JOURNAL OF BACTERIOLOGY, Sept. 1981, p. 757-767
0021-9193/81/090757-11$02.00/0

Vol. 147, No. 3

Mitochondrial Biogenesis During Fungal Spore Germination: Respiration and Cytochrome *c* Oxidase in *Neurospora crassa*

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Received 17 March 1981/Accepted 5 June 1981

The germination of conidiospores of wild-type *Neurospora crassa* was found to be dependent upon the function of the cytochrome-mediated electron transport pathway. The cyanide-insensitive alternate oxidase did not contribute significantly to the respiration of these germinating spores. The dormant spores contained all of the cytochrome components and a catalytically active cytochrome *c* oxidase required for the activity of the standard respiratory pathway, and these preserved components were responsible for the accelerating rates of oxygen uptake which began immediately upon suspension of the spores in an incubation medium. Mitochondria of the dormant spores contained all of the subunit peptides of the functional cytochrome *c* oxidase; nevertheless, de novo synthesis of these subunits began at low rates in the first stages of germination. Reactivation of the respiratory system of germinating *N. crassa* spores seems not to be dependent initially upon the function of either the mitochondrial or cytoplasmic protein-synthesizing systems. The respiratory activity of spores of three mutant cytochrome *c* oxidase-deficient strains of *N. crassa* also was found to depend upon the function of the cytochrome electron transport pathway; the dormant and germinating spores of these strains contained a catalytically active cytochrome *c* oxidase. Cytochrome *c* oxidase may be present in the dormant and germinating spores of these strains as the result of a developmental-phase-specific synthesis of and requirement for the enzyme.

The germination of dormant fungal spores into cells with a vigorous metabolism characteristically includes the rapid emergence of a strong aerobic respiration. In previous studies in our laboratory we examined the means by which the development of this respiratory activity is regulated early in germination and whether the mitochondrial respiratory membrane of the dormant spore is functionally complete or must be modified or reassembled to allow for the development of respiratory competence (5, 7-9, 13).

Studies of spores of the tropical fungus *Botryodiplodia theobromae* have shown that, whereas spore germination was dependent upon the function of the standard, cytochrome-mediated electron transport system (5), the mitochondria of the dormant spores did not contain cytochrome *c* oxidase (7-9) or oligomycin-sensitive ATPase (26). These respiratory enzyme complexes subsequently were shown (8, 26) to be assembled into the respiratory membrane of germinating spores by two different biogenetic mechanisms (see Discussion) to yield a competent respiratory system.

In contrast to the observations that germinating *Botryodiplodia* spores have an essential requirement for a functional standard electron transport system, the prevailing view (18) of the respiratory requirements of germinating conidiospores of *Neurospora crassa* is that during the earlier stages of germination these spores depend primarily upon the function of an alternate, non-cytochrome pathway of electron transport. It had been assumed that this pathway, mediated by a poorly characterized alternate oxidase (see reference 11 for a review), fulfills the respiratory demand of the spores until the cytochrome-mediated electron transport system becomes functional, late in the germination sequence.

In the present study, we re-examined the roles of the cytochrome-mediated and alternate electron transport systems in germinating spores of *N. crassa*, and we found that respiration in these spores was completely dependent upon the function of the standard electron transport system and that cytochrome *c* oxidase is preserved in the mitochondria of the dormant spores in an assembled complex which is catalytically active. Another question asked in this study was whether the function of this cyanide-sensitive respiratory pathway is always required for *N. crassa* spore germination or whether some strains could germinate using the alternate path-

way for respiratory activity. To answer this question definitively, we sought to find a *N. crassa* strain which utilized the alternate pathway as its respiratory pathway during spore germination, as might be expected of *N. crassa* mutant strains which have been characterized as being cytochrome *c* oxidase deficient. Instead, we found that the germination of spores of three such strains, which respire primarily through the alternate oxidase in logarithmic-phase growth, depended upon the function of the cytochrome electron transport system and that all of the mutant spores contained catalytically active cytochrome *c* oxidase.

