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Energy Status of *Neurospora crassa* Mutant *nap* in Relation to Accumulation of Carotenoids

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***N. crassa* mutant strain *nap* showed reduced growth rate, decreased electric membrane potential, and elevated intracellular ATP content in comparison to the wild type. Blue light induced a hyperpolarization of the membrane potential in both strains. The analysis of oxidative and phosphorylation activities of mitochondria isolated from the two strains has revealed that *nap* utilized more efficient oxidative pathways. The higher intracellular ATP content in the *nap* was presumably due to impaired transport systems of the plasma membrane, and to a lesser extent to the functioning of the fully competent respiratory chain. The excess ATP possibly accounts for carotenoid accumulation in the mutant.**

Key words: *Neurospora crassa,* mutant nap, membrane potential, ATP content, mitochondrial respiratory chain

The combined influence of nutrients and stress factors determine whether and at what rate an organism will grow and differentiate. Cells of *N. crassa*, like many other fungi, possess a blue-violet light sensitive photoreceptor mechanism, providing for the regulation of several differentiation events such as the formation of vegetative spores, the resetting of the circadian clock and the biosynthesis of carotenoids in mycelia (Linden *et al.*, 1997). It has been shown that the joint action of starvation and light results in the additive expression of caroteno- and conidiogenesis genes in wild-type (WT) *N. crassa* (Sokolovsky and Belozerskaya, 2000). A promising model for studying the mechanisms of the combined action of starvation and light on *Neurospora* carotenogenesis is the *N.crassa* mutant *nap*. It has been shown that the constitutive carotenoid content in *nap* is two times higher than in the WT (Belozerskaya *et al.*, 1998). The tendency is retained after light treatment. The unique component of acidic carotenoids in both strains is neurosporaxanthin. Only minor differences in the neutral carotenoids have been revealed in WT and *nap* (Belozerskaya *et al.*, 1998).

One gene mutant *nap* was isolated by Jacobson and Metzenberg (Jacobson and Metzenberg, 1968); it was cloned and sequenced and its product was identified as the amino acid permease (*Neurospora* GenBank, AF001032). *nap* has been reported to be pleiotropically deficient (by about 30-60%) in glucose, amino acid and uridine transport (Rao and DeBask, 1973). MgATP-dependent [^{14}C] SCN^- uptake by *nap* plasma membrane preparation exhibited about 50% lower activity than the WT membranes showing the decrease in the E_m value. H^+-ATPase specific activity was about one-half of the WT membranes (Brooks *et al.*, 1983). In view of this characteristics, together with the registration of the E_m with intracellular microelectrodes, we studied intracellular ATP content - the substrate of plasmalemma H^+-ATPase - in wild-type *Neurospora* and mutant *nap*. We also carried out a comparative investigation of mitochondria respiratory chains in both strains.

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Materials and Methods

Chemicals

BSA, EDTA, EGTA, sorbitol, mannitol, ADP, ATP, Tris, pyruvate, malate and Novozym 234 were purchased from Sigma (USA), Coomassie G-250 and NADH from Serva (Germany), dithiothreitol from Reanal (Hungary). Other reagents of analytical grade (of highest quality available) were obtained from domestic suppliers.

Fungal strains and growth conditions

N. crassa WT (RL3-8A) [FGSC#2218] and the transport mutant *nap* [FGSC 1604] were generously provided by Fungal Genetics Stock Center (FGSC, University of Kansas, Kansas City, USA). Mycelium was grown for 22-26 h at 28°C in Petri dishes on cellophane discs covering Vogel's agar medium with 2% sucrose (Potapova *et al.*,

1984). For mitochondria isolation the equivalent aerated liquid cultures were used.

E_m measurements.

A standart microelectrode technique was used to study the E_m value in both strains and its deviations upon the influence of light (300-500 nm, 20 W/m^{-2}) (Potapova *et al.*, 1984). A region of cellophane with hyphae taken at a distance of 10 mm from the colony edge was placed in a measuring chamber; the aerial hyphae were washed off with distilled water, and the measuring cell was filled with a solution containing 1.2 mM Na_2HPO_4, 8.8 mM NaH_2PO_4, 8.8 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 85 mM sucrose, pH 6 (Belozerskaya *et al.*, 1988). All the preparatory procedures and dark control measurements were taken under red light of low intensity and at a temperature of 21-23°C. The solution (2 ml per min) in the chamber was changed between measurements every 15-20 min. Standard microelectrodes with glass fibers (tip diameter <0.3 μm) filled with 2.5 M KCI were used. The potentials were recorded by a two-channel FET cathode follower (input resistance $<10^9$ ohms) and a double beam Disa oscilloscope (Denmark). The microelectrodes were moved with an MM-1 micromanipulator attached to an MBI-15 microscope.

