Journal of Bioenergetics and Biomembranes, Vol. 26, No. 4, 1994

Preparation of Highly Phosphorylating Mitochondria from the Yeast *Schizosaccharomyces pombe*

Jean-Michel Jault,[1,3] Jane Comte,[2] Danièle C. Gautheron[1] and Attilio Di Pietro[2,4]

Received November 14, 1993

Schizosaccharomyces pombe yeast cells grown on either fermentable or respiratory media were efficiently converted to stable spheroplasts by the α-(1 $\rightarrow$ 3)-glucanase Novozym 234 in the presence of 1.2 M sorbitol. Lysis of spheroplasts by gentle homogenization in dilute sorbitol resulted in the preparation of mitochondria with a structure similar to that observed within the starting yeast cells. The isolated mitochondria exhibited high oxidation rates with various respiratory substrates, NADH being the most efficient. The mitochondria appeared well coupled since the second State 4 rate observed after ADP consumption was identical to the initial one. The State 3 rate in the presence of ADP was completely inhibited by low oligomycin concentrations, similarly to the concomitant ATP synthesis of 900 nmol/min $\times$ mg protein. These NADH oxidation and dependent ATP-synthesis activities are much higher than those previously described for mitochondria isolated from *Schizosaccharomyces pombe*, and similar to the highest values reported for *Saccharomyces cerevisiae*.

KEY WORDS: Isolated mitochondria; yeast *Schizosaccharomyces pombe*; spheroplasts; oxidative phosphorylation; respiration; ATP synthesis.

INTRODUCTION

Preparation of yeast mitochondria requires either mechanical fracture of the cell wall by glass beads (for a review, see Mattoon and Balcavage, 1967; Mackler and Haynes, 1973) or its enzymatic digestion. A comparative study of both methods in *Saccharomyces cerevisiae* showed that the enzymatic method provided mitochondria of better integrity, as monitored by higher respiratory rates and respiratory control ratios (Guérin *et al.*, 1979). The cell wall, which consists of high amounts of β-linked glucan (Phaff, 1971; Fleet and Phaff, 1973), can be digested by gastric juice from the snail *Helix pomatia* which contains β-(1 $\rightarrow$ 3)- and β-(1 $\rightarrow$ 6)-glucanases in addition to mannanase, cellulase, chitinase, lipase, polygalacturonase, and protease activities (Phaff, 1971). Some β-glucanase-enriched fractions were later obtained as helicase, glusulase, β-glucuronidase/arylsulfatase or zymolyase. These fractions were successfully used to prepare stable spheroplasts, whose lysis at low osmolarity under strong homogenization subsequently enabled the preparation of mitochondria (Kovac *et al.*, 1972; Briquet *et al.*, 1976; Guérin *et al.*, 1979; Daum *et al.*, 1982). This method was also successfully applied to other strains such as *Saccharomyces carlbergensis* (Ohnishi *et al.*, 1966) and *Candida parapsilosis* (Guérin *et al.*, 1989).

A major problem with *Schizosaccharomyces pombe* is that its cells wall also contains α-(1 $\rightarrow$ 3)-glucan (Fleet and Phaff, 1973; Bush *et al.*, 1974; Manners and Meyer, 1977), which is resistant to the above β-glucanases (for a recent review, see Fleet, 1991). This problem can be overcome by first treating the yeast cells with 2-deoxyglucose, an inhibitor of cell-wall synthesis, and then with the β-glucanases

[1] Campus de La Doua, Laboratoire de Biologie et Technologie des Membranes et des Systèmes Intégrés, Villeurbanne, Université Claude Bernard-Lyon I, France.
[2] Campus de Gerland, Institut de Biologie et Chimie des Protéines, Lyon, Université Claude Bernard-Lyon I, France.
[3] Present address: Department of Chemistry, University of California at San Diego, La Jolla, California.
[4] To whom correspondence should be addressed.

0145-479X/94/0800-0447$07.00/0

(Foury and Goffeau, 1973; Labaille *et al.*, 1977). The use of 2-deoxyglucose, however, presents a number of limitations due to (i) its toxicity on cell growth (Megnet, 1965), (ii) the fragility of the spheroplasts, and (iii) the loss in mitochondrial respiratory capacity related to a lowered amount of cytochrome *c* (Goffeau *et al.*, 1975). Mitochondria isolated under these conditions exhibit much lower rates of respiration and of ATP synthesis as compared to *Saccharomycetoidae*. More recently, an enzyme extract from *Trichoderma harzianum*, named mutanase or Novozym 234 and containing α-(1 $\rightarrow$ 3)-glucanase activity, in addition to minor β-(1 $\rightarrow$ 3)-glucanase, cellulase, and neutral protease activities, was found to produce spheroplasts and protoplasts from various yeast strains that were suitable for cell fusion and genetic transformation (Stephen and Nasim, 1981; Beach *et al.*, 1982; Dickinson and Isenberg, 1982; Boutry *et al.*, 1984; Sipiczki *et al.*, 1985; Mann and Jeffery, 1986), or for patch-clamp experiments (Vacata *et al.*, 1992).

The present paper describes the preparation of well-coupled and highly phosphorylating mitochondria from *Schizosaccharomyces pombe* using Novozym 234 to produce spheroplasts, which are stable at high osmolarity but sensitive to low osmolarity, combined with gentle homogenization during spheroplast lysis. This versatile method is applicable to different growth conditions and gives mitochondrial respiration rates and ATP synthesis much higher than previously described using 2-deoxyglucose treatment, and similar to the highest values reported for *Saccharomycetoidae*.

EXPERIMENTAL

Chemicals

ADP,[5] NADH, and bovine serum albumin (fatty acid free) were purchased from Boehringer Mannheim. Novozym 234 was from Novo Industries, Copenhagen, Denmark, and Zymolyase 20T came from Seikagaku, Kogyo Co., Japan. Hexokinase (type F-300) and Pipes came from Sigma; glutaraldehyde and osmium tetroxide were from Polysciences Inc., Warrington, Pennsylvania; sodium periodate, sodium cacodylate, and uranyl acetate were from Merck. [^{32}P] orthophosphate (10 mCi/ml) was from the Commissariat à l'Energie Atomique, France.

