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OXIDATIVE PHOSPHORYLATION IN YEAST

VIII. OSMOTIC AND PERMEABILITY PROPERTIES OF MITOCHONDRIA ISOLATED FROM WILD-TYPE YEAST AND FROM A RESPIRATION-DEFICIENT MUTANT

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SUMMARY

1. Close similarities between yeast and mammalian mitochondria were found with respect to (a) osmotic response in impermeable solutes, sorbitol and KCl, (b) substrate translocation, (c) properties of the adenine nucleotide translocation system. A separate transport system for succinate, distinct from the dicarboxylate translocator, may be present in yeast mitochondria.

2. Substrate translocation was found to be preserved in pro-mitochondria from anaerobically-grown cells and in mitochondria from a respiration-deficient mutant. Adenine nucleotide translocation was demonstrated not to be affected by the cytoplasmic mutation. Along with previous data of other investigators, these results allow a general conclusion that neither the presence of a functional respiratory chain nor mitochondrial protein synthesis are prerequisite for the proper assemblage of the translocation systems in the mitochondrial membrane and for determining its permeability characteristics.

INTRODUCTION

In contrast to the vast amount of knowledge on mammalian mitochondria (as may be documented by review articles[1–4]) little is known as yet about the osmotic and permeability properties of yeast mitochondria. Swelling in hypotonic solutions[5], restricted permeability to H^+, K^+ and to external pyridine nucleotides[5,6], the presence of atractyloside-sensitive adenine nucleotide translocation[7] and the lack of energy-dependent Ca^{2+} transport[8,9] have been observed in experiments with *Saccharomyces* mitochondria. Pro-mitochondria from anaerobically-grown yeast, lacking a functional respiratory chain and mitochondria from a cytoplasmic respiration-deficient mutant were also found to be relatively impermeable for H^+ and K^+ (refs 5 and 10). Atractyloside sensitivity of ATP-dependent processes in pro-mito-

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

chondria has been demonstrated[10] suggesting the preservation of the adenine nucleotide translocator in these incomplete mitochondria.

A more detailed knowledge of the basic properties of yeast mitochondria seems mandatory if their potentialities for studies of energy conservation and genesis of the mitochondrial membrane are to be fully exploited. The results presented in this paper indicate close similarities between mitochondria isolated from mammals and from wild-type yeast. By extending previous observations on pro-mitochondria and mitochondria or respiration-deficient mutants, they provide evidence that the phenotypic of genotypic absence of a functional respiratory chain does not substantially affect either the osmotic behavior or the permeability properties of mitochondria.

EXPERIMENTAL

Preparation of cells and mitochondria

Wild-type yeast *Saccharomyces cerevisiae* DT XII and its acriflavin-induced respiration-deficient counterpart DT XIIA were used. The methods of aerobic culture and preparation of mitochondria were similar to those employed in previous studies[5,9–12], except that tonicities of the medium used in isolation of protoplasts and mitochondria were higher: 1.5 M sorbitol for protoplasts and 0.6 M mannitol for mitochondria. Mitochondria were kept at 0 °C as a dense suspension in 0.8 M mannitol and used for experiments within 4 h.

Anaerobically-grown cells were harvested after 24 h growth in closed vessels with mercury traps in a medium containing 0.26 % Tween 80 and 0.0012 % ergosterol, and 0.5 % glucose as carbon source. The medium was flushed with argon before and after inoculation and the washing of cells and preparation of protoplasts were done with solutions containing 25 μg/ml cycloheximide and 1 mg/ml erythromycin.

Measurement of volume changes

This was done either by determining difference between wet and dry weight of mitochondria or by following changes in absorbancies of mitochondrial suspensions at 520 nm in the Spekol (Zeiss, Jena) photometer attached to a recorder.

