. It is therefore different from the energy-linked Ca $^{2+}$ uptake observed in liver and other animal mitochondria, but similar to the metabolism-independent Ca^{2+} binding described by ROSSI *et al.*[2] and REYNAFARJE AND LEHNINGER[3]. In fact, inhibition of Ca^{2+} binding was observed in the

presence of butacaine, which has been reported to inhibit the metabolism-independent binding of Ca^{2+} by liver mitochondria[13] (Fig. 6).

Since *Saccharomyces cerevisiae* mitochondria lack energy-conserving capacity at Site 1, the possibility was considered that Site 1 alone might support some energy-linked uptake of Ca^{2+}. Mitochondria from *Torulopsis utilis*, which possess Site-1 activity, were therefore prepared. However, experiments in Fig. 7 show that in Torulopsis mitochondria Ca^{2+} uptake was not inhibited by uncouplers, and was therefore not energy-linked.



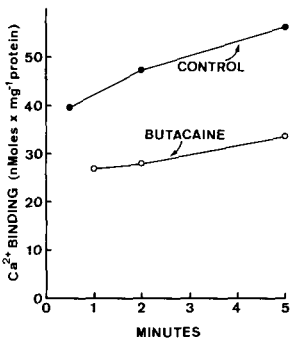
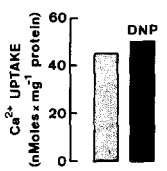


Fig. 5. ADP- and KCN-insensitive Ca^{2+} binding by mitochondria from *S. cerevisiae*. Technical details in MATERIALS AND METHODS. Final volume, 8 ml; temp., 25°. Concentrations: mitochondria, 12 mg of protein; 2,4-dinitrophenol (DNP), 0.1 mM; KCN, 2 mM; Ca^{2+}, 130 nmoles/mg protein.

Fig. 6. Effect of butacaine on Ca^{2+} binding by *S. cerevisiae* mitochondria. Conditions as in Fig. 5. Concentrations: mitochondria, 10 mg of protein; butacaine, 0.1 mM; Ca^{2+}, 160 nmoles/mg of protein.

Recent studies have shown that a large fraction of the endogenous Ca^{2+} of liver and other mitochondria is maintained in a dynamic steady state at the expense of the limited amount of energy transformed by the respiratory chain under resting conditions[14,15]. Indeed, uncouplers promptly discharge 40–70 % of endogenous Ca^{2+}



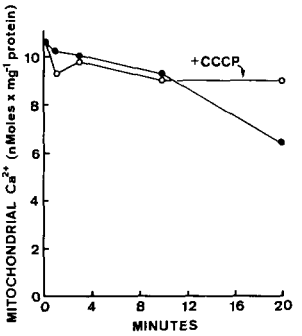
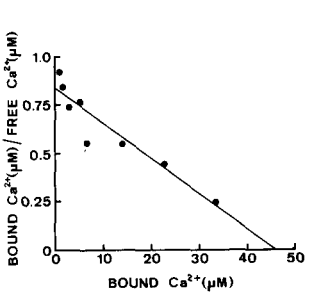


Fig. 7. 2,4-Dinitrophenol-insensitive binding of Ca^{2+} by mitochondria from *T. utilis*. Conditions as in Fig. 5. Mitochondrial concentration, 6 mg of protein. Ca^{2+} concentration, 265 nmoles/mg of protein.

Fig. 8. Stability of endogenous Ca^{2+} in mitochondria from *S. cerevisiae*. Incubation at 25°, in 0.6 M mannitol, 16 mM succinate. Final volume, 5 ml. Protein concentration, 12.5 mg of protein; *m*-chlorocarbonylcyanide phenylhydrazone (CCCP) concentration, 1.0 μM. The determination of Ca^{2+} is described in MATERIALS AND METHODS. The points were obtained from separate tubes run for the times shown, each containing 5 ml of medium and 12.5 mg of mitochondrial protein.

from liver mitochondria. Under the conditions used in the present study, yeast mitochondria isolated from cells grown in a variety of media, including those not supplemented with Ca^{2+}, were found to contain about 9–12 nmoles of Ca^{2+} per mg of protein; however, as is shown in Fig. 8, this Ca^{2+} is very stably bound and not discharged by uncoupling agents.

High-affinity binding of Ca^{2+} by yeast mitochondria. REYNAFARJE AND LEHNINGER[3] have found that liver and other animal mitochondria can bind Ca^{2+} at high-affinity sites, which are few in number (approx. 1–5 nmoles/mg protein), but possess high affinity (K_m = 0.1–1.0 μM)[3]. Such binding can be observed at 0° in the presence of respiratory inhibitors; however, this type of binding is inhibited by uncoupling agents. Low-affinity Ca^{2+} binding sites, on the other hand, are more numerous (30–50 nmoles per mg protein) and of much lower affinity (K_m = 30–60 μM). The high-affinity Ca^{2+} binding sites were postulated to reflect the existence in the mitochondrial membrane of a translocase or carrier molecule highly specific for Ca^{2+}, Sr^{2+}, and Mn^{2+} (ref. 3).



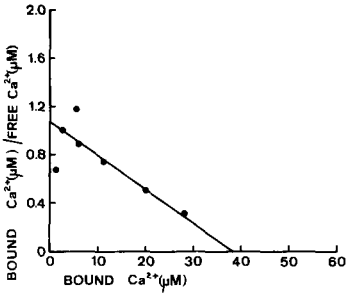


Fig. 9. Monophasic Scatchard plot of Ca^{2+} binding by *S. cerevisiae* mitochondria. Technical details are described in the MATERIALS AND METHODS. Final volume, 2 ml. Mitochondrial protein concentration, 2 mg/ml.

