How to Isolate Tightly-Coupled Mitochondria from *Neurospora crassa* mycelium. A Fast and Reproducible Procedure

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Background

Substation genetic and molecular information has been obtained after 70 years of intense research with *Neurospora* which provided foundations for studying mitochondrial activities and biogenesis.[Davies, 2002; Borkovich *et al*., 2004]. The basic understanding of mitochondrial operation has been enhanced tremendously in recent years by advances in structural biology that span the resolution spectrum. X-ray crystallography of large soluble and integral membrane protein complexes is providing atomic-level detail of the mitochondrial respiratory machinery, promising to unravel the mechanisms of electron transfer, proton pumping, and phosphorylation. However, a frustrating limitation is the lack of a technique allowing for the isolation of fairly intact mitochondria at different growth stages [see Sebald *et al*., 1979; Lambowitz *et al*., 1972a; Lambowitz and Slayman, 1972; Schwitzguebel and Palmer, 1982; Cramer *et al*., 1983; Bowman and Bowman, 1988]. Therefore, methods for the rapid and large-scale isolation of physiologically intact organelles from Neurospora mycelium ought to be of general value. Here, we report an improved procedure for preparation of fairly intact mitochondria from *N. crassa* mycelium at different growth phases. We empirically optimized the composition and concentrations of the lytic enzymes, the time required for spheroplast formation, and the composition of washing and grinding media. In addition, the whole procedure was significantly simplified and shortened to 2.5 h. Thus, the distinctive advantages of the protocol proposed are its simplicity, reproducibility, and versatility. The mitochondria preparations isolated from mycelium harvested from exponential and stationary growth phases respired actively with Krebs cycle intermediates, exogenous NADH, and some other substrates. They met all known criteria of physiological intactness; they controlled metabolic states upon successive additions of ADP, displayed good respiratory control (reflecting the degree of oxidation to phosphorylation coupling) and were close to the theoretically expected ADP/O values (reflecting the efficiency of the oxidative phosphorylation) (see Table 1). RC and ADP/O ratios upon oxidation of all examined substrates were higher than previously reported [Lambowitz *et al*., 1972b]; moreover, RC ratios, obtained upon oxidation of 2-oxoglutarate attained record values, far exceeding those reported not only for *N. crassa* mitochondria, but also for mitochondria from other fungi. Ficoll-purified mitochondria displayed 2-fold higher respiratory rates, retaining almost the same RC and ADP ratios (not shown). Mitochondria contained the fully competent respiratory chain with all three points of energy conservation. Oxidation of all examined substrates by mitochondria from WT was mediated by two alternative terminal oxidative

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Abbreviations: SHAM) salicylhydroxamic acid; RC) respiratory control; HEPES) (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]); PMSF) phenylmethylsulfonyl fluoride.

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systems, with the more pronounced engagement of the alternate oxidase in the stationary growth phase. Mitochondria were fully functional for at least 2.5 h when stored on ice. The utility of the protocol described was substantiated in investigation of Complex I of the *N. crassa* respiratory chain [Grivennikova *et al*., 2003] and in comparative studies of the respiratory chain in *N. crassa* wild type (WT) and in the transport mutant *nap* [Isakova et al., 2002; Belozerskaya et al., 2003].

Procedure

N. crassa WT (RL3-8A) [FGSC#2218] strain was generously provided by Fungal Genetics Stock Center (FGSC, University of Kansas, Kansas City, USA). Mycelia were grown in aerated liquid cultures as described previously [Sokolovsky *et al*., 1983], in Vogel's N medium supplemented with 2% sucrose. Cells harvested at exponential or stationary growth stages (3.2 to 9.3 or 16.0 to 27.0 g wet weight per litre, respectively) were separated from the culture medium by filtration through three layers of muslin, washed three times with ice-cold water, suspended (1 ml per g wet biomass) in spheroplast medium containing 1.0 M sorbitol, 50 mM EDTA, 10 mM HEPES-buffer, pH 7.5, helicase (complex of lytic enzymes from the snail *Helix pomatia*, 40 mg per g wet weight), and Novozyme T-234 (3 mg per g wet weight), and incubated under mild stirring at 28-30^{o}C for 6-8 min. Spheroplast formation was examined under a light microscope. Incubation with lytic enzymes was terminated by adding 8 volumes of ice-cold spheroplast medium supplemented with 0.3 mM PMSF, an inhibitor of proteinases.