Measurement of substrate permeability

Two methods were employed: Swelling in isotonic ammonium salts[13] and determination of exchange of labelled substrates for unlabelled substrates across the mitochondrial membrane. In the latter procedure, mitochondria were prelabelled at 10 °C with ^{14}C-labelled anion (spec. act. 400 cpm/nmole) and, after a short incubation, separated from the incubation medium by rapid filtration on membrane filters. The mitochondrial pellet was washed with 5 ml of ice-cold 0.4 M sorbitol in 5 mM histidine chloride (pH 6.4). The filters were placed in scintillation vials, dried, suspended in scintillation liquid and the radioactivity was determined by scintillation counting.

Measurement of adenine nucleotide translocation

Two procedures of "forward exchange" and of "back exchange" as described by Pfaff and Klingenberg[14] were employed. In the former procedure, mitochondria

were incubated with labelled nucleotide at a low temperature and then separated from the medium by rapid filtration. Occasionally, 200 μM atractyloside was used to stop the exchange[14,15] which allowed the shortest incubation times of 4 s. The mitochondrial pellet on the filter was washed with 5 ml of ice-cold 0.4 M sorbitol in 10 mM Tris–maleate buffer (pH 6.4) and, after drying, the radioactivity of the pellet was counted.

Atractyloside was also used to differentiate between the exchange specific binding of nucleotides to the carrier and the unspecific binding in mitochondria. This discrimination, determination of numbers of binding sites and of dissociation constants of the carrier–nucleotide complexes were performed according to Weidemann *et al.*[16]. To reduce the ratio between the exchangeable and the carrier-bound portion of adenine nucleotides in the determination of carrier sites and dissociation constants, mitochondria from the wild-type strain were depleted of endogenous nucleotides by preincubation for 15 min at 30 °C in a buffered isotonic medium enriched with 10 mM phosphate and 5 mM $MgCl_2$ (ref. 16). These depleted mitochondria were employed for the measurements.

In the "back exchange" procedure, mitochondria were preincubated with [^{14}C]ATP (3 μCi/100 mg mitochondrial protein) of spec. act. 210 mCi/mmole, at 0 °C for 45 min in 0.8 M mannitol. After washing twice with 0.6 M mannitol in 2 mM EDTA (pH 7.0) the mitochondria gave 2000–4000 cpm per mg protein. The labelled mitochondria were incubated with nonradioactive adenine nucleotide and the exchange was arrested by 200 μM atractyloside. Mitochondria were separated from the medium by filtration or by centrifugation for 5 min at 10000 $\times$ g. Radioactivity of the pellet on the filter was determined as above. When centrifugation was employed, the pellet after centrifugation was solubilized by 0.5 ml of 10 % deoxycholate and the distribution of radioactivity was measured both in the solubilized pellet and in the centrifugation supernatant. Percentage of exchange, rate constants and the translocation activity were calculated according to Pfaff and co-workers[14,17].

Determination of adenine nucleotide pool

Mitochondria (5–20 mg protein) were extracted with 1 M $HClO_4$ at 0 °C for 5 min and adenine nucleotides were determined in the extract fluorimetrically[18] using the appropriate Boehringer test kits.

Determination of intra- and extramitochondrial spaces in the mitochondrial pellet

Mitochondria were mixed with a medium containing [^{14}C]sucrose or tritiated water and then separated by centrifugation. 0.5 ml of 10 % deoxycholate was added both to the pellet and to 0.1 ml of supernatant. Aliquots were pipetted for determination of radioactivity. In parallel samples, dextran-impermeable space was determined spectrophotometrically after mixing the mitochondria with blue dextran.

Other procedures

Protein was determined by the biuret method[19]. Radioactivity of ^{14}C- and ^{4}H-labelled compounds was counted in a Packard/Tricarb scintillation counter using the SLS 31 (Spolana, Neratovice) scintillation liquid.