ATP measurements

ATP extraction from the mycelium was performed with dimethylsulfoxide (DMSO) [10 mg wet wt per 1 ml of DMSO]. The ATP was totally extracted from the mycelium after 1 min incubation in DMSO solution. ATP extracts were stable in DMSO for about 3-4 h at room temperature. ATP concentration was measured with the aid of bioluminisent ATP-reagent based on immobilized glow-worm luciferase (Ugarova *et al.*, 1992). Change of luminiscent intensity was registered using a luminometer 1250 (LKB, Sweden).

Isolation of mitochondria

Cells harvested at exponential or stationary growth stages were washed three times with ice-cold distilled water, suspended in a minimal volume of medium (1 ml per g wet wt) containing 1.0 M sorbitol, 50 mM citrate-phosphate buffer, pH 5.8-6.0, 50 mM EDTA, helicase (complex of lytic enzymes from gut juice of Helix pomatia, 40 mg per 1 g of wet wt), and Novozym T-234 (3 mg per 1 g of wet wt), and incubated under gentle stirring at 28-30°C for 6-8 min. Spheroplast formation was examined under a light microscope. The spheroplasts were pelleted by centrifugation at 4000 g for 10 min, washed twice with 1.2 M sorbitol containing 0.25% (w/v) BSA (pH value was adjusted to 5.8), and disrupted with a glass-glass Dounce homogenizer for 2 min. The grinding medium contained 10 mM Tris-HCl, pH 7.2, 0.4 M mannitol, 0.5 mM EDTA, 1.0 mM EGTA, and 0.4% BSA. 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, centrifugated for 10 min at 3,200 g and the mitochondrial fraction was obtained by differential centrifugation as described (Bazhenova *et al.*, 1998). The mitochondria thus obtained remained fully active for at least three hours when kept on ice. The yield of mitochondria was about 4 to 5 and 0.5 to 1 mg of protein per 1 g of initial wet mycelium harvested from exponential and stationary growth phases, respectively.

Oxygen consumption

Oxygen consumption in mitochondrial suspensions was monitored amperometrically with a Clark-type electrode in a medium containing 0.6 M mannitol, 2 mM Tris-phosphate, pH 6.6, 2 mM EDTA, 1% BSA, 4 to 20 mM respiratory substrate, and mitochondria corresponding to 0.5 mg ml^{-1} of mitochondrial protein. Respiratory control and ADP/O ratios were calculated as recommended by Chance and Williams (1956). Protein content was estimated by the Bradford method with BSA as a standard.

Results

Growth, membrane potential and ATP content

The mutant *nap* of *N. crassa* with the lowered transport through the plasma membrane developed slower than the wild type. The specific growth rate was 0.29 h^{-1}, being in the WT of 0.39 h^{-1}. As a result, the mutant culture reached the stationary growth phase three hours later than the WT. Thus, the lowered rate of metabolite transport through cell membrane apparently manifests itself in the decreased growth rate.

A 20% decline in membrane voltage was observed in the mutant strain in comparison to the WT (Table 1). The data support previous results obtained with the aid of potassium isotiocyanide which showed the decrease in E_m value of *nap* (Brooks *et al.*, 1983). In spite of the obtained differences the plasma membrane of the mutant retained the ability to respond to the light treatment by the E_m shift. The shift was equal to that of the WT. Similar to the wild type, plasma membrane hyperpolarization of *nap* was transient with the maximum on the 25-30 min of illumination.

The lowered E_m resting value of the mutant as well as the 50% decrease in the H^+-ATPase activity of *nap* (Brooks *et al.*, 1983) provoked experiments on comparison of intracellular ATP (substrate of plasmalemma H^+-ATPase) in the WT and the mutant. ATP content after DMSO extraction and determination with immobilized glow worm luciferase in the WT was higher than previously obtained for the *N. crassa* WT (Slayman, 1973) probably due to more stable conditions of extraction and determination (Table 1). ATP content was about 1.5 times higher in *nap* than in the WT.

