STABLE PHOSPHORYLATING SUBMITOCHONDRIAL PARTICLES FROM BAKER'S YEAST*

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The genetic control and the enzymic and morphological variability of mitochondria from baker's yeast (Saccharomyces cerevisiae) have been extensively studied (1-4). Little is known, however, about oxidative phosphorylation in these organelles. In mammalian systems, studies of oxidative phosphorylation have been greatly facilitated by the availability of phosphorylating submitochondrial particles which are less complex and in general also more stable than mitochondria. The preparation from baker's yeast of phosphorylating mitochondria (5-7) as well as of non-phosphorylating submitochondrial particles (8) has been reported, but submitochondrial particles catalyzing oxidative phosphorylation have not been described.

The present communication outlines a procedure for the isolation of stable phosphorylating submitochondrial particles from commercially grown baker's yeast. In addition, evidence is presented that the mitochondria as well as the submitochondrial particles as isolated by the present procedure lack the phosphorylation site between DPNH and cytochrome b.

Experimental Procedure: Baker's yeast cells (680 g), obtained from the National Yeast Corp., Belleville, New Jersey, are washed by suspending three

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times with 3 l of distilled water and once with 2 l of 0.25 mannitol, 20 mM Tris SO_4 (pH 7.4), 1 mM EDTA (MTE - medium). Between each washing step the cells are reisolated by centrifugation. The cells are evenly suspended in MTE-medium to a final volume of 960 ml; 20-28 ml aliquots (see below) are poured into 75 ml glass homogenizing flasks** and shaken for 20 seconds with exactly 50 g of glass beads ** (0.45-0.50 mm diameter) at 4000 cycles per minute in a Bronwill MSK Mechanical Cell Homogenizer** cooled with liquid carbon dioxide. In order to obtain satisfactory cell breakage (25-40%) it is important to fill the flasks to not more than 2/3 of capacity. When all of the cell suspension has been processed, the turbid fluid is decanted from the glass beads, which are rinsed several times with MTE-medium. The washings are combined with the homogenized suspension to give a final volume of 1900 ml. Residual glass beads, unbroken cells and large debris are removed by two successive centrifugations at 2000 x g for 10 minutes (Lourdes VRA rotor; 3500 rpm). The supernatant fluid from the second centrifugation at 2000 x g is referred to as "homogenate". The pH is 6.1-6.4 and is not further adjusted. All subsequent centrifugation steps are carried out in a Spinco Model L ultracentrifuge. The homogenate (1400 ml; 30.5-42.7 g protein) is centrifuged for 15 minutes at 10,000 rpm in the No. 21 rotor. The sediment is homogenized gently in 300 ml MTE-medium, centrifuged for 15 minutes at 11,000 rpm in the No. 30 rotor, and resuspended in 60 ml MTE-medium. Centrifugation for 15 minutes at 15,000 rpm in the No. 40 rotor yields 0.35-1.1 g of "heavy" mitochondria, which are suspended in 10 mM Tris SO_4 (pH 7.4), to give a final protein concentration of 20 mg/ml. Aliquots of 8.0 ml are exposed to ultrasonic oscillation for 40 seconds at 0- 4° in a 20 kc ultrasonic disintegrator (Measuring and Scientific Equipment Ltd.), fitted with the small probe. The suspension is then centrifuged for 15 minutes at 17,000 rpm in the No. 40 rotor. The supernatant fluid is centrifuged for 30 minutes at 50,000 rpm in the No. 50 rotor to sediment the phosphorylating submitochondrial par-

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ticles. Approximately 15-20% of the mitochondrial protein is recovered in this fraction. The particles are suspended in 10 mM Tris SO_4 (pH 7.4) to give a protein concentration of 20-30 mg/ml and stored at -55° in several aliquots. Protein is determined by the biuret procedure (9).

Results and Discussion: The oxidation rates and P/2e ratios of mitochondria and submitochondrial particles with different substrates and acceptors are listed in Table I. The isolated mitochondria oxidize pyruvate-malate at 1/3 to 1/4 the rate of succinate. If stored at -55° they slowly, but consistently lose phosphorylation capacity. In contrast, the submitochondrial particles do not oxidize pyruvate-malate and are stable at -55° for at least 3 weeks if freezing and thawing is avoided. They form a single band upon equilibrium centrifugation in a sucrose density gradient. In the electron microscope, on negative staining with 2% phosphotungstate, they appear as vesicles with diameters between 0.05 and 0.2 μ. No contamination with unbroken "heavy" mitochondria (diameter 0.7-1.5 μ) can be seen. Phosphorylating "light" mitochondria, isolated from the washings of the "heavy" mitochondria by centrifugation for 15 minutes at 35,000 x g, differ from the submitochondrial particles described here in that they are rather unstable upon storage, are not homogeneous as determined by density gradient centrifugation and oxidize pyruvate-malate at a significant rate.

