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Isolation and Characterization of Tightly Coupled Mitochondria from Wild Type and *nap* Mutant *Neurospora crassa*

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Abstract—A fast and reproducible procedure was elaborated for isolation of tightly coupled mitochondria from wild type and *nap*-mutant *Neurospora crassa* cells harvested at different growth stages. The isolated mitochondrial preparations had controlled metabolic states and were tightly coupled, i.e., displayed good respiratory control and had close to the theoretically expected maximal ADP/O ratios upon oxidation of Krebs cycle intermediates and exogenous NADH. They contained the fully competent respiratory chain with all three points of energy conservation. Oxidation of all examined substrates by mitochondria from both wild type and mutant cells was mediated by two alternative terminal oxidative systems, albeit to varying extent, with the more pronounced engagement of the alternative oxidase in the stationary growth phase and with a minor contribution of this non-phosphorylating pathway in the substrate oxidation by mutant mitochondria. Oxidation of NAD-dependent substrates by mitochondria from the two cell types was accommodated via both rotenone-sensitive and rotenone-insensitive pathways, while the level of rotenone-insensitive pathway in mutant cells was lower than in wild type cells. It is suggested that a more limited contribution of alternative non-phosphorylating oxidative pathways to the total respiration in mutant cells, as compared with wild type cells, could, at least partially, explain an elevated ATP level in these cells. However, the absence of principal differences in the arrangement of the respiratory chain in mitochondria of wild type and mutant cells implies that the elevated ATP level in the *nap* mutant is largely related to reduced ATP expenses for transport processes in these cells.

Key words: fungi, *Neurospora crassa*, *nap* mutant, mitochondria, respiratory chain

The mitochondrial respiratory chain of *Neurospora crassa* wild type is similar to that of many higher organisms; electron transport is inhibited by cyanide and antimycin A [1, 2] and oxidation is coupled to phosphorylation at three discrete points [3]. Therefore, this fungus was used as a promising (suitable) model to study complex I of the respiratory chain [4, 5]. A number of mutants of *N. crassa* with grossly altered energy status have been isolated [3, 6, 7]. One of these is the transport mutant *nap* (neutral and acidic amino acid permeability) [8], obtained by mutation in a single gene [9] and appropriately named for its reduced permeability for neutral amino acids. The mutant displayed slower growth rate [9]; reduced (by 30 to 60%) rate for transport of amino acids, uridine, and glucose [10, 11]; diminished membrane potential generated across the plasma membrane [12]; reduced activity of the plasma membrane H^+-ATPase [13], and elevated 1.5-fold, as compared to wild type cells, intracellular ATP content [9].

Because the H^+-ATPase, maintaining the H^+ gradient across the fungal plasma membrane, which is the driving force for transport processes, consumes up to 50% of the cellular ATP [14], it seems likely that the observed increase in the ATP content in mutant cells may, at least partially, relate to the reduced rate of transport processes in these cells. However, the increased ATP content may also be a result of enhanced ATP production in these cells. To test this possibility we examined the particular arrangement of the respiratory chain in *N. crassa* cells of the two phenotypes, as in the overwhelming majority of fungal aerophiles, the mitochondrial respiratory chain is the principle mechanism for intracellular ATP formation. Here we elaborated a procedure for isolation of physiologically intact, tightly coupled mitochondria from *N. crassa* cells harvested at different growth stages. To our knowledge, such information is not available for either the *N. crassa nap* mutant or for the wild type.

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

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MATERIALS AND METHODS

Reagents. Reagents of the highest quality available were used. BSA, EDTA, sorbitol, mannitol, ADP, AMP, Tris, HEPES, PMSF, pyruvate, malate, 2-oxoglutarate, succinate, and Novozym 234 were purchased from Sigma (USA); Coomassie G-250 from Serva (Germany); dithiothreitol from Reanal (Hungary). Other reagents of analytical grade were obtained from domestic suppliers.

Biomass preparation. *N. crassa* wild type (RL3-8A) [FGSC#2218] and *nap* mutant (Sta-73a) [FGSC#1604] strains were generously provided by Fungal Genetics Stock Center (FGSC, University of Kansas, Kansas City, USA). Cells were cultured as described previously [12, 15]. Macroconidia of the two strains grown at 28°C for 7 days on solid Vogel N medium [16] supplemented with 2% sucrose were used as inoculum for 200 ml of the equivalent liquid medium.

Cells were harvested at the exponential or stationary growth phases corresponding to 3.2 to 9.3 or 16.0 to 27.0 g wet weight per liter, respectively.

