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Evidence for the involvement of a rapidly turning over Protease in the degradation of cytochrome oxidase in Neurospora crassa.

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Summary:

Treatment of N. crassa cultures with cycloheximide followed by washing and incubation in drug-free fresh medium results in a rapid decline in cytochrome oxidase activity. This is associated with the degradation of higher molecular weight subunits of cytochrome oxidase under these conditions. The protease activity associated with the mitochondrial preparation decreases during cycloheximide treatment and rapidly returns to normal levels on subsequent washing and transfer to drug-free fresh medium. It is suggested that the steady-state level of cytochrome oxidase is governed by a rapidly turning over cytoplasmically synthesized mitochondrial protease.

Introduction:

Cytochrome c oxidase (EC 1.9.3.1.) located in the inner membrane of the mitochondrion is an ideal enzyme to study the interaction between the nuclear and mitochondrial genetic systems. In yeast and Neurospora the enzyme has been shown to consist of seven subunits, three of which (I - III) are encoded by the mitochondrial genome and the other four (IV - VII) coded for by the nuclear genome (1).

The nuclear coded subunits of cytochrome oxidase have been implicated in the regulation of the synthesis and stability of the mitochondrial subunits (1,2). It has been demonstrated that in the absence of the nuclear coded subunits, the mitochondrial

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subunits are not integrated into the functional complex and are degraded (3,4). Poyton and Kavanagh (5) have shown that a 55,000 dalton precursor to the nuclear-coded subunits of yeast cytochrome oxidase exists in the cytosol which stimulates specifically the synthesis of the mitochondrial subunits. However, Ohashi and Schatz (6) have recently shown that the stimulatory agent in the cytosol is GMP and that the postulated 55,000 dalton precursor has no role in the stimulation. In fact, detailed studies from different laboratories have failed to detect this 55,000 dalton precursor to the nuclear coded cytochrome oxidase subunits (7 - 9).

In N.crassa, Rücker and Neupert (3) have shown that mitochondrial translation continues even when the cytoplasmic protein synthesis is shut off by cycloheximide for periods as long as two hours. Our present studies reveal that cycloheximide in fact protects cytochrome oxidase from degradation and that the steady state level of this enzyme is perhaps governed by a cytoplasmically synthesized, rapidly turning over mitochondrial protease.

Materials and Methods:

N.crassa (E m 5297a, Wild) was grown in 250 ml flasks containing 25 ml of basal medium in stationary cultures (10). After 36 hours growth, the mycelia were shaken in fresh media containing chloramphenicol (2 mg/ml or cycloheximide (10 µg/ml) Phase I. At the end of 4 hours of shaking, the mycelia were washed thoroughly and transferred to fresh media with or without the inhibitors and the incubation continued (Phase II). Mycelia were removed at different intervals of time during Phase I and Phase II for the isolation of mitochondria.

Mitochondria were isolated from the mycelia (11) and cytochrome c oxidase was assayed as described by Wharton and Tzagoloff (12) except that dithionite was used to reduce cytochrome c.

Degradation of cytochrome oxidase in vitro was followed by prelabelling the myclelial protein with ^{14}C- chlorella protein

hydrolysate and then following the loss of radioactivity in the enzyme subunits, specifically immunoprecipitated with the cytochrome oxidase antibody. The preparation of the antibody and the conditions of immunoprecipitation have been described elsewhere (11).

Azocasein was used as the substrate to assay the protease activity of the mitochondrial preparation. The assay mixture in a total volume of 0.5 ml contained 5 mg of azocasein, 2 - 3mg mitochondrial protein and 20 mM citrate buffer (pH 6.0). After incubation for 1 hour, the reaction was stopped by the addition of trichloroacetic acid (10% w/v, final concentration) and the absorbance of the supernatant was measured at 366 nm.

Results and Discussion:

It is known that inhibition of protein synthesis on mitoribosomes by chloramphenicol leads to a decrease in cytochrome oxidase level. When chloramphenicol pretreated cultures are washed and transferred to drug-free fresh media, there is an enhanced rate of protein synthesis on mitoribosomes. It has been suggested that while choramphenicol blocks the synthesis of proteins on mitoribosomes, the nuclear coded mitochondrial proteins would accumulate under these conditions and these in turn would stimulate the synthesis of the former when chloramphenicol is removed. This has been taken as an indirect evidence for the control of protein synthesis on mitoribosomes by nuclear coded proteins synthesized on cytoplasmic ribosomes (1,2,13).

The results of the present study indicate that treatment of N.crassa cultures with chloramphenicol for four hours results in a decrease of cytochrome oxidase activity by 50% (Phase I). When chloramphenicol pretreated cultures are washed and transferred to fresh media, there is a rapid regeneration of enzyme activity as shown by the slope of the line depicting Phase II (Fig. 1).