MATERIALS AND METHODS

Strains of *N. crassa*. The wild-type strain used was 74-OR23-1A. The following cytochrome *c* oxidase-deficient mutant strains were donated by H. Bertrand (University of Regina, Saskatchewan, Canada): *mi-3*, *pan-2* (pantothenate requiring); *cya-4-23*, *nic-1*, *al-2* (albino, nicotinamide requiring); and *cya-5-34*. All of the mutant strains have the same general genetic constitution as 74A/74a, with the exception of the marker mutations listed above.

Growth of cells. Conidiospores of *N. crassa* were obtained from all strains after growth for 8 days on V-8 juice agar (5), supplemented with vitamins (when required) at a final concentration of 10 μg/ml of medium. The spores were harvested from the parent mycelium with a paraffinic oil (6) as a suspending medium to prevent hydration and activation of protein synthesis and respiration (R. Brambl, manuscript in preparation); these spore suspensions were free of contaminating mycelial fragments.

Spores were incubated in 500-ml baffled flasks containing 100 ml of Vogel minimal medium (22) with 2% sucrose (and vitamins as required; final concentration, 10 μg/ml); the concentration of the spores was 1 mg/ml of medium. Germination and incubation were carried out at 30°C in a gyratory incubator shaker or a gyratory water bath incubator at 300 rpm. Inhibitors were added to the medium immediately before spore inoculation. The inhibitor concentrations (in micrograms per milliliter of medium) were: sodium cyanide (25), salicyl hydroxamate (SHAM; 120), cycloheximide (100), chloramphenicol (4,000), ethidium bromide (50), and erythromycin (100). All of the inhibitors were dispensed from freshly prepared stock solutions; SHAM and erythromycin were dissolved in 95% ethanol, and control incubations showed that this solvent did not affect spore physiology.

Preparation of mitochondria. Mitochondria were obtained in an identical manner from the spores of each strain. The mitochondria used in the enzyme activity measurements were prepared as follows: 300 mg of spores were combined with 27 g of 1-mm glass beads and 7.5 ml of MTE buffer (0.25 M mannitol, 20 mM Tris-SO_4 [pH 7.4], 1 mM EDTA) and disrupted in a Braun MSK mechanical homogenizer (Bronwill Scientific Inc., Rochester, N.Y.) for 30 s with simultaneous cooling with a stream of CO_2. The homogenized spore suspension was rinsed from the MSK flask with about 15 ml of MTE buffer, and the suspension was centrifuged for 10 min at 1,200 × *g* (*g* values are g_{av}). Three distinct layers were apparent in the centrifuge tube after the first centrifugation; the supernatant fluid used for further work was contained between a thick lipid layer and the pellet of cell debris and was removed by aspiration. The supernatant fluid was centrifuged again as above to remove any remaining lipid material, and it was then centrifuged for 30 min at 25,300 × *g*. The resulting crude mitochondrial pellet was suspended in 2.0 ml of MTE buffer containing 20% glycerol for immediate use in the measurement of cytochrome *c* oxidase activity.

Mitochondrial fractions from dormant spores used for the spectral studies were obtained in the following manner: 3.5 to 4.0 g of spores were combined with 27 g of glass beads and 15 ml of STE buffer (0.44 M sucrose, 0.05 M Tris-chloride, [pH 7.5], 1.0 mM EDTA) and were disrupted and centrifuged as above. The final pellet was suspended in 4.0 ml of 10 mM sodium phosphate buffer (pH 8.0), which contained 1% Triton X-100 and 0.3 M KCl; the resulting suspension was centrifuged for 30 min at 25,300 × *g*. The clarified mitochondrial extract (which contained 6 to 10 mg of protein per ml) was used immediately for spectrophotometry. Protein was determined by the method of Lowry et al. (15).

Oxygen uptake rate determinations. Oxygen consumption was measured polarographically as previously described (5) with the oxygen electrodes calibrated in air-saturated distilled water at 30°C. Full-scale deflection corresponded to a concentration of 5.0 μl of oxygen per ml of medium (at 30°C). Dilutions of the suspensions of germinating spores with fresh aerated incubation medium were made as necessary (determined in trial experiments) to ensure that oxygen consumption by the spores would not exceed full-scale deflection over the 10-min recording period.

Enzyme activity measurements. Enzyme activity was measured according to Smith and Conrad (20) at room temperature (23 to 25°C). The 1-ml reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.4), 1% Tween 20, and 25 μM *Neurospora* ferrocytochrome *c*, purified and reduced as previously described (7, 21). Each assay mixture contained approximately 15 to 30 μg of crude mitochondrial protein. Corrections were made for the absorbance (at 550 nm) of the mitochondria added to each reaction mixture.