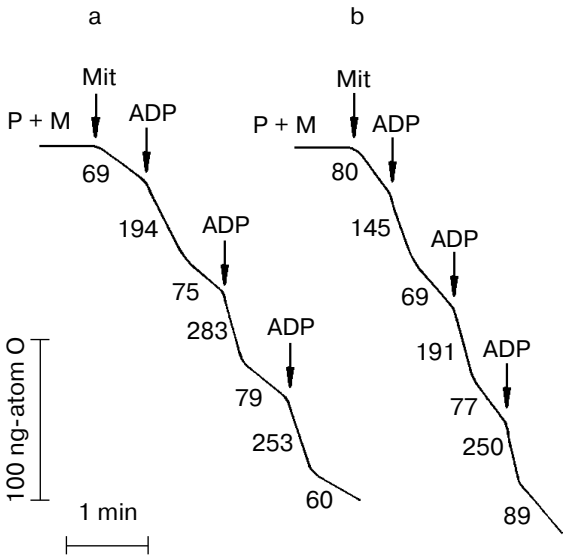
Isolation of mitochondria was performed according to a protocol specially designed for *N. crassa*. Cells were separated from the culture medium using three layers of muslin, washed with ice-cold water, and suspended in spheroplasting medium (1 ml per g wet biomass) containing 1.0 M sorbitol, 50 mM EDTA, 10 mM HEPES-buffer, pH 7.5, helicase (complex of lytic enzymes from the snail *Helix pomatia*, 40 mg per g wet weight), and Novozym T-234 (3 mg per g wet weight). The cell suspension was incubated under mild stirring at 28-30°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 medium containing 1.0 M sorbitol, 50 mM EDTA, 10 mM HEPES-buffer, pH 7.5, and 0.3 mM PMSF, an inhibitor of proteinases. Spheroplasts were pelleted by centrifugation for 10 min at 1800*g*, washed twice with 10 mM HEPES-buffer, pH 7.5, containing 1.0 M sorbitol and 0.25% (w/v) BSA, suspended in 10 mM Tris-HCl-buffer, pH 7.2, supplemented with 0.4 M mannitol, 1.0 mM EDTA, 0.4% BSA, and 0.3 mM PMSF, and disrupted with a Dounce homogenizer for 2 min. The homogenate was mixed with an equal volume of the same buffer, except that 0.4 M mannitol was substituted for 0.6 M mannitol. The mitochondrial fraction was obtained by differential centrifugation as described earlier [17]. The mitochondria thus obtained met all known criteria of physiological intactness and kept unchanged energy parameters for at least 2.5 h. 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.

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 by Chance and Williams [18]. When required, we added rotenone (an inhibitor of NADH-dehydrogenase and point I of energy conservation in the respiratory chain), KCN (an inhibitor of the cytochrome oxidase), or salicylhydroxamic acid (SHAM, an inhibitor of the alternative (non-phosphorylating) cyanide-resistant oxidative pathway).

Protein content of mitochondrial fraction was estimated by the method of Bradford [19] with BSA as standard.

RESULTS AND DISCUSSION

The assessment of features of energy metabolism in *N. crassa* wild type and *nap* mutant cells required us to develop a procedure to isolate physiologically intact, tightly coupled mitochondria from cells of the two types harvested at different growth phases. It is worthwhile to note that it was not a trivial task, because, to our knowledge, so far functionally intact mitochondria from stationary grown *N. crassa* cells have not been isolated and no information was available about mitochondria from *nap* mutant cells. 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 commonly accepted procedure for isolation of mitochondria from fungi based on pretreatment of cells with dithiothreitol with subsequent treatment of weakened cells with lytic enzymes, routinely extending over 5.5 h, was significantly simplified and shortened to 2.5 h. Thus, the distinctive advantages of the protocol proposed for isolation of functionally competent mitochondria of *N. crassa* cells from different growth stages are its simplicity and versatility. The figure depicts the result of typical experiments on amperometric registration of oxygen consumption by respiring on pyruvate + malate mitochondria from *N. crassa* wild type (figure, panel (a)) and mutant (figure, panel (b)) cells harvested at exponential growth phase. The typical transition from ADP-induced state 3 respiration to state 4 respiration (after phosphorylation of the ADP added) are seen. An ability of mitochondria to control distinctive metabolic states upon successive additions of ADP, good respiratory control (RC) ratios (reflecting the degree of oxidation to phosphorylation coupling), close to the theoretically expected ADP/O values (reflecting the efficiency of the oxidative phosphorylation process) indicated good preservation of energy transduction in the mitochondrial preparations obtained. We also used some additional test for intactness of the outer and inner mitochondrial membranes. Up to 40% of the mito-