Fig. 10 Monophasic Scatchard plot of Ca^{2+} binding by mitochondria from *T. utilis*. Technical details are described in the MATERIALS AND METHODS. Final volume, 2 ml. Mitochondrial protein concentration, 4 mg/ml.

Yeast mitochondria were examined for their high-affinity Ca^{2+} binding capacity. The rectilinear Scatchard plot presented in Fig. 9 shows that Saccharomyces mitochondria possess only one class of Ca^{2+} binding sites: in a large series of experiments, these binding sites were found to vary between 40 and 50 nmoles per mg of mitochondrial protein and their apparent K_m for Ca^{2+} was in the range 10–20 μM, corresponding to the low-affinity sites observed in liver mitochondria. High-affinity Ca^{2+} binding sites were therefore completely absent from mitochondria of *S. cerevisiae*. They were absent also from Torulopsis mitochondria, as shown in Fig. 10.

DISCUSSION

The results presented in this paper have shown that isolated intact yeast mitochondria are unable to accumulate Ca^{2+} from the suspending medium during respiration and thus differ from the mitochondria isolated from animal tissues.

Respiration-linked Ca^{2+} transport is therefore not as general and characteristic a mitochondrial attribute as the phosphorylation of ADP. Although active Ca^{2+} accumulation has been found in all mammalian mitochondria so far examined[16], it is not always present in mitochondria from plants and lower organisms. No stimulation of respiration is induced by addition of Ca^{2+} to mung bean or beet root mitochondria[17]. Indeed, other reactions typical of energy-linked Ca^{2+} transport are also absent from mung bean mitochondria (L. MELA, personal communication); this fact is in agreement with the view that most of the Ca^{2+} in plant tissues is immobilized. There are, however, some exceptions to this generalization: in some plant mitochondria, such as in maize, active transport of Ca^{2+} does take place[18].

In yeast cells, no functional or physiological role for Ca^{2+} has been reported. However, in view of the slight stimulatory effects of Ca^{2+} on both growth and respiration that we observed, and because of the significant amounts of Ca^{2+} found in yeast mitochondria from cells grown in unsupplemented media, some functional role for Ca^{2+} in the yeast cell must still be considered possible. LOMANDER[5] has suggested that Ca^{2+} may be important in the regulation of nitrogen metabolism, possibly at the level of the mitochondrion.

Although intact yeast mitochondria, as well as mitochondria of some higher plants, fail to accumulate Ca^{2+} in a respiration-dependent process, it may not be concluded that they totally lack the capacity to pump Ca^{2+}. They may contain the requisite apparatus to move Ca^{2+} and other cations against a gradient, but, for lack of a Ca^{2+}-specific translocase or carrier in the membrane, Ca^{2+} may be unable to penetrate the membrane. The fact that all mitochondria tested to date, appear to show the capacity to accumulate K^+ on addition of valinomycin, which acts as a mobile K^+ carrier across the membrane, implies that all mitochondria may equally well be able to accumulate Ca^{2+} if they possess a carrier capable of getting Ca^{2+} across the membrane. The possibility might also be considered that the mechanism and the components for the energy-linked reactions of Ca^{2+} are present in yeast mitochondria, but with an affinity far lower than in mitochondria from liver or kidney. Additions of concentrations of Ca^{2+} far exceeding those routinely used in the studies of energy-linked Ca^{2+} transport in mitochondria could thus trigger the energy-linked transport mechanism. Experiments on this possibility are presently being carried out.

Evidence that high-affinity Ca^{2+} binding is a reflection of the function of a Ca^{2+}-specific membrane carrier has been presented elsewhere[3]. All mitochondria possessing high-affinity Ca^{2+} binding capacity are also able to accumulate Ca^{2+} in a stoichiometric, respiration-dependent process. If the membrane of yeast mitochondria can be made permeable to Ca^{2+} experimentally, they might also show capacity for energy-linked Ca^{2+} accumulation. Experiments are under way to test this working hypothesis, whose feasibility is supported by past work on the oxidation of external NADH by liver mitochondria following induction of permeability. The basic driving force for pumping of all cations by mitochondria, including Ca^{2+}, Sr^{2+}, Mn^{2+}, and K^+, among others, may be a respiration-dependent H^+ translocation occurring either as a result of the action of vectorial protonmotive loops of electron carriers as discussed by MITCHELL AND MOYLE[20], or by vectorial hydrolysis of high-energy chemical intermediates.

In recent studies designed to test the chemiosmotic hypothesis for oxidative

phosphorylation, pulses of O_2 (or ATP) were added to anaerobic liver mitochondria; they were observed to result in the acidification of the extramitochondrial medium[20]. These findings were interpreted to mean that respiration (or ATP hydrolysis) can bring about proton transport across the mitochondrial membrane. However, an alternative explanation for these experiments has been proposed; namely, that the acidification of the medium is due to energy-linked re-incorporation of Ca^{2+} (or Mg^{2+}) lost by mitochondria during the anaerobic incubation prior to the addition of O_2 or ATP[21,22]. In view of the absence of energy-linked transport activities in intact yeast mitochondria and the stability of the bound Ca^{2+}, such mitochondria would appear to be ideal for reexamining the occurrence of H^+ ejection following pulses of O_2. Such experiments are under way.

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