Yeast Cultures

The *Schizosaccharomyces pombe* wild strain 972h$^-$ was grown on either YD (2% yeast extract, 1% glucose) or YDG (2% yeast extract, 0.1% glucose, 3% glycerol) medium, at pH 4.5. The cultures (600–1,200 ml) were inoculated at about 1×10^6 cells/ml from precultures and allowed to grow at 30°C for the indicated times, up to either exponential or stationary phase; a final density of $60–100 \times 10^6$ cells/ml was obtained, as determined by cell counting with a Thoma hemacytometer after dilution in 20 mM EDTA.

Spheroplast Production

The yeast cells were harvested and centrifuged at $1,100 \times g$ for 5 min at 20°C; the pellet was resuspended in milliQ ultrapure water and centrifuged ($1,100 \times g$; 2-fold), and then adjusted to 10^9 cells/ml in 1.2 M sorbitol and 10 mM citrate, pH 5.8. Enzymes producing lysis of cell walls were added as indicated and incubation was performed at 30°C. Aliquots were withdrawn at intervals and counted for residual unlysed cells upon dilution in 20 mM EDTA containing either 1.2 or 0.3 M mannitol. Spheroplasts were identified as the osmotically sensitive cells which were intact at the high concentration of mannitol, but were lysed at the low concentration. When more than 90% cells were judged to have become spheroplasts, the mixture was centrifuged for 3 min at $1,250 \times g$ and 4°C. The pellet was washed three times after careful resuspension in 1.3 M sorbitol and 50 mM Tris-HCl, pH 7.5, and centrifugation. Spheroplasts were finally suspended in either 0.4 M sorbitol, 0.2% (w/v) bovine serum albumin, and 10 mM imidazole-HCl, pH 6.4 (YD culture), or 0.6 M mannitol, 4 mM EGTA, 0.2% bovine serum albumin, and 50 mM imidazole-HCl, pH 6.4 (YDG culture).

Preparation of Mitochondria

Spheroplasts were lysed by gentle homogenization for 3–15 s in a Waring blender set at low speed

[5] ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; ATPase, adenosine 5′-triphosphatase, E.C. 3.6.1.3; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid; F_1, soluble mitochondrial ATPase; NADH, reduced nicotinamide adenine dinucleotide; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, 2-amino-2-(hydroxymethylpropane)-1,3-diol; YD medium, 2% (w/v) yeast extract + 1% (w/v) glucose; YDG medium, 2% (w/v) yeast extract + 0.1% (w/v) glucose + 3% (v/v) glycerol.

and equipped with a power reducer (Triplex 541-C model 3 set on position 3). The homogenate was either directly centrifuged for 5 min at 1,250 × *g* and 4°C (YDG culture) or first mixed with an equal volume of 1 M sorbitol, 25 mM KH_2PO_4, 4 mM EGTA, 0.2% bovine serum albumin, and 10 mM imidazole-HCl, pH 6.4, and then centrifuged (YD culture). The pellet was resuspended in the same medium and submitted to a second Waring blender homogenization and centrifugation. Both supernatants were pooled and centrifuged for 15 min at 12,000 × *g* at 4°C. The supernatant was centrifuged again and both pellets were carefully resuspended in a low volume of 0.6 M mannitol, 2 mM EGTA, 0.2% bovine serum albumin, and 10 mM imidazole-HCl, pH 6.4. The mitochondria were kept on ice and their activities were measured during the same day. Proteins were estimated by either the biuret method (Gornall *et al.*, 1949) or the procedure of Lowry *et al.* (1951) using bovine serum albumin as a standard. The method resulted in preparations of mitochondria up to 20 mg protein/10^{10} yeast cells.

Electron Microscopy

Yeast cells were washed twice in ultrapure water and one time in 0.1 M Pipes, 2 mM EGTA, and 1 mM $MgCl_2$, pH 6.9, and harvested by centrifugation for 5 min at 3,000 × *g*. They were subsequently prefixed for 2 h at room temperature with 2% (v/v) glutaraldehyde in the same buffer and washed twice. The cells were then incubated in 1% (w/v) sodium metaperiodate for 1 h at 30°C (Van Tuinen and Riezman, 1987), washed in the above buffer, incubated in 50 mM ammonium chloride for 30 min at room temperature, and washed twice with 0.1 M sodium cacodylate, pH 6.8. The preparation of spheroplasts was adapted from the method of Banta *et al.* (1988). The prefixed washed cells were incubated in 1.2 M sorbitol, 50 mM Tris-HCl, and 5 mM EDTA, pH 8.0, for 10 min at 30°C, centrifuged, and suspended in 1.2 M sorbitol and 10 mM citrate, pH 5.8, containing Novozym 234 (0.1 mg/ml) and Zymolyase 20T (0.5 mg/ml). After 2 h at 30°C, the spheroplasts were centrifuged and washed twice with 0.1 M cacodylate, pH 6.8. Yeast cells and spheroplasts were postfixed overnight with 0.5% (w/v) osmium tetroxide and 0.1 M cacodylate, pH 6.8, at 4°C in the dark, washed with ultrapure water, prestained with 2% (w/v) aqueous uranyl acetate for 1 h at room temperature in the dark, and washed twice in water. Mitochondrial pellets were prefixed for 1 h at 4°C with 3% glutaraldehyde in 0.3 M mannitol, and 0.2 M cacodylate, pH 6.5, washed overnight, and postfixed for 1 h at room temperature in 2% osmium tetroxide and 0.2 M cacodylate, pH 6.5. After progressive dehydration with ethanol and embedding in Epon 812, polymerization was achieved overnight at 37°C and subsequently for three days at 60°C. Ultrathin sections were stained with 7% uranyl acetate and lead citrate as described previously (Vial *et al.*, 1981) and examined using a Jeol 1200EX electron microscope operating at 80 kV at the Centre de Microscopie Electronique Appliquée à la Biologie et la Géologie, Université Claude Bernard-Lyon I.