Amperometric registration of oxygen consumption by respiring on pyruvate + malate mitochondria from *Neurospora crassa* wild type (a) and *nap* mutant (b) cells harvested at exponential growth phase. The incubation medium contained 0.6 M mannitol, 2 mM Tris-phosphate, pH 6.6, 1 mM EDTA, 20 mM pyruvate, 5 mM malate, and mitochondria corresponding to 0.3-0.5 mg of protein per ml. The figures along the curves indicate respiratory rates (ng-atom O/min per mg protein). Designations: P + M, pyruvate + malate; Mit, mitochondria. The states 3 and 4 respiration are taken to be the respiratory rate induced by the addition of ADP and after the phosphorylation of the ADP added, respectively. Respiratory control ratios (determined as ratios of state 3 respiration to state 4 respiration) upon successive additions of ADP attained: a) 2.59, 3.58, 4.22; b) 2.10, 2.48, 2.81. ADP/O ratios upon successive additions of ADP reached: a) 2.30, 2.54, 2.45; b) 2.60, 2.71, 2.89.

chondrial Mg^{2+} pool and adenylate kinase are known to be located in the intermembrane space [20]. The ability of mitochondria from both types of *N. crassa* cells to support in Mg^2-free incubation medium the same respiratory rate in state 3 respiration in the presence of AMP or ADP as phosphate acceptors (not shown) indicated that they preserved the bulk of intramitochondrial Mg^{2+} and adenylate kinase and hence, retained the intact outer mitochondrial membrane. Oxidation of all examined substrates occurred without additional exogenous cofactors such as cytochrome *c* or NAD (for oxidation of NAD-linked substrates). Oligomycin, an inhibitor of energy transfer, being added in state 3 respiration, diminished the respiration rate to state 4 respiration. Combined action of cyanide (2.5 mM) and SHAM (2 mM), inhibitors of alternative terminal oxidases, blocked the respiration rate by 99-100%. Thus, the mitochondrial preparations obtained met all known criteria of physiological intactness, which allowed us to embark on a comparative characterization of mitochondria from *N. crassa* wild type and mutant cells. Tables 1 and 2 summarize oxidative and phosphorylating activities of mitochondria from cells of two types harvested at different growth stages.

Mitochondria from both strains oxidized a wide range of respiratory substrates including intermediates of the TCA cycle and exogenous NADH. Succinate and exogenous NADH were the most readily utilized substrates. The substrates examined were oxidized by mitochondria from wild type cells with almost the same rates, regardless on the prevailing growth phase. As can be inferred from the analysis of ADP/O ratios, in *N. crassa*

Table 1. Oxidative and phosphorylating activities of *Neurospora crassa* mitochondria isolated from wild type cells at different growth phases*

Substrate	Respiration rate in state 3, ng-atom O/min per mg protein	RC	ADP/O	Inhibition by rotenone, %	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	53.7 ± 2.6	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	63.2 ± 10.9	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 from 3 to 9 independent experiments.

Table 2. Oxidative and phosphorylating activities of *Neurospora crassa* mitochondria isolated from *nap* mutant cells at different growth phases*

Substrate	Respiration rate in state 3, ng-atom O/min per mg protein	RC	ADP/O	Inhibition by rotenone, %	Inhibition by KCN, %	Inhibition by (KCN + SHAM), %
Exponential growth phase						
2-Oxoglutarate	131 ± 16	4.78 ± 0.15	3.15 ± 0.26			
Pyruvate + malate	134 ± 64	2.77 ± 0.17	2.45 ± 0.12	60.2 ± 2.2	85.7 ± 2.9	99-100
Succinate	538 ± 47	1.65 ± 0.09	1.51 ± 0.01		96.4 ± 1.7	99-100
NADH	470 ± 37	1.81 ± 0.08	1.76 ± 0.19		95.5 ± 2.01	99-100
Stationary growth phase						
2-Oxoglutarate	171 ± 1	4.26 ± 0.24	3.81 ± 0.10			
Pyruvate + malate	124 ± 49	2.68 ± 0.08	2.74 ± 0.14	80.0 ± 3.9	90.3 ± 3.1	99-100
Succinate	394 ± 27	1.60 ± 0.10	1.85 ± 0.14		90.7 ± 3.2	99-100
NADH	462 ± 60	1.88 ± 0.44	1.80 ± 0.14		96.5 ± 0.05	99-100

* Average from 3 to 9 independent experiments.

cells of two phenotypes harvested from both exponential and stationary growth phases, all three points of energy conservation in the respiratory chain were operative, and, additionally, phosphorylation at the substrate level (substrate phosphorylation) was functional upon oxidation of 2-oxoglutarate. It is worthy of note that RC ratios obtained upon oxidation of 2-oxoglutarate were very high (record), far exceeding values reported not only for *N. crassa* mitochondria, but also for mitochondria from other fungi (and yeasts).