Cytochrome absorption difference spectra. Oxidized versus reduced cytochrome difference spectra were recorded as previously described (5) at room temperature with a Cary 118CX spectrophotometer equipped with a scattered transmission accessory and high-intensity light source. Each cuvette contained 1 ml of mitochondrial extract.

Radiolabeling and immunochemical techniques. The germinating spores were pulse-labeled as described elsewhere (8). The antiserum to cytochrome *c* oxidase used in this study was developed against the purified enzyme which was dissociated into its component polypeptides (8). The conditions for lysis of the mitochondria, for the immunoprecipitation reaction, and for processing and electrophoresis of the immunoprecipitates are detailed elsewhere (8, 13).

All of the experiments described in this report were

repeated three or more times, and the examples cited represent typical results.

RESULTS

The germination of spores of the *N. crassa* wild-type strain in the presence of respiratory inhibitors was measured to help determine which pathway of electron transport accounted for the majority of the respiratory activity during the early stages of spore germination. Germination of the spores was monitored in the presence of cyanide, an inhibitor of the standard, cytochrome-mediated electron transport pathway, in the presence of SHAM, an inhibitor of the alternate respiratory pathway, and in the absence of either inhibitor. A small percentage (5 to 6%) of the uninhibited wild-type spores developed germ tubes between 90 and 120 min, with the majority (90% or above) having germinated by 300 min. The addition of SHAM at the start of incubation yielded a curve for germination almost indistinguishable from the curve for uninhibited spores. Since the alternate respiratory pathway presumably was unable to function under these conditions and since the germination rate was unaltered in the presence of this drug as compared to the uninhibited control, these results suggest that the function of the alternate pathway is not required for wild-type *N. crassa* spore germination. The presence of cyanide in the incubation medium resulted in a complete block of wild-type spore germination, indicating that cyanide-sensitive respiratory activity, or the activity of the cytochrome electron transport pathway, was required for the germination of wild-type *N. crassa* spores.

We examined the respiratory activity of spores of three respiration-deficient strains of *N. crassa* in an attempt to determine whether this cyanide-sensitive respiratory activity is always required for *N. crassa* spore germination. The three mutant strains chosen for this study, *mi-3*, *cya-4-23*, and *cya-5-34*, have been reported previously to be deficient in cytochrome *c* oxidase, the terminal enzyme of the normal respiratory pathway, as judged by studies carried out on their logarithmic-phase cells (2, 16, 17). Previous characterizations of these mutant strains indicated that all three strains used the alternate pathway as their major respiratory pathway during logarithmic-phase growth, that cytochrome *a* was not detectable in logarithmic-phase mycelium, and that the mitochondria in each of the mutant strains were severely deficient in or completely lacking one or more of the polypeptide subunits of cytochrome *c* oxidase (1, 3, 4, 17, 19, 23). Our intention here was to determine whether spores of these mutant strains used the alternate respiratory pathway as their major respiration route during spore germination or whether they contained cytochrome *c* oxidase and used the standard electron transport pathway for respiratory activity, as did the wild-type spores.

The first experiment performed on the mutant strains was to test the effect of respiratory inhibitors upon spore germination rates. Each mutant strain had a characteristic spore germination rate in the absence of any inhibitor; the *cya-5-34* strain had a rate similar to that of the wild-type strain, whereas the *cya-4-23* and *mi-3* rates typically were less rapid (attaining 45 and 75% germination by 300 min, respectively). The addition of SHAM resulted in an approximately 50% reduction in the germination of all three mutant strains by 300 min of incubation. Cyanide caused a nearly complete inhibition of spore germination in all of the mutant strains. These results indicate that the mutant strain spore germination, like the wild-type spore germination, was dependent upon the function of the standard, cyanide-sensitive electron transport pathway.

This conclusion was tested by the measurement of the oxygen uptake rates of each of the wild-type and mutant strains in the absence or presence of the respiratory inhibitors, cyanide and SHAM. This method provides a more direct means of assessing the respiratory activities by the two pathways since they can be distinguished by their differential sensitivities to the two inhibitors.

