A NOVEL METHOD FOR THE RAPID PREPARATION OF COUPLED YEAST MITOCHONDRIA

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1. Introduction

Different methods have been developed for the preparation of yeast mitochondria [1–3]. In the methods described, two main techniques for the breakage of the yeast cells are used; in one of them, enzymes are used first to digest the cell wall, and then a rather gentle method is used for the breakage itself. In the other method, no treatment of the cell wall is used, and the cells are broken directly in the MSK cell homogenizer. The first technique is long and tedious, and the integrity of the mitochondria obtained is variable. With the use of the MSK homogenizer, although the procedure is faster, the mitochondria obtained are usually uncoupled.

The Ribi cell fractionator (Ivan Sorvall, Inc.) has been used successfully for the breakage of different kinds of cells; this method of disintegration has been claimed to be adequate for the preparation of forespores; these fragile structures are obtained intact after breaking bacteria with this instrument [4]. Dr Anthony J. Andreoli, who has used this method widely, suggested its use for the preparation of yeast mitochondria, provided the instructions are followed to polish the valve needle and seat, as recommended by the manufacturers. This paper describes the results of using the Ribi cell fractionator as an adequate disruption method for the reproducible preparation of coupled yeast mitochondria by means of a procedure that takes approximately two hours.

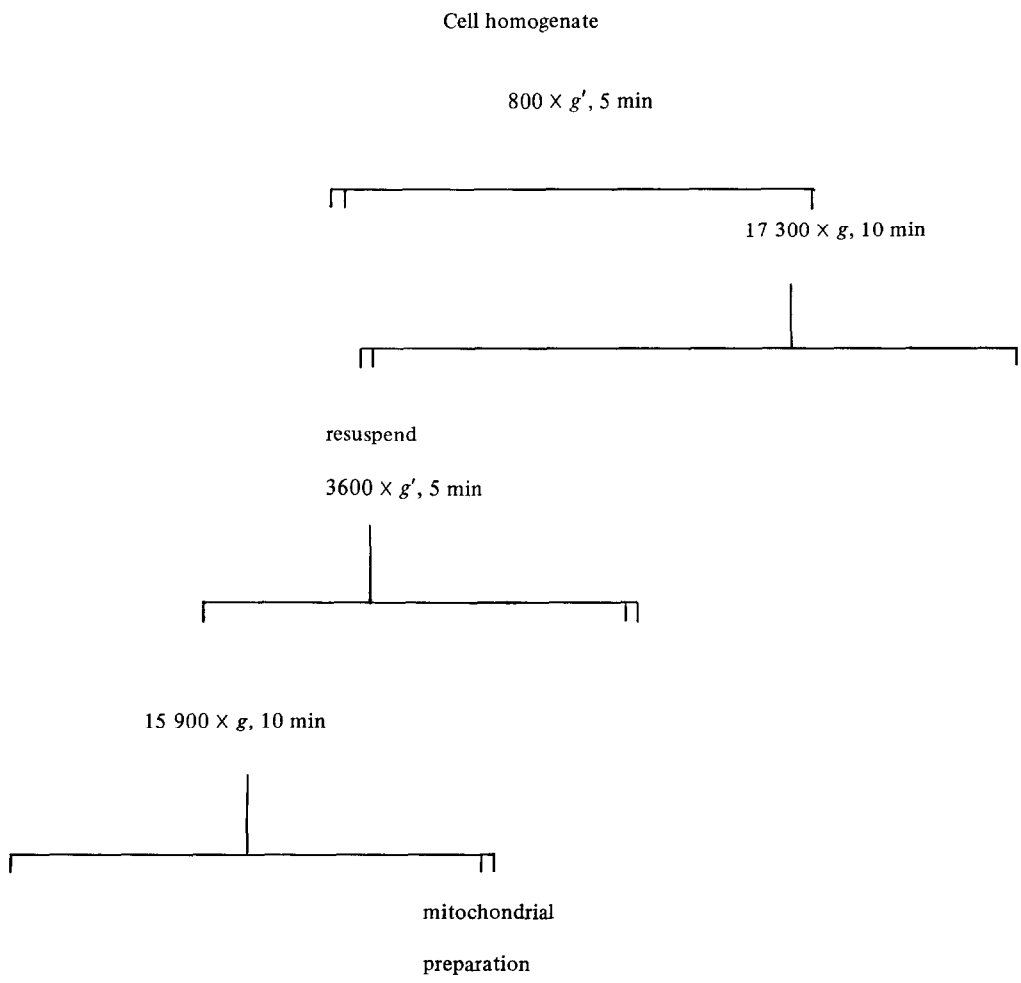
2. Materials and methods

2.1. *Preparation of yeast*

Baker's yeast was obtained commercially (La Azteca S. A.). The cells were never used more than 10 days after purchased. Just before use, 25 g of packed cells were resuspended in 1 liter of the medium described by De Kloet et al. [5], to which approximately 3 ml of an antifoaming agent (FG-10, Dow Corning) were added. The flask was placed in a constant temperature room (30°C) and aerated constantly by means of a piece of porous glass, at an approximate rate of 1.2 l/min. After 8–10 h, yeast cells were centrifuged, washed once with water and resuspended in 500 ml of water. The suspension was placed in a cylinder and aerated overnight (around 15 h) at the same rate of 1.2 l/min. The cells were then centrifuged, washed twice with water and weighed. Twenty grams wet cells were resuspended in 50 ml of preparation medium (0.6 M mannitol, 0.2% albumin, 10 mM imidazole, pH 6.8) and centrifuged. The pellet was resuspended again in the same medium to give 75 ml of suspension for each 20 grams of cells, wet weight. The albumin used (Bovine Albumin, Fraction V, Sigma) was always defatted by the procedure of Chen [6], and this was found to be essential to obtain coupled mitochondria.