The following steps were performed at 0-2^{o}C. Spheroplasts were pelleted by centrifugation for 10 min at 1800g, washed twice with spheroplast medium supplemented with 0.25% (w/v) BSA, suspended in the isolation buffer containing 0.4 M mannitol, 1.0 mM EDTA, 10 mM Tris-HCl-buffer, pH 7.2, 0.4% BSA, and 0.3 mM PMSF, and disrupted for 2 min in a 20-ml all-glass Dounce homogenizer (Kontens, Vineland, NY, USA) using twenty up and down strokes with a high clearance pestle (total clearance 0.12 mm). The resulting homogenate was diluted 1:1 with the washing buffer containing 0.6 M mannitol, 1.0 mM EDTA, 10 mM Tris-HCl-buffer, pH 7.2, 0.4% BSA and centrifuged for 13 min at 1600 g. The pellet was discarded, while the supernatant was centrifuged for 20 min at 7,000g. The mitochondrial pellet obtained was washed in the same washing buffer and used for energy parameters measurements. The yield of mitochondria attained 4.0-5.0 or 0.5-1.0 mg protein from 1 g initial wet biomass harvested at exponential or stationary phase, respectively. The mitochondria thus obtained kept unchanged energy parameters for at least 2.5 h. In some experiments, mitochondria were purified in Ficoll gradient. For this purpose, 9 ml of mitochondrial suspension were layered over a two-step Ficoll gradient (7.5 and 13.0 % Ficoll in the washing buffer) and centrifuged at 10,000 g for 30min). The fraction accumulating at the interface between the two Ficoll layers was collected, slowly diluted 1:4 with the washing buffer, and centrifuged at 10,000 g) for 20 min. The pellet was resuspended in the minimal volume of the washing buffer supplemented with 0.5 % BSA.

Oxygen consumption in mitochondrial suspensions was monitored amperometrically with a Clark-type closed platinum electrode. The incubation medium contained 0.6 M mannitol, 2 mM Tris-phosphate, pH 6.6, 2 mM EDTA, 1% BSA, 4 to 20 mM respiratory substrate, and 0.2-0.3 mM ADP. Respiratory control (RC) and ADP/O ratios were calculated as

recommended [Chance and Williams, 1956]. When indicated, 10 μM rotenone (an inhibitor of NADH-dehydrogenase and point I of energy conservation in the respiratory chain), 2 mM KCN (an inhibitor of the cytochrome oxidase), or 2 mM salicylhydroxamic acid [SHAM, an inhibitor of the alternative (non-phosphorylating) cyanide-resistant oxidative pathway] were added.

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Table 1. Oxidative and phosphorylative activities of *Neurospora crassa* mitochondria isolated from wild type mycelium harvested at different growth phases *

Substrate	Oxidation consumption in state 3 respiration, ng-atom O in min per mg pr.	RC	ADP/O	Inhibition by KCN, %	Inhibition by KCN + SHAM,%
Exponential growth phase					
2-Oxoglutarate	147±1	6,17±1,15	3,35±0,33		
Pyruvate + malate	148±53	2,46±0,27	2,41±0.1	66,0±3,2	98-100
Succinate	491±67	2,27±0,17	1,86±0,25	92,0±6,5	99-100
NADH	416±50	1,97±0,11	1,78±0,18	95,8±4,6	99-100
Stationary growth phase					
2- Oxoglutarate	126±10	5,22±0,28	2,93±0,26		
Pyruvate + malate	116±9	2,29±0.03	2,59±0,03	80,0±3,1	99-100
Succinate	497±60	1,75±0,05	1.80±0.01	85,0±6,7	99-100

NADH	591±41	1,92±0,12	1,75±0,09	82,0±8,0	99-100

*Average mean from 4 to 6 independent experiments