The effect of cyanide and SHAM upon the oxygen uptake rates of the wild-type spores is shown in Fig. 1. In the absence of either inhibitor, accelerating rates of oxygen uptake were observed throughout the 300-min germination period, with a typical rate of 285 μl of oxygen consumed per mg of spores per 10 min at 300 min of germination. No lag was observed in the acceleration of oxygen uptake rates at the start of the germination sequence; the spores began to consume oxygen immediately after being suspended in the incubation medium. SHAM had no observable effects on the oxygen consumption rates as compared to the uninhibited control (Fig. 1). Since oxygen uptake via the alternate respiratory pathway presumably was blocked in this experiment, most of the oxygen consumption measured should have been due to activity of the cytochrome-mediated pathway of respiration. These results support the findings of the first experiment, which showed that SHAM also had no effect on the germination rate of the wild-type spores. In contrast, cyanide severely inhibited oxygen consumption throughout the germination sequence (Fig. 1). The results of these oxygen uptake experiments support the

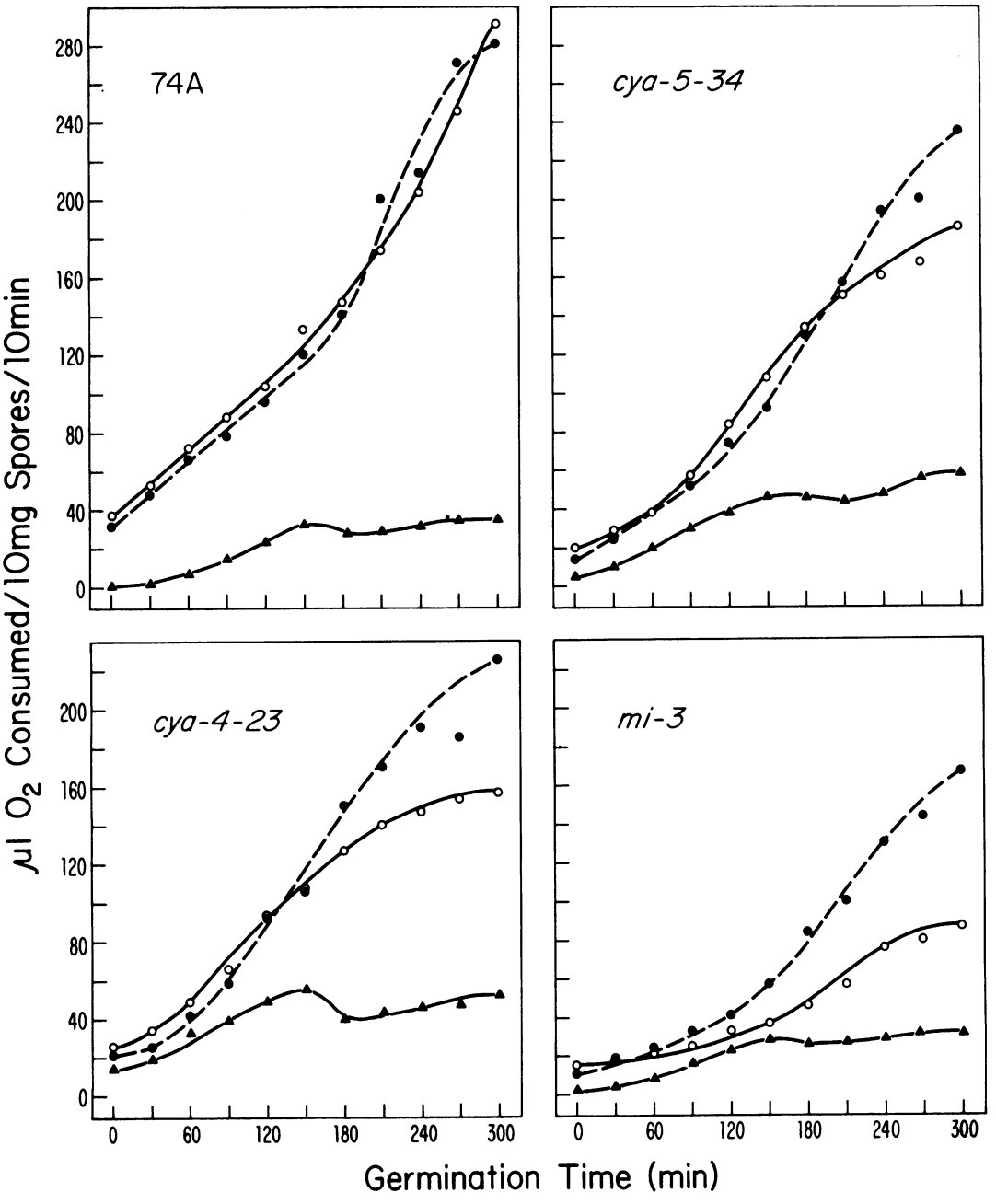

FIG. 1. *Rates of oxygen consumption by spores incubating in nutrient medium* (●) *and in nutrient medium with SHAM* (○) *or cyanide* (▲).

conclusion that respiration via the cytochrome electron transport system is required for germination of wild-type *N. crassa* spores.

Fig. 1 also shows the oxygen uptake rates of spores of the mutant strains in the presence of the respiratory inhibitors. The oxygen uptake rates of the *cya-4-23* and *cya-5-34* spores were not affected by SHAM until approximately 180 and 240 min of the germination sequence, respectively, after which time the drug caused slight decreases in the rates of oxygen consumption. The presence of SHAM had a more severe effect upon the oxygen uptake rates of the *mi-3* strain, causing a nearly 50% decrease in the rate

of oxygen uptake by 300 min of germination. Under cyanide inhibition, however, oxygen consumption in all of the strains was sharply reduced (Fig. 1), indicating that the major portion of the oxygen consumed in these strains was via the standard electron transport pathway and not through the alternate pathway of respiration.