The source of chemicals is indicated in parentheses: ATP (Serva); ADP (Boehringer); atractyloside, antimycin A, dithioerythritol (Sigma); tetramethyl-*p*-phe-

nylenediamine (Serva); ^{14}C-labelled ATP, succinate and malate (Amersham); blue dextran (Pharmacia, Uppsala); $^{3}H_2O$ (Institute for research, production and uses of radioisotopes, Prague); *n*-butylmalonate[20] and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)[21] were synthetized in the laboratory. Other chemicals originated from Lachema, Brno; most of them were purified in the laboratory to reach analytical purity.

Membrane filters with an average diameter of 0.6 μm (Synpor 4) were purchased from Synthesia, Pardubice.

RESULTS

Osmotic properties

Mitochondria from wild-type yeast and from the respiration-deficient mutant behaved like osmometers when suspended at 0 °C and pH 6.4 in sorbitol or KCl solutions of varying tonicities (Fig. 1). This indicates that, under the conditions employed, sorbitol and KCl do not penetrate the interior of mitochondria. Similar response was observed with pro-mitochondria of anaerobically-grown wild-type yeast in support of the previous finding of Groot *et al.*[10].

Yeast mitochondria, as prepared by the procedure employed in this work, showed osmotic stability in sorbitol and KCl only at pH values lower than 7. At higher pH values, they swelled rapidly even in 0.6 osM solutions. Similarly, their respiratory control fell rapidly and irreversibly at pH values higher than 7 indicating inherent instability of the mitochondria at alkaline pH.

As can be seen from Fig. 1, the extent of osmotic response of mitochondria from the respiration-deficient mutant was much smaller than that of wild-type mitochondria. This was mainly due to a relatively low fraction of intact mitochondria in the mitochondrial preparation from the mutant yeast as indicated both by space measurements (Fig. 2) and electron microscopy.

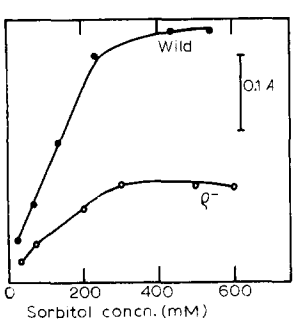

Fig. 1. Osmotic responses of yeast mitochondria to varying tonicities of the suspending medium. Mitochondria (2 mg of protein) were suspended at 2 °C in 3 ml of sorbitol buffered with 10 mM Tris–maleate buffer (pH 6.4). Concentrations of sorbitol are indicated on the abscissa. Similar curves were obtained when KCl was used instead of sorbitol. The two curves correspond to aerobically grown wild-type yeast and respiration-deficient (ρ^-) mutant, respectively.

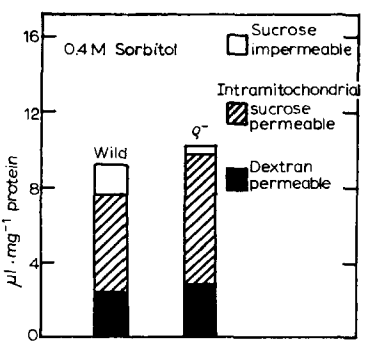

Fig. 2. Sucrose- and dextran-permeable spaces in pellets of wild-type and respiration-deficient (ρ^-) mitochondria. The spaces were determined by a procedure outlined in Experimental.

Substrate permeability

When wild-type mitochondria were suspended in approximately isotonic solutions of ammonium salts of phosphoric and organic acids, swelling responses similar to those found with mammalian mitochondria (for review, see refs 3 and 4) were recorded (Fig. 3). Rapid swelling ensued in ammonium phosphate indicating the presence of a phosphate carrier. For swelling with malate to occur, addition of phosphate was necessary indicating a malate for phosphate exchange. For swelling in citrate or tartrate, both phosphate and malate were required pointing to the coupling between phosphate, dicarboxylate and tricarboxylate exchanges[3,4,22].