Assay of Mitochondrial Respiration

Oxygen consumption was measured polarographically with a Clark electrode (Hansatech D.W.). The cell was thermostated at 30°C and contained 1 ml of an oxygenated medium composed of 0.65 M mannitol, 2 mM $MgCl_2$, 16 mM KH_2PO_4, 10 mM imidazole, pH 6.4, and 50–100 μg of mitochondrial protein. State 4 rate was measured for several minutes upon addition of the respiratory substrate. Addition of ADP allowed the measurement of State 3 rate, corresponding to phosphorylating conditions, and of the second State 4 rate reached after complete ADP consumption. The respiratory control ratio was defined as the State 3 rate/second State 4 rate ratio. To calculate the ADP/O ratio, the amounts of oxygen consumed during State 3 respiration in the presence of known amounts of ADP were estimated from the polarographic recordings. Where indicated, specific inhibitors (8 μM rotenone, 5 μM antimycin, 3 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP), and up to 1.4 μg oligomycin) were added to the medium. In the latter case, the residual inhibited rate was measured after 1-min incubation.

Assay of Mitochondrial ATP Synthesis

The amount of ATP synthesized under conditions of State 3 respiration was measured in a 0.2-ml medium containing 0.65 M mannitol, 16 mM potassium phosphate, 5 mM $MgCl_2$, 50 mM imidazole, at pH 6.4–9.0, 20 mM glucose, 10 units hexokinase, 1 mM NADH, and 10^6 cpm [^{32}P] orthophosphate/μmol. The medium was thermostated at 30°C and oxygenated for at least 10 min before addition of mitochondria (50–100 μg protein). The reaction was initiated by addition of 1 mM ADP and stopped after

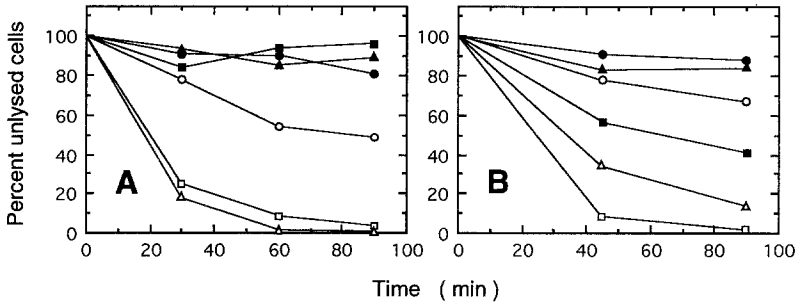

Fig. 1. Efficiency of cell-wall lytic enzymes to produce spheroplasts from differently cultured cells. Yeast cells grown on either YD medium (A) for 18 h (98×10^6 cells/ml) or YDG medium (B) for 23 h (76×10^6 cells/ml) were suspended in 1.2 M sorbitol and 10 mM citrate, pH 5.8, with either 1 mg Novozym 234/ml (squares) or 0.5 mg Zymolyase 20T/ml (circles), or with a mixture of both enzymes (triangles): 0.5 mg Novozym 234 + 0.1 mg Zymolyase 20T/ml (A) or 0.1 mg Novozym 234 + 0.5 mg Zymolyase 20T/ml (B). At the indicated times, aliquots were diluted in either 1.2 M (closed symbols) or 0.3 M (empty symbols) mannitol and counted with the hemacytometer.

1–2 min by mixing a 125-μl aliquot with 13-ml of isobutanol/benzene extraction medium (Pullman, 1967; Penin *et al.*, 1986). Under these conditions, ATP synthesis was linear with the reaction time. The radioactivity of the glucose-6-[^{32}P]phosphate produced was determined with a Beckman LS 7000 liquid scintillation counter. When indicated, 3 μM CCCP or 2.5–10 μg oligomycin was added before ADP.

RESULTS

Preparation of Mitochondria from *Schizosaccharomyces pombe* Spheroplasts

The efficiency of various cell-wall lytic enzymes, either alone or mixed together, to produce spheroplasts from *Schizosaccharomyces pombe* yeast cells is shown in Fig. 1. When the culture was performed on glucose (panel A), either Novozym 234 alone (1 mg/ml) or a mixture consisting of Novozym 234 (0.5 mg/ml) and Zymolyase 20T (0.1 mg/ml) resulted in the transformation of more than 95% yeast cells into spheroplasts during a 90-min treatment. The spheroplasts were quite stable in 1.2 M of either sorbitol or mannitol but very sensitive to low osmolarity (0.3 M). In contrast, Zymolyase 20T alone (0.5 mg/ml) was only partially efficient since less than 50% spheroplasts were obtained. Different sensitivities of the yeast cell wall were observed when the culture was performed on glycerol (panel B). The use of 1 mg/ml Novozym 234 was too extensive since more than 60% spheroplasts spontaneously lyzed at high osmolarity. The mixture constituted of 0.1 mg Novozym 234/ml and 0.5 mg Zymolyase 20T/ml was preferable since nearly all the spheroplasts were stable. Zymolyase 20T alone (0.5 mg/ml) appeared even less efficient than for glucose-grown cells since a very limited proportion of cells was converted to spheroplasts. As a consequence, we routinely used Novozym 234 alone (1 mg/ml) for glucose-grown cells, or a mixture of Novozym 234 (0.1 mg/ml) and Zymolyase 20T (0.5 mg/ml) for glycerol-grown cells.