2.2. *Disruption of cells*

The cell suspension described was treated in a Model RF-1 Ribi cell Fractionator (Ivan Sorvall Inc., Norwall, Conn.) at 13 500 psi and at 4–10°C. After



Scheme 1. Centrifugation pattern used to isolate yeast mitochondria. After the centrifugations at 17 300 and 3600 × g', the lipids adhered to the walls of the tube were carefully wiped with a paper towel.

disruption, the viscous preparation was treated at room temperature with a few crystals of deoxyribonuclease (Type I, DN-100, Sigma) during approximately 15 min (unitl viscosity decreased).

It is essential for the disruption to have polished the valve components of the press, as recommended by the manufacturer.

2.3. *Centrifugation*

The homogenate was submitted to the centrifugation pattern described in Scheme 1. In the last step, the lipids adhered to the walls of the centrifuge tube were removed carefully with a paper towel. The yield of mitochondria was from 452–720 μg/protein/g of yeast with a mean of 587/μg/g in seven experiments. Protein was measured by the method of Lowry et al. [7]. The mitochondria were resuspended in the same preparation medium and were usually employed within 3–4 h after preparation.

2.4. *Respiration*

Respiration was measured by means of a Clark electrode (Yellow Springs), using the following mediun 0.6 M mannitol, 10 mM sodium phosphate (pH 6.5), and 2.8 mg/ml of defatted albumin. Other additions and substrates are described under each experiment.

3. Results and discussion

Figure 1 and table 1 show some general properties of the mitochondria obtained by the technique described in this paper. The major characteristics are: ADP produces a higher stimulation than FCCP (trifluoromethoxycarbonylcyanide phenylhydrazone); even though the concentration of the uncoupler was varied, the maximal response was always lower than that obtained for ADP with all substrates tested. The best substrate for these mitochondria was ethanol, both from the point of view of the respiratory controls and the ADP ratios observed. This behavior with respect to different substrates seems to be the consequence of enzymatic capabilities of the mitochondria. In the case of pyruvate-malate, for instance, if 10 mM pyruvate 1 mM malate is used, coupling seems to be decreased, but this is probably because the maximal respiratory capacity is decreased; in fact, with the pyruvate concentration kept the same, increasing only the malate concentration (tracing F), with a similar state 4 respiratory rate, a higher response to ADP was obtained.

It is also noticeable that NADH gives always the higher respiratory rates and the lowest coupling of all substrates used. This again can be explained by the properties of the NADH oxidation system(s); it seems that part at least of this activity is not coupled