To gain additional information about structural organization (arrangement) of the respiratory chain in mitochondria from wild type and mutant cells, we applied an approach using respiratory inhibitors. Oxidation of succinate and exogenous NADH by mitochondria from exponentially grown cells of the two types was almost totally inhibited by 2.5 mM KCN, an inhibitor of the cytochrome oxidase, indicating that the cytochrome oxidase was predominant, if not the only terminal oxidase upon oxidation of these substrates at this growth phase. Shift to the stationary growth phase was accompanied by appearance of cyanide-resistant respiration in both cell types, albeit to lesser extent in mutant cells. Distinctions between mitochondria from wild type and mutant cells were also noted, respiring on pyruvate + malate. In mitochondria from mutant cells, the contribution of the cyanide-resistant oxidative pathway upon oxidation of these substrates did not depend on the prevailing growth stage and was as low as 9-14%, while in mitochondria from wild type cells this level attained 31-37% in exponentially growing cells and 17-23% in cells of the stationary growth phase. In all cases, the cyanide-resistant respiration was inhibited by 2 mM SHAM, indicating that the terminal oxidation was accommodated by both the cytochrome oxidase and the alternative oxidase. The higher level of the cyanide-resistant oxidation upon utilization of pyruvate + malate, as compared to succinate and exogenous NADH, may reflect activation of the alternative oxidase by α-keto acids, notably by pyruvate [21], probably through the formation of thiohemiacetal by interaction between the cysteine sulfhydryl and the α-keto acids [22], as postulated for plant mitochondria [23]. Thus, in *N. crassa* wild type cells, two alternative oxidative pathways coexisted, i.e., the cytochrome pathway and the alternative oxidase, and the electron flux was partitioning between them depending on the prevailing growth phase and the substrate used. By contrast, in *nap* mutant cells, the electron flux was mediated mainly by the cytochrome oxidase with a minor contribution of the alternative oxidase. Having considering that the electron flow through the alternative oxidase is not coupled to ATP synthesis or generation of equivalent energy form ($\Delta\psi$) and presumably serves as a mechanism for overflow of excessive reducing equivalents [24], the data suggest that *N. crassa* mutant cells utilized substrates energetically more efficiently.

Titration of mitochondrial respiration with rotenone, the classical inhibitor of complex I of the respi-

ratory chain, also showed a considerable difference between cells of the two types. In mitochondria from wild type cells, rotenone (20 μg/mg protein) inhibited oxidation of NAD-dependent substrates by 54-63%, regardless of the growth stage. In mitochondria from exponentially growing mutant cells, rotenone inhibited the oxidation of the same substrates by 60% and the level of rotenone-sensitivity increased to approximately 80% in mitochondria from cells harvested at the stationary growth phase. This implies that in both cell types, oxidation of NAD-dependent substrates was partially mediated by rotenone-sensitive, energy-producing complex I, and partially by non-phosphorylating rotenone-insensitive pathway (i.e., rotenone-insensitive NADH-ubiquinone dehydrogenase), which is in keeping with data available in the literature [25, 26]. In mutant cells, a greater expression of complex I upon the shift to the stationary growth phase was observed.

The revealed features in the structural arrangement of the respiratory chain in *N. crassa* wild type and mutant cells, i.e., a lesser engagement of the alternative (not coupled to ATP synthesis) oxidative pathways and invariant functioning of all three points of energy conservation in the respiratory chain in mutant cells, was in a harmony with a 10%-increase in the molar growth yield (reflecting the efficiency of substrate utilization) in these cells as compared to wild type cells. Interestingly, similar data were obtained for chemostat-grown *Candida utilis* cells, when growth was limited by one or many growth factors [27]. When three growth factors were limited, the molar growth yield was higher by 10% as compared with growth limitation by only one growth factor, suggesting the utilization of more efficient pathways for substrate consumption; the data correlated with a greater expression of complex I in the respiratory chain. This suggests *nap* mutant cells as a promising model for starving culture. It seems very likely that the increased in intracellular ATP content in *nap* mutant cells [9] has its origin not only in diminished ATP consumption [10, 11], but also in enhanced ATP production due to the utilization of more energetically efficient, coupled to phosphorylation, pathways for substrate consumption. However, the absence of principal differences in the arrangement of the respiratory chain in mitochondria of wild type and mutant cells implies that the elevated ATP level in the *nap* mutant is largely related to the reduced ATP expenses for transport processes in these cells.