Interestingly, swelling in ammonium succinate did not require phosphate. This suggested that a separate succinate carrier, distinct from the dicarboxylate carrier, may be present in yeast mitochondria as it is in mitochondria from housefly flight muscle[23]. This was supported by the observations that succinate uptake was only moderately inhibited by *n*-butylmalonate and that succinate did not compete for malate uptake (Table I).

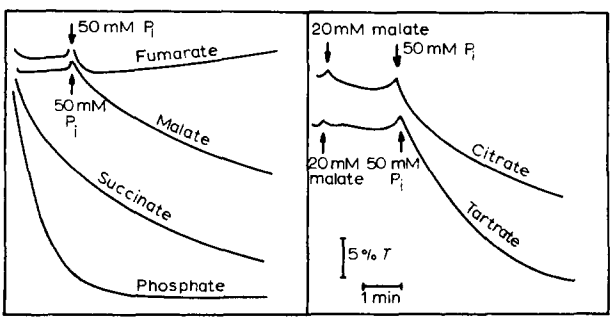

Fig. 3. Swelling of yeast mitochondria in ammonium salts of organic acids. Mitochondria of aerobically grown wild-type yeast (2.5 mg protein) were added to 3-ml solutions of ammonium salts of organic acids as indicated on the curves and the decrease in absorbance was recorded. The solutions had pH values of 6.2–6.4 and contained 1 μg antimycin A per mg protein. Ammonium fumarate, malate and succinate were 0.1 M, ammonium citrate and tartrate were 0.08 M and ammonium phosphate was 0.15 M. In addition, ammonium phosphate or malate at the concentrations shown in the figure were added as indicated by arrows.

TABLE I

INHIBITION OF ANION TRANSLOCATION IN YEAST MITOCHONDRIA

Mitochondria from aerobically grown wild-type yeast (1.3 mg protein) were incubated at 10 °C, for 90 s in a medium containing (in 0.3 ml): 0.4 M sorbitol, 1 mM EDTA, 5 mM histidine chloride, 0.1% bovine serum albumin, 2 μg antimycin A and the potassium salt of the labelled organic acid at concentrations indicated in the table, final pH 6.4. Values given are averages from several experiments.

Substrate	*Inhibitor*	*Inhibition* (%)
[^{14}C]Succinate, 1 mM	*n*-Butylmalonate, 20 mM	20
[^{14}C]Malate, 0.66 mM	*n*-Butylmalonate, 20 mM	90
	Succinate, 1 mM	5
	Malonate, 1 mM	46

When measured with radioactive substrates, anion uptake in yeast mitochondria was found to be very rapid and to exhibit saturation kinetics with apparent K_m values of 0.55 and 0.35 mM for succinate and malate, respectively. Less substrate was taken up by yeast mitochondria than by mammalian mitochondria, even at relatively low external pH. This may be explained by a relatively low proportion of sucrose-impermeable space in yeast mitochondria (Fig. 2) in which the accumulation of substrate should occur.

As may be inferred from Fig. 4, mitochondria of the respiration-deficient mutant and pro-mitochondria isolated from anaerobically-grown wild-type yeast exhibited similar properties with respect to anion translocation as did mitochondria from aerobically grown wild-type yeast.

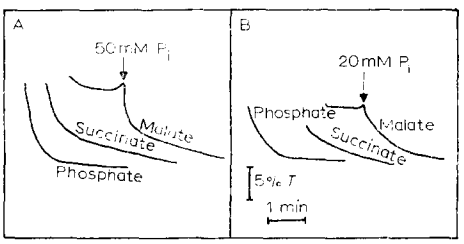

Fig. 4. Substrate translocation in pro-mitochondria and in mitochondria from the respiration-deficient mutant. Conditions as in Fig. 3. (A) Pro-mitochondria (0.85 mg protein·ml^{-1}). (B) Mitochondria from respiration-deficient mutant (1.0 mg protein·ml^{-1}).