The germination and oxygen uptake rates of wild-type conidia were also monitored in the presence of inhibitors of cytoplasmic and mitochondrial protein synthesis to help determine whether new synthesis of products from either genetic system might be required for the reactivation of the respiratory system preserved in the dormant spores. The biosynthesis and assembly of the standard electron transport pathway are known to require joint contributions from the nuclear and mitochondrial genetic systems (for a review, see reference 24). For example, the terminal enzyme of the cytochrome electron transport system, cytochrome *c* oxidase, contains seven subunit polypeptides, four of which are synthesized on cytoplasmic ribosomes and three of which are synthesized on mitochondrial ribosomes (24). In these germinating spores of *N. crassa*, chloramphenicol severely disrupts the synthesis of the three mitochondrial subunits of the enzyme (R. Brambl, unpublished data).

Chloramphenicol caused a slight decrease (10%) in the spore germination rate as compared to the rate for the uninhibited control, whereas ethidium bromide caused a more significant decrease (50%) in the extent of germination by 300 min of incubation; the effect of erythromycin on the rate of spore germination was similar to that of chloramphenicol. Treatment of the germinating spores with cycloheximide, a cytoplasmic protein synthesis inhibitor, resulted in a complete block of germination. These results indicate that, whereas mitochondrial protein synthesis apparently was not required for wild-type *N. crassa* spore germination, cytoplasmic ribosome function was essential.

The cyanide-sensitive oxygen uptake rates of the wild-type spores in the presence of the protein synthesis inhibitors is shown in Fig. 2. Ethidium bromide had little effect on oxygen consumption until approximately 180 min of the germination sequence; by 300 min, the drug reduced the rates of oxygen consumption by 50%. Chloramphenicol and erythromycin caused only a slight inhibition (about 15%) of the cyanide-sensitive oxygen uptake rate at 300 min (data not shown). Small amounts of oxygen were consumed by spores treated with cycloheximide, the inhibitor that completely blocked spore germination, and this finding suggests that the development of respiratory activity in these spores