Also when cells were grown on raffinose, an alternative fermentable substrate, Novozym 234 alone

Table I. Dependence of Mitochondrial Respiration Coupling on the Homogenization Strength[a]

Homogenization conditions				
Power reducer	Speed (rpm)	Time (s)	Revolution number	Respiratory control ratio[b]
No	13,500	15	3,375	1.2
No	13,500	3	675	2.3
Yes	3,144	5	262	3.0

[a] Yeast cells grown on YD medium were harvested at a cell density of 90×10^6 cells/ml and treated with 1 mg Novozym 234/ml for 90 min at 30°C as described in Fig. 1A. The spheroplasts were lysed under various homogenization conditions using a Waring blender set at low speed and sometimes equipped with a power reducer set on position 3. The rotation speed was measured using a tachometer (Jaquet 628 630).

[b] Mitochondria were prepared as described under Experimental and their respiration was assayed in 0.65 M mannitol, 10 mM imidazole, 2 mM $MgCl_2$ and 16 mM KH_2PO_4, pH 6.4, in the presence of 1 mM NADH. State 3 and second State 4 rates were measured upon addition of 0.1 mM ADP and enabled the calculation of the respiratory control ratio.

(1 mg/ml) or a mixture consisting of Novozym 234 (0.5 mg/ml) and Zymolyase 20T (0.5 mg/ml) were quite suitable for spheroplast production (not shown here). In contrast, other cell-wall lytic enzymes such as helicase (from Industrie Biologique Française) or β-glucuronidase/arylsulfatase (from Boehringer Mannheim) were not able to produce spheroplasts from *Schizosaccharomyces pombe* cells no matter what culture conditions were used.

The conditions of homogenization in a Waring blender to disrupt the spheroplast plasma membrane were critical for mitochondrial membrane integrity as monitored by the respiratory control ratio in the presence of NADH (Table I). The use of a power reducer, to lower speed and revolution number of the blender, markedly increased the respiratory control ratio from 1.2 to 3.0 under the respiration conditions used. Lower-quality mitochondria were obtained when the homogenization was performed with a manual Potter-Elvehjem apparatus since a value of 2.1 was obtained under the same respiration conditions.

Electron microscopy of the different fractions obtained from glucose or glycerol cultures is shown in Fig. 2. Structural integrity was maintained for both types of cells since plasma membranes, nuclei, vacuoles, lipid droplets, and mitochondria all appeared well preserved. The spheroplasts showed a characteristic spherical shape with an altered cell wall which resulted in a better contrast due to the facilitated diffusion of fixing and staining reagents. Isolated mitochondria appeared to be devoid of contamination by other subcellular components. Their internal structure was rather heterogenous: some mitochondria showed numerous well-developed cristae, others had

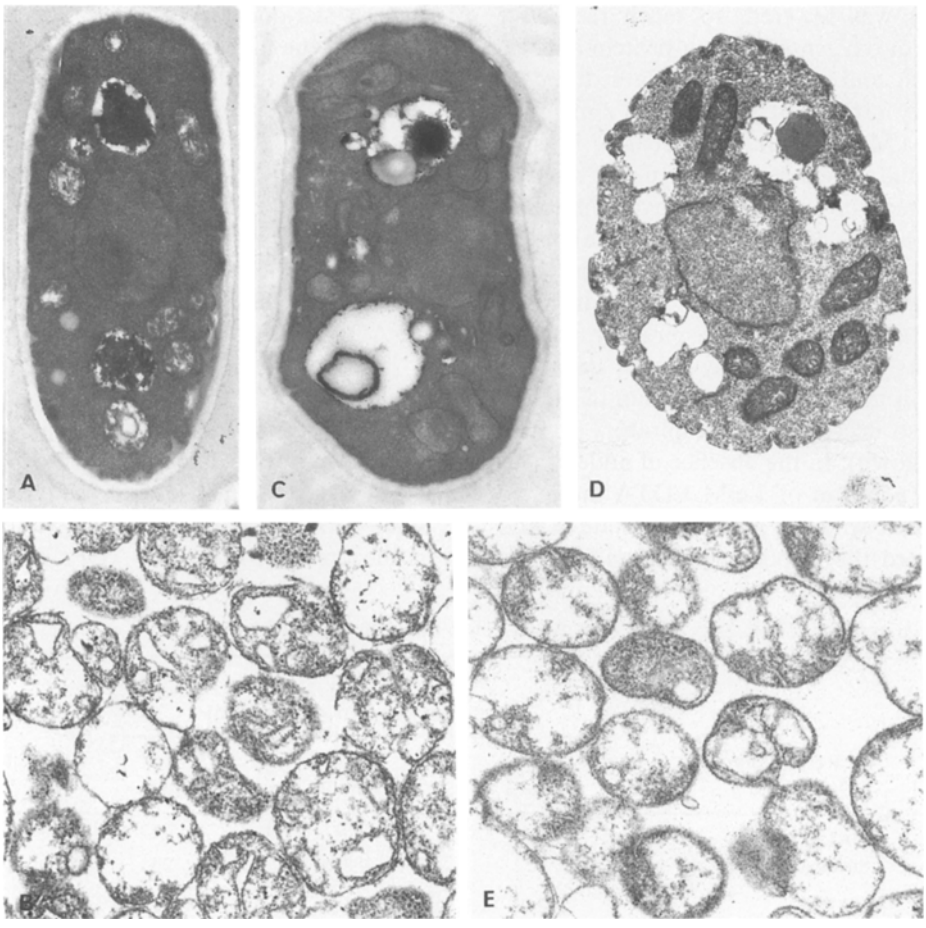

Fig. 2. Electron microscopy of yeast cells, spheroplasts, and isolated mitochondria. The different fractions obtained from a 16-h YD culture (A–B) at 95 × 10^6 cells/ml or a 24-h YDG culture (C–E) at 89 × 10^6 cells/ml were submitted to the successive treatments detailed under Experimental: yeast cells × 15,000 (A, C); spheroplasts × 15,000 (D); isolated mitochondria × 37,500 (B, E).

cristae of reduced size, and others showed peripheral concentric membranes. A similar heterogeneity was already present in the corresponding starting yeast cells.