Cycloheximide on the other hand brings about a slight increase in cytochrome oxidase activity during a 4 hour incubation

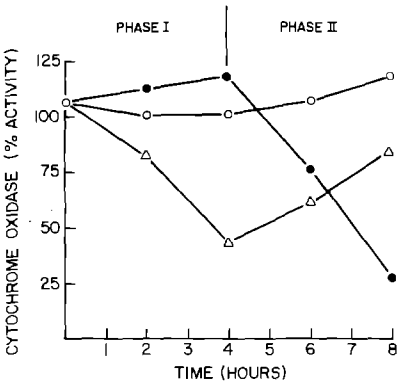

Fig. 1.
Effect of cycloheximide and chloramphenicol on cytochrome oxidase activity in N.crassa:
Chloramphenicol (2mg/ml) and cycloheximide (10 µg/ml) were added to 36 hr. old cultures and incubated for 2 - 4 hr. (Phase I). One set of cultures was washed at the end of 4 hr., transferred to fresh basal media without any additions and incubated for another 2-4 hr. (Phase I). One set of cultures was washed at the end of 4 hr., transferred to fresh basal media without any additions and incubated for another 2 - 4 hr. (Phase II). Mitochondria were isolated from mycelia removed at different time points and cytochrome oxidase activity assayed.

(O) Normal mycelia (Phase I)
(●) Cycloheximide treated mycelia (Phase I)
(Δ) Chloramphenicol treated mycelia (Phase I)

period of the cultures (Phase I). However, when cycloheximide pretreated cultures are washed and transferred to drug-free fresh media, cytochrome oxidase activity shows a sharp decline (Phase II--Fig. 1). Chloramphenicol plus cycloheximide pretreated cultures show a similar response during phase II (data not shown).

The possible involvement of protein degradation in accounting for the rapid decline in cytochrome oxidase activity has been checked under these conditions. For this purpose, mycelia are labelled with [^{14}c - chlorella protein hydrolysate for 1 hour and then transferred to a medium containing cycloheximide. After 4 hours, one set of cultures is washed and transferred to drug-free fresh media. After another 4 hour incubation,

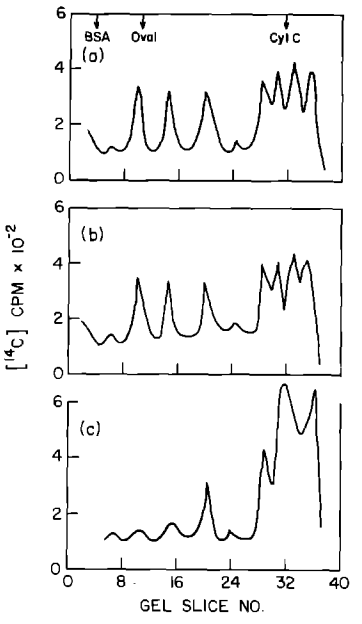

Fig. 2.
Degradation of cytochrome oxidase in cycloheximide pretreated cultures of Neurospora crassa.
N.crassa cultures were labelled for one hr. with 5µci of [14C] chlorella protein hydrolysate per culture. One set of cultures were transferred to a medium containing 10 g/ml of cycloheximide and incubation continued for 4 hr. and mycelia removed at the end of the incubation period. Another set of cycloheximide treated cultures were washed and transferred to fresh medium and incubation continued for 4 hr. Other experimental details are given in the text.

(a) Normal
(b) Cycloheximide treated for 4 hr.
(c) Cycloheximide pretreated and transferred to fresh media.

mitochondria are isolated and the radioactivity associated with cytochrome oxidase is assessed after immunoprecipitation and SDS - gel electrophoresis. The results presented in Fig. 2 indicate that cycloheximide treatment results in a higher retention of label in the mitochondrial subunits (I - III) of cytochrome oxidase than that of the untreated controls (Phase I). On subsequent washing and transfer of cycloheximide-pretreated mycelia to cycloheximide free fresh media, there is a striking loss of label from the mitochondrial subunits with concomitant

Table 1

Effect of cycloheximide on the protease activity of the mitochondrial preparation

Treatment	Protease activity E366/mg protein
Normal	0.88
Cycloheximide	0.25
Cycloheximide pretreated and transferred to fresh medium	1.16

N.crassa cultures were treated with cycloheximide (10 µg/ml medium) for 4 hr. One set of cultures was removed. Another set of cultures was washed and transferred to fresh media and incubated for 3 hr. Protease activity of the mitochondrial preparation was assayed as described in the text.

accumulation of label in the small molecular weight polypeptides. The accumulation of label in the small molecular weight polypeptides precludes the examination of the label retention in the cytoplasmic subunits of cytochrome oxidase (IV - VII) under these conditions.

The protease activity associated with the mitochondrial preparations decreases during cycloheximide treatment of the cultures and returns to normal levels when such cultures are washed and incubated in drug-free fresh media (Table 1).

It is reasonable to suggest that cycloheximide inhibits the elaboration of a cytoplasmically synthesized mitochondrial proteolytic machinery and thus protects the cytochrome oxidase from degradation. The removal of cycloheximide results in a sharp increase in the levels of the proteolytic machinery which rapidly degrades the cytochrome oxidase subunits leading to a rapid decline in enzyme activity. It is relevant to point

out that Michael et al (14) have shown that the protease activity associated with the mitochondrial preparation from Neurospora decreases exponentially with a half-life of 20 min during incubation of cells with cycloheximide and that the proportion of low molecular weight mitochondrial translation products decreases during cycloheximide treatment.

Thus, the coordination between nuclear and mitochondrial genetic systems may not only be in terms of cytoplasmically synthesized mitochondrial proteins governing the rate of synthesis of proteins on mitoribosomes, but also in terms of the former controlling the degradation rates of the latter.

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