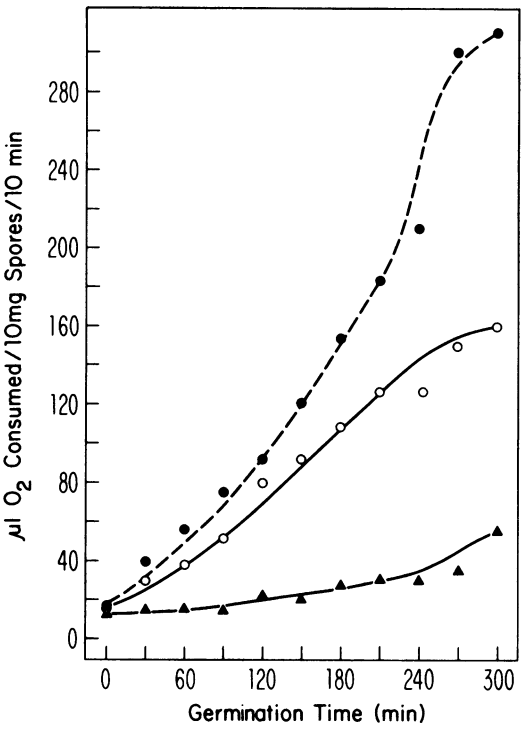

FIG. 2. *Rates of oxygen consumption by wild-type (74A) spores incubating in nutrient medium* (●) *and in nutrient medium with cycloheximide* (▲) *or ethidium bromide* (○). *Chloramphenicol gave uptake kinetics nearly identical to those of untreated spores.*

may not be completely dependent upon cytoplasmic protein synthesis as it is in other fungal spores (5).

Because we found that cyanide-sensitive respiration was required for the germination of the *N. crassa* spores, we sought to establish whether the cytochrome components of the cyanide-sensitive respiratory pathway were present in the mitochondria of the dormant spores of each of the strains or whether some of these components had to be assembled early in spore germination to yield a functional respiratory membrane. The mitochondria of dormant spores of each strain therefore were examined to determine whether they contained all of the cytochrome species required for the function of the cytochrome electron transport pathway and whether they possessed an assembled, catalytically active cytochrome *c* oxidase.

Room temperature difference spectrophotometry was used to measure the respiratory cytochrome content of the mitochondria of the dormant and germinating spores. Figure 3 shows the pattern of elaboration of the mitochondrial cytochromes in cells of the wild-type strain from dormant spores (0 h) to logarithmic-phase

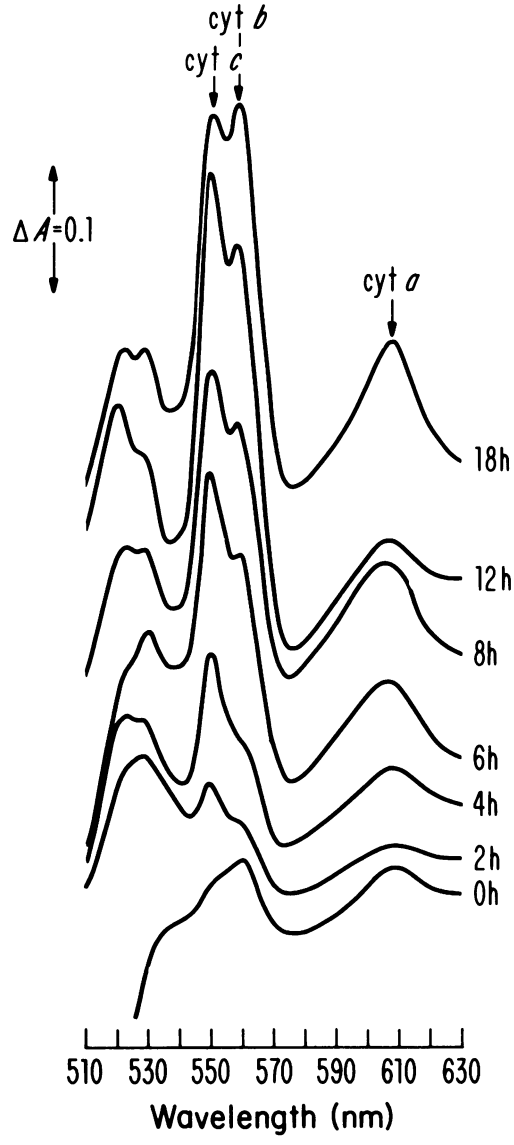

FIG. 3. *Oxidized versus reduced cytochrome absorption difference spectra (room temperature) showing the elaboration of the mitochondrial cytochromes in the wild-type (74A) strain at different stages through spore germination and mycelial growth. 0 h refers to dormant spores. A, Change in absorbance.*

growth. Absorbance peaks corresponding to cytochromes *a*, *b*, and *c* were found at approximately 610, 560, and 550 nm, respectively; the 18-h cytochrome spectrum is representative of a typical, mature cytochrome pattern for logarithmic-phase cells of wild-type *N. crassa*. This experiment shows that in mitochondria of the dormant spores (0 h), all of the cytochrome species necessary for the function of the standard electron transport pathway are present.