Oxidative Phosphorylation by Isolated Mitochondria

Various parameters were found to modify markedly the respiration rates and respiratory control ratios in the presence of different substrates. Both the State 3 rate and the respiratory control ratio in the presence of 2 mM NADH measured with mitochondria isolated from a 14-h-grown culture were very dependent on the osmolarity of the respiratory medium. The same bell-shaped curve was obtained with maximal values of 190 nmol oxygen/min × mg protein (State 3 rate) and 3.0 (respiratory control ratio) at 0.6 M sorbitol. The values dramatically fell when osmolarity was lowered, to reach respective values of 100 nmol oxygen/min × mg protein and 1.6 at 0.2 M sorbitol, and were moderately reduced at higher osmolarity (160 nmol oxygen/min × mg protein and 2.7 at 1 M sorbitol). In all cases, NADH oxidation was totally insensitive to rotenone, whereas antimycin inhibited State 4 and oligomycin inhibited State 3. In contrast, 3 μM CCCP increased about 4-fold the State 4 rate. The respiration medium contained 2 mM $MgCl_2$ for two reasons: (i) its omission lowered both State 3 rate and respiratory control ratio, and (ii) its replacement by $CaCl_2$ caused a greater increase in the second State 4 than in State 3 and therefore also decreased the respiratory control ratio (data not shown). In the absence of added divalent cations, the addition of 1 mM EDTA increased the second State 4 without modifying State 3 and therefore decreased the respiratory control ratio. In contrast, EGTA did not produce any effect on the respiration rates and was therefore included, rather than EDTA, in the buffers used to prepare mitochondria from spheroplasts.

The growth phase of glucose cultures greatly modified mitochondrial respiration. Table II shows that the respiratory rates both with NADH and succinate + pyruvate was much higher when the cells were harvested at stationary phase (17 h) as compared to exponential phase (14 h). Both the State 4, State 3, and second State 4 rates were roughly doubled, and therefore the respiratory control ratio remained unchanged. When the yeast cells were grown on raffinose, a fermentable substrate not susceptible to catabolite repression, the mitochondrial respiration rates were similar to the high values observed for glucose at stationary phase. A set of respiratory substrates was checked with mitochondria prepared from glucose stationary phase cells (Table III). NADH produced the highest rates, both at State 3 and State 4 respiration, the second State 4 being identical to the initial one. Appreciable, although lower, respiration was also observed with Krebs-cycle intermediates provided 2 mM pyruvate was present, whereas the intermediate alone or pyruvate alone was only poorly oxidized. Respiratory control ratios of 2.3–4.0 were obtained when pyruvate was added to either succinate, α-ketoglutarate, malate, or citrate. In all cases, the ADP/O ratio was between 1.3 and 1.95.

Respiration rates using NADH as substrate were 2-fold higher when mitochondria were prepared from a glycerol culture as compared to glucose or raffinose. Values of 168–265 and 448–769 nmol oxygen/min × mg protein were respectively obtained for State 4 (or the identical second State 4) and State 3 in the presence of 1 mM ADP. The dependence of

Table II. Mitochondrial Oxidation Rates as a Function of the Growth Phase on Glucose Medium[a]

Growth phase	Respiratory substrate[b]	Oxidation rate (nmol oxygen/min × mg protein) State 4	State 3	Second State 4	Respiratory control ratio
Exponential	NADH	60	191	63	3.0
	Succinate + pyruvate	22	88	30	2.9
Stationary	NADH	110	334	112	3.0
	Succinate + pyruvate	43	199	66	3.0

[a] Mitochondria were prepared using the power reducer, as in Table I, from glucose-grown yeast harvested after either 14 h during the exponential phase (60 × 10^6 cells/ml), or 17 h during the stationary phase (95 × 10^6 cells/ml).

[b] The respiration medium contained either 1 mM NADH, as in Table I, or 16 mM succinate and 2 mM pyruvate.

Table III. Mitochondrial Oxidation Rates of Various Respiratory Substrates[a]

Respiratory substrate	Oxidation rate (nmol oxygen/min × mg protein) State 4	State 3	Second State 4	Respiratory control ratio	ADP/O ratio[b]
1 mM NADH	84–126	286–388	93–131	2.8–3.2	1.55–1.95
16 mM succinate	22	24			
2 mM pyruvate	20	20			
16 mM succinate + pyruvate	37–59	199–233	64–76	2.4–3.4	1.3–1.9
33 mM α-ketoglutarate + pyruvate	22	121	36	3.3	1.5
33 mM malate + pyruvate	28	160	40	4.0	1.5
16 mM citrate + pyruvate	23	127	55	2.3	1.4

[a] Mitochondria were prepared from glucose-grown yeast harvested at stationary phase (100 × 10^6 cells/ml after a 17-h culture) as in Table II, and assayed in the presence of various respiratory substrates.
[b] The ADP/O ratio was determined in the presence of 0.1 mM ADP.

State 3 on ADP concentration is illustrated in Fig. 3A: it increased linearly up to about 60 μM ADP and then reached a plateau value; the half-maximal effect was produced at 35 μM ADP. The maximal State 3 values was highly sensitive to oligomycin (Fig. 3B) since a near-complete inhibition was produced by a low concentration of oligomycin (14 μg/mg protein) with a half-maximal effect around 6 μg oligomycin/mg protein.

A high ATP synthase activity of about 900 nmol ATP/min × mg protein was determined in the presence of 1 mM NADH at pH 6.8–7.0, the optimal pH value (not shown here). The activity was very dependent on the medium pH since it abruptly fell at alkaline values to become completely abolished at pH 9.0 and decreased to 740 nmol ATP/min × mg protein at pH 6.5. ATP synthesis was strictly due to oxidative phosphorylation since it was more than 96% inhibited by either oligomycin or CCCP.