Table 1. Membrane potential and intracellular ATP content of the *N. crassa* wild type (WT) and mutant *nap*[1]

Strain	FGSC no.	Allele	E_m, mV		ATP constitutive
			Dark	Light, 30 min	μmol per g dry wt
WT	2218	RL3-8A	145 ± 9	180 ± 11	13.8 ± 1.0
nap	1604	-	120 ± 13	150 ± 14	20.2 ± 1.5

[1]Average value from 10-20 measurements for every table position ± standard deviation (sd)

Mitochondria respiratory chain

The increased intracellular ATP content in the mutant not only might be a result of impaired plasma membrane transport systems, it could also arise from a more efficient ATP production by the respiratory chain. To test this assumption, tightly-coupled mitochondria were isolated from the mycelium of the two *N. crassa* strains harvested at both logarithmic and stationary growth phases.

The isolated mitochondria met all known criteria of intactness as they controlled metabolic states and displayed high respiratory rates, and good respiratory control (Tables 2 and 3). Mitochondria from WT and *nap* respired actively with the Krebs cycle intermediates and exogenous NADH, with succinate and exogenous NADH being the most readily utilized substrates. From the analysis of ADP/O ratios it was evident that all three phosphorylation sites were active in hyphae of WT and *nap* harvested during both logarithmic and stationary growth phases.

Oxidation of all examined substrates by mitochondria from both WT and mutant hyphae was mediated by two alternative terminal oxidative systems, i.e., the cytochrome pathway and the alternate oxidase (Tables 2 and 3). Albeit to varying extent, the alternate oxidase was mainly related to the stationary growth phase. Poor activity of this pathway was found upon substrate oxidation by mutant mitochondria. It has been well documented that the nonphosphorylating alternate oxidase was used only to accomodate the surplus electron flux (Lambowitz *et al.*, 1972; Lambers, 1980) Thus, a minor contribution of the alternate pathway of substrate utilization to total respiratory system in *nap* suggests that over all phosphorylation efficiency of the mutant mitochondria appeared to be higher than that of the WT.

Rothenone titrations showed that oxidation of NAD-linked substrates by mitochondria from the two strains was carried out via both rotenone-sensitive and rotenone-insensitive pathways (Tables 2 and 3). The activity of the rothenone-insensitive pathway in *nap* was lower than in the WT. These data gave additional proof to the conclusion that in the mutant strain the cytochrome system runs its maximum. Thus it seems likely that a 1.5-fold increase in the intracellular ATP content in *nap* was caused not only by reduced ATP consumption, but also, at least par-

Table 2. Oxidative and phosphorylation activities of *N. crassa* mitochondria isolated from wild type (WT) mycelium at different growth phases[1]

Substrate	Oxygen consumption in state 3 respiration, ng-atom O in min per mg pr.	RC[2]	ADP/O	Inhibition by rotenone, %	Inhibition by KCN, %	Inhibition by SHAM[3], %
Exponential growth phase						
Pyruvate+malate	148±53	2.46±0.27	2.41±0.1	53.7±2.6	66.0±3.2	31.0-37.0
Succinate	491±67	2.27±0.17	1.86±0.25	0	92.0±6.5	6.0-14.0
NADH	416±60	1.97±0.11	1.78±0.15	0	95.8±4.6	3.0-4.0
Stationary growth phase						
Pyruvate + malate	116± 9	2.29±0.03	2.59±0.03	63.2 ±10.9	80.0±3.1	17.0-23.0
Succinate	497±60	1.75±0.05	1.80±0.01	0	85.0±6.7	15.0-20.0
NADH	591±41	1.92±0.12	1.75±0.09	0	82.0±8.0	18.0-22.0

[1]Average mean from 4 to 6 independent experiments, [2]RC respiratory control, [3]SHAM salicyl hydroxamic acid

Table 3. Oxidative and phosphorylation activities of *N. crassa* mitochondria isolated from *nap* mycelium at different growth phases[1]