The DPNH oxidase system of mitochondria and of submitochondrial particles is not inhibited by 2 μM rotenone, which is in agreement with the results obtained by Ohnishi and Hagihara (7). The succinoxidase system of the submitochondrial particles is resistant to thienyl-1,1,1-trifluoro-2,4-butanedione in concentrations as high as 4 mM. Higher concentrations lead to a parallel decrease of DPNH oxidase and succinoxidase activities. The cytochrome content of mitochondria and submitochondrial particles is given in Table II. It can be seen that the submitochondrial particles contain higher concentrations of the cytochromes a + a_3 and b than the mitochondria and that relatively little cytochrome c is lost during the preparation of the

TABLE I

Oxidation rates and P/2e ratios with various substrates and acceptors observed with mitochondria and submitochondrial particles from baker's yeast

Reaction measured	Mitochondria		Submitochondrial particles	
	Specific activity*	P/2e	Specific activity*	P/2e
Succinate $\longrightarrow$ O_2	0.20	1.15	0.29	0.55
α-glycerophosphate $\longrightarrow$ O_2	0.28	1.02	0.37	0.45
Ethanol $\longrightarrow$ O_2	0.16	1.20	0.15**	0.38**
Pyruvate-malate $\longrightarrow$ O_2	0.046	1.20	0.00***	-
Site I (DPNH $\longrightarrow$ Fumarate)	0.004	0.00	0.007	0.00
(DPNH $\longrightarrow$ CoQ_1	3.14	0.00	4.57	0.00
Site II	0.16	0.36	0.22	0.18
Site III	0.32	0.70	0.44	0.20

* μmoles of substrate oxidized per minute per mg of protein.
** In the presence of 0.2 μmole DPN^+ and 300 μg yeast alcohol dehydrogenase.
*** In the presence of 0.2 μmole DPN^+.

All measurements were carried out at 22-24° in the presence of 2.0 μmoles $MgCl_2$, 0.48 μmole EDTA, 5.0 μmoles Tris SO_4, pH 7.4, 32 μmoles glucose, 1.0 μmole ATP, 30 units dialyzed hexokinase, 2.0 mg dialyzed bovine serum albumin, 16 μmoles $^{32}P_i$, pH 7.4 (0.8-2.0 X 10^5 c.p.m./μmole). The final volume was always adjusted to 1.03 ml. by the addition of 0.25 M sucrose. The following concentration of substrates was used: succinate, 10 mM; α-glycerophosphate, 6 mM; pyruvate and malate, 1 and 7 mM; ethanol, 15 mM. Oxidative phosphorylation with oxygen as acceptor was assayed polarographically at a particle concentration of 620 μg/ml. Oxidative phosphorylation at site I with DPNH as electron donor was assayed spectrophotometrically at 340 mμ, either with CoQ_1 as acceptor in the presence of 100 μg particles as described elsewhere (10) or with fumarate (4 mM) as electron acceptor (11) in the presence of 1.6 mM KCN and 518 μg particles. Phosphorylation in these two systems was corrected for that observed in the absence of CoQ_1 and fumarate, respectively. Site II was assayed by measuring phosphorylation coupled to the reduction of 0.8 μmole of ferricyanide by succinate in the presence of 1.6 mM KCN. The concentration of particles was 280 μg/ml. Reduction of ferricyanide was followed spectrophotometrically at 420 mμ (ε mM = 1.0). In calculating P/2e ratios in this system, two corrections were applied: The amount of ferricyanide reduced was corrected for that reduced in the same length of time in a reaction insensitive to 1 μg antimycin/mg particle protein. The antimycin-insensitive reduction (15-19% of that in the absence of antimycin) was found to be non-phosphorylating. In addition, the amount of ATP formed in the absence of ferricyanide was subtracted from that formed in its presence. This second correction amounted to 5-7%. Site III was measured polarographically in the presence of 300 μg particles, 2 μg antimycin, 0.1 μmole tetramethyl-diamino-p-phenylenediamine and 2 μmoles ascorbate, pH 7.4. Incorporation of $^{32}P_i$ into glucose-6-P was determined as described by Conover et al. (12).

TABLE II

Cytochrome content of mitochondria and submitochondrial particles from S. cerevisiae

Cytochrome	Wavelength pair used (mμ)	ε mM	Cytochrome content (mμmoles/mg protein)		
			Mitochondria I	Submitochondrial particles II	II/I
$a + a_3$	605 - 625	16	0.33	0.44	1.33
b	562 - 575	20	0.30	0.40	1.33
$c + c_1$	551 - 540	19	0.49	0.51	1.04

The measurements were carried out essentially as described elsewhere (13). The values represent the amount of cytochrome reducible by DPNH.

particles. With both mitochondria and submitochondrial particles, the same P/2e ratio is obtained with flavin-linked substrates and DPN-linked substrates (Table I). These findings suggest that the first phosphorylation site is not operative in these yeast particles. Similar results with yeast mitochondria have been reported previously (5,7). To obtain more direct evidence on this point the measurement of the individual phosphorylation sites was undertaken. The assays of sites II and III presented little difficulty, whereas the assay of site I was complicated by the unusual properties of the DPNH-cytochrome b segment of yeast which are described in the accompanying paper. In the submitochondrial particles from yeast, the coenzyme Q_1 assay for oxidative phosphorylation at site I (10) did not reveal any ATP formation, although ATP formation at site II and III was readily apparent. The experiments reported in the accompanying paper suggest, however, that yeast particles, in contrast to beef heart particles (10), reduce coenzyme Q_1 by DPNH exclusively at the level of DPNH dehydrogenase. Oxidative phosphorylation at site I was therefore also measured with fumarate as electron acceptor (11). Although the rate of this reaction in

yeast particles is very low (0.6-0.9% of the rate of DPNH oxidase) and permits only qualitative conclusions, it is clear that no phosphorylation accompanied this oxidation step.

Experiments designed to detect an ATP-dependent reduction of DPN^+ by succinate or an ATP-dependent hydrogen transfer from DPNH to TPN^+ were unsuccessful. The yeast particles prepared as described here thus lack a functional first phosphorylation site. The present experiments, however, do not rule out the existence of this site in yeast mitochondria prepared by more gentle procedures or from different yeast strains.

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