DISCUSSION

The present work describes the preparation of coupled highly phosphorylating mitochondria from the yeast *Schizosaccharomyces pombe* after spheroplast production by Novozym 234 and gentle homogenization. The method is applicable to yeast cells from different culture conditions using glucose, glycerol or raffinose growth medium during either exponential or stationary phase. The mitochondrial activities for NADH oxidation and related ATP synthesis are much higher than those previously reported using mitochondria from the same yeast

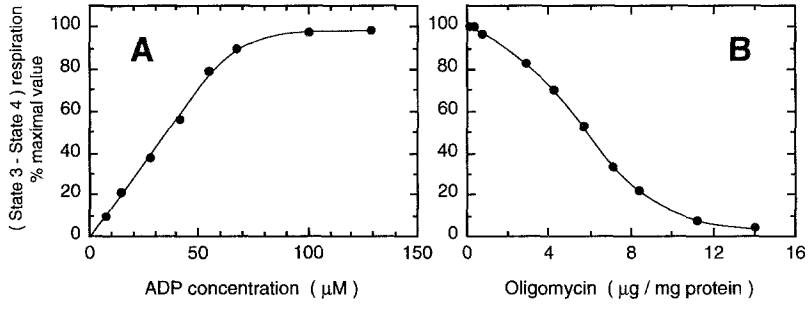

Fig. 3. ADP-dependent oligomycin-sensitive rate of NADH oxidation. Yeast cells were grown on YDG medium and harvested after 23 h, during exponential growth, at 80 × 10^6 cells/ml. Spheroplasts were produced by 0.1 mg Novozym 234 and 0.5 mg Zymolyase 20T/ml for 90 min, as illustrated in Fig. 1B, and mitochondria were prepared as previously using gentle homogenization with the power reducer. The respiratory medium contained 1 mM NADH and increasing ADP concentrations allowing estimation of the corresponding State 3 and State 4 rates (A). Increasing amounts of oligomycin were mixed with the medium before addition of 1 mM ADP, and the residual rates were measured (B).

but prepared by different procedures, and similar to the highest values reported with *Saccharomyces cerevisiae* whose cell wall is of different composition.

The results indicate that use of Novozym 234 is a prerequisite for successful production of spheroplasts from *Schizosaccharomyces pombe* cells. It can be used either alone at 1 mg/ml or as a mixture with Zymolyase 20T at lower concentration. Its efficiency is linked to its α-(1 $\rightarrow$ 3)-glucanase activity since the β-glucanases enriched preparations such as Zymolyase 20T, helicase, and β-glucuronidase/arylsulfatase were quite inefficient. The sphereoplasts produced by Novozym 234 are stable at high osmolarity (1.2 M of either sorbitol or mannitol), which agrees with the results of Sipiczki *et al.* (1985). This contrasts with the observation of Mann and Jeffery (1986) that spheroplasts undergo spontaneous lysis, even when a further-purified Novozym preparation was used. However, these authors used a considerably higher enzyme concentration and a medium of different composition with yeast cells harvested in exponential phase where they appear to be more fragile. Indeed, the efficiency of lysis by Novozym 234 depends on the growth conditions as shown here by the higher sensitivity to 1 mg Novozym 234/ml for cells grown on glycerol as compared to glucose. A similar differential sensitivity to β-glucanases was observed for 2-deoxyglucose-treated cells (Foury and Goffeau, 1973). *Saccharomyces cerevisiae* cells from exponential phase are also known to be more easily converted to protoplasts than the stationary-phase cells (Phaff, 1971).

The preparation of coupled mitochondria from *Schizosaccharomyces pombe* spheroplasts requires a much gentler homogenization at low osmolarity than the *Saccharomycetoidae* ones where strong and long homogenization conditions were used previously (Ohnishi *et al.*, 1966; Kovac *et al.*, 1968, 1972; Guérin *et al.*, 1979). Indeed, we show here that the best results are obtained by using a power reducer with the Waring blender homogenizer for lysis of Novozym 234-generated spheroplasts, whereas more drastic conditions using either vigorous magnetic stirring (Labaille *et al.*, 1977) or vortexing with glass beads (Boutry and Douglas, 1983) were applied to differently prepared spheroplasts. This might be related to the relative fragility of *Schizosaccharomyces pombe* mitochondria that are much more sensitive to osmolarity than those from *Saccharomyces carlbergensis* (Ohnishi *et al.*, 1966). Although many mitochondrial preparations use Dounce homogenization to break cells or spheroplasts (Daum *et al.*, 1982), we preferred to use a Waring blender homogenizer since spheroplasts from *Schizosaccharomyces pombe* appeared to be essentially broken by the hypotonicity of the medium, the main role of homogenization being to disperse mitochondria and prevent them from being drawn sedimenting with nuclei and cell debris. Under our conditions, the Waring blender homogenizer gave higher yields and more reproducible results than a manual Potter-Elvehjem apparatus.

We have omitted β-mercaptoethanol from our homogenization medium, which was found to be essential in the case of *Saccharomyces cerevisiae* (Kovac *et al.*, 1972; Guérin *et al.*, 1979). It was previously reported to produce no beneficial effects in *Schizosaccharomyces pombe*, as was observed for dithiothreitol (Fleet and Phaff, 1973), and to favor cell lysis (Sipiczki *et al.*, 1985). We have included EGTA instead of EDTA since the latter lowers the mitochondrial respiratory control ratio with NADH. EGTA was also used in a *Saccharomyces cerevisiae* preparation where Mg^{2+} ions were proposed to control the mitochondrial inner-membrane permeability (Arselin-De Chateaubodeau *et al.*, 1976).