Substrate	Oxygen consumption in state 3 respiration, ng-atom O in min per mg pr.	RC[2]	ADP/O	Inhibition by rotenone, %	Inhibition by KCN, %	Inhibition by SHAM[3], %
Exponential growth phase						
Pyruvate+malate	134±64	2.77±0.17	2.45±0.12	60.2±2.2	85.7±2.9	12.0-16.0
Succinate	538±47	1.65±0.09	1.51±0.01	0	96.4±1.7	4.0-6.0
NADH	470±37	1.81±0.08	1.76±0.19	0	95.5±2.1	2.8-4.0
Stationary growth phase						
Pyruvate + malate	124±49	1.9±0.10	2.8±0.2	80.0±3.9	90.3±3.1	8.0-12.0
Succinate	394±27	1.6±0.1	1.8±0.3	0	90.7±3.2	8.0-12.0
NADH	462±60	2.0±0.05	1.8±0.25	0	96.5±0.5	3.0-4.0

[1]Average mean from 4 to 6 independent experiments, [2]RC respiratory control, [3]SHAM salicyl hydroxamic acid

tially, by enhanced ATP production due to high phosphorylation efficiency of the *nap* mitochondria.

Discussion

The mutant *nap* with damaged transport systems in the plasma membrane showed a decreased E_m value in comparison with the wild type. We propose that through the decreased voltage, slowing off the pumps drives less transport inward through the channels and cotransporters, which, in turn, decreases the growth rate up to 33%.

Despite the different resting E_m values (1.2 times lower in *nap*) (Table 1), light-induced plasma membrane hyperpolarization was almost the same in both strains tested. Thus, upregulation of transport via adjustment of pump velocity occurred in both strains upon light treatment. Similar properties of the plasma membranes were elucidated in *Phycomyces blakeesleanus* (Weiss and Weisenseel, 1990).

Previous experiments demonstrated that constitutive carotenoid content as well as the amount of carotenoids after light exposure was two times higher in *nap* (Belozerskaya *et al.*, 1998). The data clearly show that impairment of the plasma membrane transport encouraged carotenogenic activity in the mutant. Thus, joined action of starvation and light resulted in carotenoid increase in *nap* in comparison to the WT.

The existence of light-induced plasma membrane hyperpolarization in both strains on the one hand, and the ability to accumulate carotenoids after the light treatment in WT and *nap* on the other, suggests that upregulation of transport might be a prerequisite of carotenoid accumulation in *Neurospora*. Two groups of data must be mentioned in this connection. First, neither intracellular ATP content, nor "energy charge" function were changed upon light treatment leading to carotenoid increase in *Neurospora* (Chernysheva *et al.*, 1980). Secondly, previous data demonstrated the lack of light-induced plasma membrane reactions in all the strains that failed to accumulate carotenoids under the light influence (*al-1, al-2, al-3*, the triple *albino* mutant, and *wc-1*) (Belozerskaya *et al.*, 1995).

The pleiotropic character of the *nap* mutation makes it difficult to interpret what changes are primary in the mutant. It seems that down regulation of metabolism due to impaired membrane transport brings about an increase in intracellular ATP, which supplies energy for accumulation of carotenoids. The assumption is consistent with the fact that in the higher plants (oak leaves) isoprene formation under stress conditions appears to be a suitable sink for dissipating excess ATP and CO_2 molecules formed in the course of intensive photosynthesis (Zeidler *et al.*, 1997). Isoprene emission up to 50% of photoassimilation could be observed in kudzu leaves under environmental stress conditions (Sharkley and Loretto, 1993).

Special features of the *nap* respiratory system such as low activity of alternate nonphosphorylating oxidation pathways and high activity of all the three phosphorylation sites were consistent with a higher (by 10%) growth yield coefficient [substrate utilization effect] (data not shown). Thus, a 1.5-fold ATP increase in *nap* may result partly from enhanced ATP production as a consequence of efficient substrate utilization. On the other hand, the overall respiratory system of *nap* mitochondria differs insignificantly from the WT. It can be calculated that a higher intracellular ATP content in the mutant is mainly due to impaired transport systems of the plasma membrane, and to a lesser extent to respiratory chain activity. Finally, our results show that the transport mutant *nap* is particularly suitable to study the joint action of different stress agents on *Neurospora* differentiation.

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