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REFERENCES

1. Weiss, H., von Jagov, G., Klingenberg, M., and Bucher, T. (1970) *Eur. J. Biochem.*, **14**, 75-82.
2. Lambowitz, A. M., and Slayman, C. W. (1971) *J. Bacteriol.*, **108**, 1087-1096.
3. Lambowitz, A. M., Smith, E. W., and Slayman, C. W. (1972) *J. Biol. Chem.*, **247**, 4859-4865, 1536-1545.
4. Hofhaus, G., Weiss, H., Galante, Y. M., and Hatefi, Y. (1981) *J. Biol. Chem.*, **256**, 9216-9220.
5 Finel, M. (1998) *Biochim. Biophys. Acta*, **1364**, 112-121.
6. Azevedo, J. E., Nehls, U., Eckerskorn, C., Heinrich, H., Rohte, H., Weiss, H., and Werner, S. (1992) *Biochem. J.*, **288**, 29-34.
7. Azevedo, J. E., Abrolat-Scharff, J., Eckerskorn, C., and Werner, S. (1993) *Biochem. J.*, **293**, 501-506.
8. Jacobson, E. S., and Metzenberg, R. I. (1968) *Biochim. Biophys. Acta*, **56**, 140-147.
9. Belozerskaya, T. A., Ershov, Y. V., Petrova, N. N., Dmitrovsky, A. A., and Kritsky, M. S. (1998) *Dokl. RAN*, **359**, 48-52.
10. Rao, T. K., and DeBusk, A. G. (1973) *Biochim. Biophys. Acta*, **323**, 619-626.
11. Wolfinbarger, L. I. (1976) *Biochim. Biophys. Acta*, **436**, 774-788.
12. Belozerskaya, T. A., Kritsky, M. S., Levina, N. N., Potapova, T. V., and Chaylachyan, L. X. (1988) *Biol. Membr. (Moscow)*, **5**, 1081-1089.
13. Brooks, K., Addison, R., and Scarboorough, G. J. (1983) *J. Biol. Chem.*, **258**, 13909-13918.
14. Slayman, C. L. (1987) *J. Bioenerg. Biomembr.*, **19**, 1-20.
15. Kritsky, M. S., Chernyshova, E. K., and Soboleva, I. S. (1977) *Appl. Biochem. Microbiol. (Moscow)*, **13**, 901-907.
16. Vogel, H. J. (1964) *Amer. Nat.*, **98**, 435-446.
17. Bazhenova, E. N., Deryabina, Yu. I., Zvyagilskaya, R. A., Eriksson, O., and Saris, N.-E. (1998) *J. Biol. Chem.*, **273**, 4372-4377.
18. Chance, B., and Williams, G. R. (1955) *Nature*, **175**, 1120-1121.
19. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
20. Jung, D. W., Panzeter, E., Baysal, K., and Brierley, G. P. (1997) *Biochim. Biophys. Acta*, **1320**, 310-320.
21. Millar, A. H., Wiskich, J. T., and Siedow, J. N. (1993) *FEBS Lett.*, **348**, 181-184.
22. Umbach, A. L., and Siedow, J. N. (1996) *J. Biol. Chem.*, **271**, 25019-25026.
23. Roads, D. M., Umbach, A. L., Sweet, C. R., Lennon, A. M., Rauch, G. S., and Siedow, J. N. (1998) *J. Biol. Chem.*, **273**, 30750-30756.
24. Lambers, H. (1982) *Physiol. Plant.*, **55**, 478-485.
25. Kerscher, S. J., Okun, J. C., and Brandt, U. (1999) *J. Cell Sci.*, **112**, 2347-2354.
26. Brandt, U. (1997) *Biochim. Biophys. Acta*, **1318**, 79-91.
27. Pozmogova, I. N., Zvyagilskaya, R. A., Shul'govskaya, E. M., Ural'skaya, L. A., Pchelkina, V. V., and Sakharova, Z. V. (1989) *Mikrobiologiya*, **58**, 20-25.