When the morphological appearance of our mitochondrial preparation was analyzed by electron microscopy, isolated mitochondria from either glucose or glycerol culture were well conserved as compared to the starting yeast cells and spheroplasts, whose morphology was similar to that shown previously (Heslot *et al.*, 1970; McCully and Robinow, 1971). The good coupling of isolated mitochondria is illustrated with NADH as the respiratory substrate where the second State 4 is identical to the initial one, whereas this was not the case with mitochondria prepared either from spheroplasts generated by 2-deoxyglucose treatment and β-glucanases (Labaille *et al.*, 1977), or by glass-bead mechanical fracture of the cells (Heslot *et al.*, 1970). With all respiratory substrates tested, the present respiratory control ratio is significantly higher and the respiration rate at State 3 is at least 4-fold higher.

The values obtained here for respiratory control ratio (2.3–4.0) and ADP/O ratio (1.3–1.9), in the presence of Krebs-cycle intermediates and pyruvate, were similar to those reported with enzymatically prepared mitochondria from *Saccharomycetoidae* (Guérin *et al.*, 1979). However, higher values were measured in the latter mitochondria for α-ketoglutarate due to an additional substrate-level phosphorylation (Ohnishi *et al.*, 1966; Rigoulet *et al.*, 1983). The

fact that the ADP/O ratio in *Schizosaccharomyces pombe* approaches 2.0, but never exceeds it, suggests the functioning of two phosphorylation sites. The insensitivity to rotenone and the inhibition by antimycin indicate that the phosphorylation site I is either nonoperative under the conditions used, or absent, as previously concluded by Labaille *et al.* (1977). The existence of phosphorylation site I in *Saccharomycetoidae* is still a matter of debate since it is generally not detected although some authors reported its presence under particular growth conditions (see the discussion in the recent review of Guérin, 1991), as previously observed with *Candida* species where three phosphorylation sites were found (Downie and Garland, 1973). Cytosolic NADH is oxidized by an external NADH dehydrogenase in *Schizosaccharomyces pombe*, as occurs for mitochondria from other yeasts and also from plants (Palmer and Ward, 1985; Møller and Lin, 1986). A NADH:Q6 oxidoreductase was previously purified in *Saccharomyces cerevisiae* and found to be subject to glucose repression and therefore to appear more abundant at stationary—as compared to exponential–phase (De Vries and Grivell, 1988).

The ATP synthesis measured here in the presence of NADH with mitochondria prepared from glycerol-grown *Schizosaccharomyces pombe* cells is very high. The maximal activity of about 900 nmol ATP synthesized/min × mg protein is nearly 3-fold higher than the highest value previously reported (Labaille *et al.*, 1977) and 12-fold higher than the ATP-Pi exchange activity of mitochondria prepared through glass-bead vortexing homogenization (Boutry and Douglas, 1983). About 6-fold lower values were previously obtained in *Candida parapsilosis*, another "petite-negative" yeast (Guérin *et al.*, 1989). In the case of *Saccharomyces cerevisiae*, values of 300–400 (Rigoulet *et al.*, 1983; Manon and Guérin, 1988) or 800–1,400 (Mazat *et al.*, 1986; Paul *et al.*, 1992) nmol ATP synthesized/min × mg protein were reported respectively with ethanol or NADH as the respiratory substrate. The NADH-dependent ATP synthesis is shown here to be very dependent on the pH. The pH optimum of 6.8–7.0 is similar to the pH optimum of ATP hydrolysis recently measured in the presence of a proton gradient in plant mitochondria, which increased to 7.5–8.0 upon dissipation of the proton gradient by ionophores (Valerio *et al.*, 1993). Higher values for the pH optimum of ATP hydrolysis were also obtained in *Schizosaccharomyces pombe* for uncoupled mitochondria (Boutry and Goffeau, 1982), sonic submitochondrial particles (Jault *et al.*, 1989), and purified F_1-ATPase (Jault *et al.*, 1991, 1993).

In conclusion, we have described a versatile method for the preparation of well-coupled and highly phosphorylating mitochondria from *Schizosaccharomyces pombe*. The method is applicable to different growth conditions, and also to strains with mutated α- or β-subunits of the ATPase–ATP synthase complex. The mutation effects were previously studied on ATP hydrolysis (Boutry and Goffeau, 1982; Falson *et al.*, 1987; Jault *et al.*, 1989); the present method will allow their subsequent study on oxidative phosphorylation.

ACKNOWLEDGMENTS

Thanks are due to M. Rigoulet and B. Guérin, from Bordeaux, and to M. Boutry and A. Goffeau, from Louvain-La-Neuve, for stimulating discussions about preparation and activities of mitochondria. R. Mayne, University of Alabama at Birmingham, is thanked for English improvement and critical reading of the manuscript, D. Thinès-Sempoux for her advice concerning electron microscopy, and A. Bosch for the photographic work. Financial support was provided by the CNRS and the Université Claude Bernard-Lyon I.

REFERENCES

Arselin-De Chateaubodeau, G., Guérin, M., and Guérin, B. (1976). *Biochimie* **58,** 601–610.

Banta, L. M., Robinson, J. S., Klionsky, D. J., and Emr, S. C. (1988). *J. Cell Biol.* **107,** 1369–1383.

Beach, T., Piper, M., and Nurse, P. (1982). *Mol. Gen. Genet.* **187,** 326–329.

Boutry, M., and Douglas, M. (1983). *J. Biol. Chem.* **258,** 15214–15219.

Boutry, M., and Goffeau, A. (1982). *Eur. J. Biochem.* **125,** 471–477.

Boutry, M., Vassarotti, A., Ghislain, M., Douglas, M., and Goffeau, A. (1984). *J. Biol. Chem.* **258,** 2840–2844.

Briquet, M., Sabadie-Pialoux, N., and Goffeau, A. (1976). *Arch. Biochem. Biophys.* **174,** 684–694.

Bush, D. A., Horisberger, M., Horman, L., and Wursch, P. (1974). *J. Gen. Microbiol.* **81,** 199–206.

Daum, G., Böhni, P. C., and Schatz, G. (1982). *J. Biol. Chem.* **257,** 13028–13033.