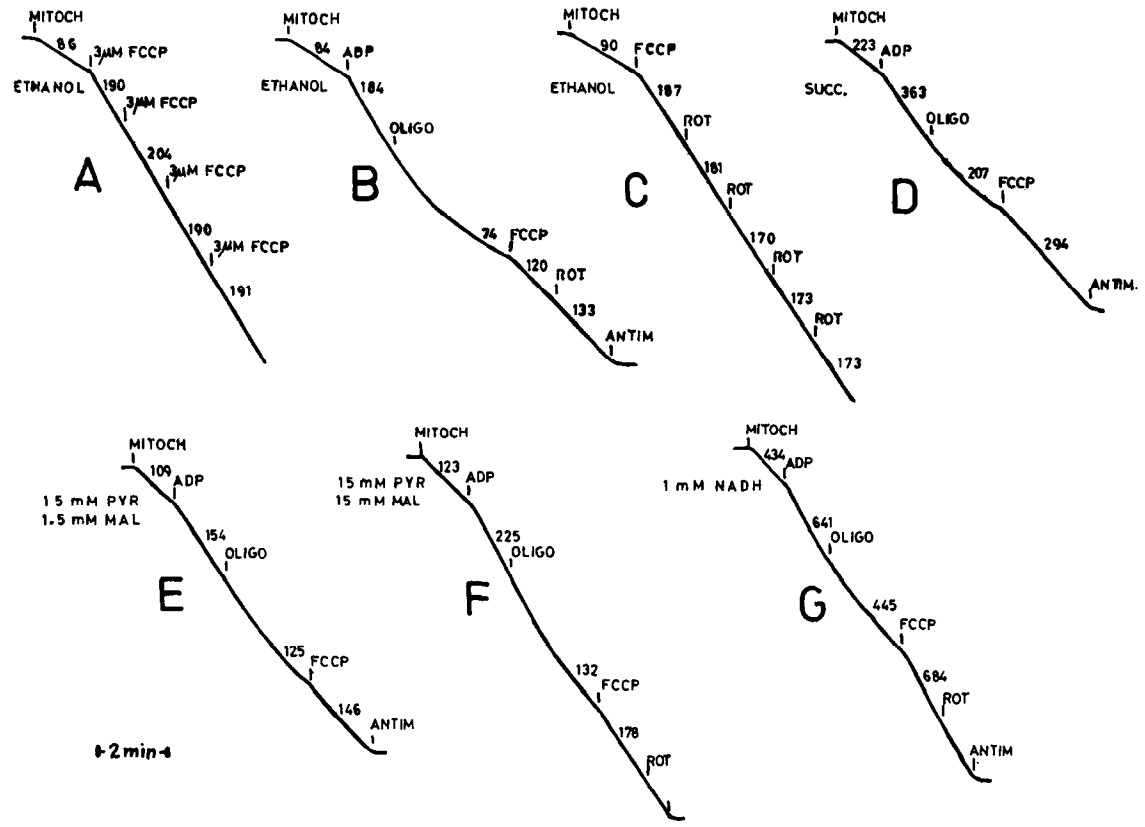


Fig.1. Behavior of yeast mitochondria with different substrates, ADP, FCCP and inhibitors. The incubation mixture was the same as for table 1, ethanol concentration was 250 mM, succinate, 15 mM, and NADH, 1.0 mM. Mitochondrial protein was 675 μg in all tracings, except in D and G, in which it was 337 and 224 μg respectively. Except for tracing A, in which each addition of FCCP was 3 μM in 1 μl of dimethylformamide (DMF), other additions were 6 μM in 2 μl of DMF. Rotenone and oligomycin additions were 1.5 μg each, in 3 μl of DMF. Antimycin A was added as 2.0 μg in 3.0 μl of DMF. ADP was 800 μM in all tracings. Figures aside tracings represent ngatoms of oxygen per minute, per mg of protein.

Table 1
Respiratory control and ADP : O ratios of yeast mitochondria with different substrates

Experiment	Substrate	First ADP addition		Second ADP addition	
		R C	ADP : O	R C	ADP : O
I	10 mM pyruvate 10 mM malate	2.90	1.77	3.70	2.11
II	10 mM pyruvate 10 mM malate	2.15	1.21	2.40	1.43
III	10 mM pyruvate 1 mM malate	2.02	–		
IV	10 mM pyruvate 1 mM malate	2.2	1.40		
I	NADH	1.83	–		
II	NADH	1.48	++		
III	NADH	1.77	–		
IV	NADH	1.46	–		
I	Succinate	2.50	1.90	2.70	2.08
II	Succinate	2.00	1.11	2.12	1.40
III	Succinate	2.47	1.40	2.57	1.51
IV	Succinate	1.83	1.33	2.39	1.62
I	Ethanol	3.91	1.82	3.23	2.2
II		3.25	2.09	2.76	1.78
III		3.08	1.55	2.74	1.55
IV		2.89	1.51	2.50	1.45

Respiration was measured in the medium described under Materials and methods. NADH and ADP were added as a solution of the disodic salt. NADH was 1.33 mM, ethanol, 250 mM, and succinate, 10 mM; each time, 160 nmol of ADP were added. All acidic substrates were added as the triethanolamine salts, taken to pH 6.5; Final volumes were 1.5 ml, and the temperature was 30°C.

to ATP generation, and that gives an extra respiratory capacity with this substrate; this could be due to the presence of broken mitochondria in the preparation. Besides, the results with NADH are the least consistent of all, mainly in the respiratory control and ADP : O ratios.

As found by other authors [1–3] these mitochondria are insensitive to rotenone. The effect of this inhibitor is null (tracing C); On the other hand, as expected, antimycin A produced in all cases a complete inhibition of respiration.

The table shows that for the measurement of the respiratory controls and ADP : O ratios, ethanol is the best substrate, followed by succinate. Pyruvate-

malate are adequate substrates when used in equimolar concentrations. With NADH also, trying to measure ADP : O ratios, erratic results were obtained, and the figures are not included in table 1. The results with ethanol and succinate are similar to those obtained by Ohnishi et al. [1] and quite reproducible.

In summary, the results presented here are a good indication that yeast mitochondria can be obtained in a coupled state and rapidly by this method. If compared with the method that uses the digestion with snail gut enzymes, it has the advantage of being much faster and reproducible. Even though several descriptions of this general technique have been published in the literature, it is a common experience that in a series of preparations, chances are low of getting a coupled one. The method described by Chambon and Labbe [3] is also a rapid one, and has the advantage of giving a higher yield of mitochondria; however, the results in the report of these authors show lower respiratory control than those reported in this paper. Discrepancies in the values of QO_2, and phosphorylating efficiency with different substrates with respect to the results of other authors may be due to characteristics of the yeast cells themselves and the preparation conditions. More detailed studies are in progress to define the contribution of each of these parameters to the properties of the mitochondria obtained.

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