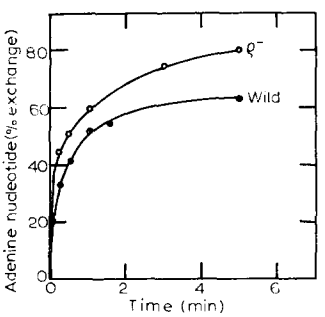

Fig. 5. Time course of adenine nucleotide exchange in yeast mitochondria. Mitochondria from aerobically grown wild-type yeast or from respiration-deficient mutant (ρ^-) (2.5 mg protein·ml^{-1} and 1.7 mg protein·ml^{-1}, respectively) prelabelled with [^{14}C]ATP were incubated with 200 μM ADP at 2 °C in a medium containing 0.4 M sorbitol, 1 mM EDTA and 10 mM Tris–maleate buffer, final pH 6.4. The "back exchange" was determined as described in Experimental.

TABLE II

SUMMARY OF PROPERTIES OF THE ADENINE NUCLEOTIDE TRANSLOCATION SYSTEM

Properties	*Mitochondrial source*:		
	Saccharomyces cerevisiae		*Rat liver*[4,16,17]
	Wild-type	*Respiration-deficient* (ρ^-)	
Number of carrier binding sites (nmoles·mg^{-1} protein)	0.10	0.06	0.13
Types of sites	High affinity	High affinity	High affinity
Dissociation constant of the carrier for ATP (μM)	0.39	0.43	0.45
Translocation activity at 2 °C; k_T, first-order constant (min^{-1})	2.8	3.1	0.7
K_m for ADP (μM)	4.75	4.40	1.3–4.0
Adenine nucleotide content (nmoles·mg^{-1} protein)	10.2	1.8	10.8

Adenine nucleotide translocation

In confirmation of the report of Ohnishi *et al.*[7], who studied adenine nucleotide translocation in mitochondria isolated from *S. carlsbergensis*, it has been found in this study that the transport system in wild-type mitochondria from *S. cerevisiae* exhibited properties similar to those of mammalian mitochondria. As shown in Fig. 5, rapid exchange of intra- for extramitochondrial adenine nucleotides could be observed both in normal and respiration-deficient mitochondria. The exchange was inhibited to 50 % and completely arrested by 30 and 150 μM atractyloside, respectively.

Table II summarizes the properties of the adenine nucleotide translocation system of wild-type and respiration-deficient mitochondria and compares them with those of mammalian mitochondria. It can be seen that the properties are similar in the three types of mitochondria, except that the translocation rate is higher in wild-type yeast mitochondria than in rat-liver mitochondria. This is in agreement with a higher respiratory rate of the yeast mitochondria.

DISCUSSION

It is apparent from this study that the mitochondrial membrane of wild-type *Saccharomyces* has properties very similar to that of mammalia. Some of the observed differences, such as instability at alkaline pH, smaller extent of volume changes and a relatively low portion of sucrose impermeable space as well as a previously found high Mg^{2+}-dependent ATPase mostly external to the atractyloside-sensitive barrier[24], may be ascribed to the imperfectness of the preparation procedure rather than to the inherent proporties of yeast mitochondria[2,3].

The results obtained with pro-mitochondria and with mitochondria from the respiration-deficient mutant extend previous relevant observations[5,10]. They allow a general conclusion that both pro-mitochondria, which phenotypically lack a complete respiratory chain and mitochondria from cytoplasmic respiration-deficient mutants which have lost the ability to carry on autonomous mitochondrial protein sysnthesis[24–27], exhibit normal osmotic and permeability properties. Thus, neither the presence of a functional respiratory chain nor mitochondrial protein synthesis are prerequisite for proper assemblage of the translocation systems in the mitochondrial membrane and for determining its permeability characteristics. This also implies that the components of translocation systems for substrate and adenine nucleotides are synthetised outside mitochondria and coded for by nuclear genes.

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