De Vries, S., and Grivell, L. A. (1988). *Eur. J. Biochem.* **176,** 377–384.

Dickinson, D. P., and Isenberg, I. (1982). *J. Gen. Microbiol.* **128,** 651–654.

Downie, J. A., and Garland, P. B. (1973). *Biochem. J.* **124,** 123–134.

Falson, P., Di Pietro, A., Darbouret, D., Jault, J.-M., Gautheron, D. C., Boutry, M., and Goffeau, A. (1987). *Biochem. Biophys. Res. Commun.* **148,** 1182–1188.

Fleet, G. H. (1991). In *The Yeasts* (Rose, A. H., and Harrison, J. S., eds.), 2nd edn., Vol. 4, Academic Press, London, pp. 199–277.
Fleet, G. H., and Phaff, H. J. (1973). In *Yeast, Mould, and Plant Protoplasts* (Villanueva, J. R., Garcia-Acha, I., Gascon, S., and Uruburu, F., eds.), Academic Press, London, pp. 33–59.
Foury, F., and Goffeau, A. (1973). *J. Gen. Microbiol.* **75,** 227–229.
Goffeau, A., Briquet, M., Colson, A.-M., Delhez, J., Foury, F., Labaille, F., Landry, Y., Mohar, O., and Mrena, E. (1975). In *Membrane Biogenesis* (Tzagoloff, A., ed.), Plenum Press, New York, pp. 63–97.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). *J. Biol. Chem.* **177,** 751–766.
Guérin, B. (1991). In *The Yeasts* (Rose, A. H., and Harrison, J. S., eds.), 2nd edn., Vol. 4, Academic Press, London, pp. 541–600.
Guérin, B., Labbe, P., and Somlo, M. (1979). *Methods Enzymol.* **55,** 149–159.
Guérin, M., Camougrand, N., Caubet, R., Zniber, S., Velours, G., Manon, S., Guélin, E., and Cheyrou, A. (1989). *Biochimie* **71,** 887–902.
Heslot, H., Goffeau, A., and Louis, C. (1970). *J. Bacteriol.* **104,** 473–481.
Jault, J.-M., Di Pietro, A., Falson, P., Gautheron, D. C., Boutry, M., and Goffeau, A. (1989). *Biochem. Biophys. Res. Commun.* **158,** 392–399.
Jault, J.-M., Di Pietro, A., Falson, P., and Gautheron, D. C. (1991). *J. Biol. Chem.* **266,** 8073–8078.
Jault, J.-M., Divita, G., Allison, W. S., and Di Pietro, A. (1993). *J. Biol. Chem.* **268,** 20762–20767.
Kovac, L., Bednarova, H., and Greksak, M. (1968). *Biochim. Biophys. Acta* **153,** 32–42.
Kovac, L., Groot, G. S. P., and Racker, E. (1972). *Biochim. Biophys. Acta* **256,** 55–65.
Labaille, F., Colson, A.-M., Petit, L., and Goffeau, A. (1977). *J. Biol. Chem.* **252,** 5716–5723.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193,** 265–275.
Mackler, B., and Haynes, B. (1973). *Biochim. Biophys. Acta* **292,** 88–91.
Mann, W., and Jeffery, J. (1986). *Bioscience Rep.* **6,** 597–602.
Manners, D. J., and Meyer, M. T. (1977). *Carbohydr. Res.* **57,** 189–203.
Manon, S., and Guérin, M. (1988). *Eur. J. Biochem.* **172,** 205–211.
Mattoon, J. R., and Balcavage, W. X. (1967). *Methods Enzymol.* **10,** 135–142.
Mazat, J.-P., Jean-Bart, E., Rigoulet, M., and Guérin, B. (1986). *Biochim. Biophys. Acta* **849,** 7–15.
McCully, E. K., and Robinow, C. F. (1971). *J. Cell. Sci.* **9,** 475–507.
Megnet, R. (1965). *J. Bacteriol.* **90,** 1032–1035.
Møller, I. M., and Lin, W. (1986). *Annu. Rev. Plant Physiol.* **37,** 309–334.
Ohnishi, T., Kawaguchi, K., and Hagihara, B. (1966). *J. Biol. Chem.* **241,** 1797–1806.
Palmer, J. M., and Ward, J. A. (1985). *Encycl. Plant Physiol.* **18,** 173–201.
Paul, M.-F., Guérin, B., and Velours, J. (1992). *Eur. J. Biochem.* **205,** 163–172.
Penin, F., Deléage, G., Godinot, C., and Gautheron, D. C. (1986). *Biochim. Biophys. Acta* **852,** 55–67.
Phaff, H. J. (1971). In *The Yeasts* (Rose, A. H., and Harrison, J. S., eds.), 1st edn., Vol. 2, Academic Press, London, pp. 135–210.
Pullman, M. E. (1967). *Methods Enzymol.* **10,** 57–60.
Rigoulet, M., Ezzahid, Z., and Guérin, B. (1983). *Biochem. Biophys. Res. Commun.* **113,** 751–756.
Rigoulet, M., Velours, J., and Guérin, B. (1985). *Eur. J. Biochem.* **153,** 601–607.
Sipiczki, M., Heyer, W.-D., and Kohli, J. (1985). *Curr. Microbiol.* **12,** 169–174.
Stephen, E. R., and Nasim, A. (1981). *Can. J. Microbiol.* **27,** 550–553.
Vacata, V., Höfer, M., Larsson, H. P., and Lecar, H. (1992). *J. Bioenerg. Biomembr.* **24,** 43–53.
Valerio, M. B., Haraux, F., Gardeström, P., and Diolez, P. (1993). *FEBS Lett.* **318,** 113–117.
Van Tuinen, E., and Riezman, H. (1987). *J. Histochem. Cytochem.* **35,** 327–333.
Vial, C., Comte, J., Font, B., and Gautheron, D. C. (1981). *Biol. Cell.* **41,** 195–202.