Figure 4 shows the room temperature difference spectra of the dormant spores of the three cytochrome *c* oxidase-deficient mutant strains. Whereas the absorbance peaks for cytochromes *b* and *c* were distinct in each case, no cytochrome *a* could be detected in the mitochondria of spores of the mutant strains. The cytochrome *b* and *c* absorbance peak heights of the mutant

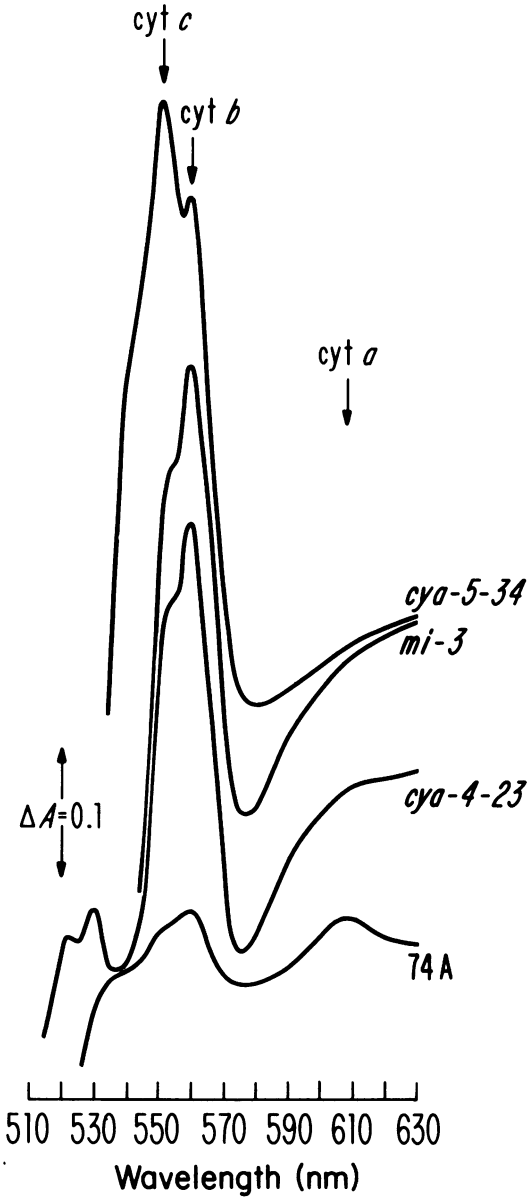

FIG. 4. *Oxidized versus reduced cytochrome absorption difference spectra (room temperature) of mitochondrial fractions prepared from the dormant spores of the wild-type (74A) and the three mutant strains of Neurospora. A, Change in absorbance.*

strains always appeared much greater than the corresponding peaks in the wild-type strain when comparisons were based on the same quantities of mitochondrial protein. Since the mutant strains, like the wild-type, were found to require a functional, cyanide-sensitive electron transport pathway during spore germination, our inability to detect cytochrome *a* in the dormant spores of the mutant strains was, therefore, an unexpected result. One possible explanation of these results is that cytochrome *a* was present in low cellular concentrations in the dormant spores of the mutant strains and that it was spectrophotometrically undetectable.

The results obtained so far indicate strongly that the function of the cytochrome electron transport system is required for germination and that the dormant spores (of the wild-type strain) contain cytochrome *a*, which probably is functional. To test this latter conclusion directly, we analyzed the mitochondria of dormant spores of the wild-type and mutant strains for the catalytic activity of the cytochrome *a*, or cytochrome *c* oxidase. We also sought to establish whether the spores of the mutant strains might contain this enzymatic activity in the absence of spectrally detectable cytochrome *a*. The in vivo substrate of cytochrome *c* oxidase, ferrocytochrome *c*, was purified from the wild-type strain (21) and was used as the substrate for the catalytic activity measurements.

As shown in Table 1, cytochrome *c* oxidase in the dormant spores of the wild-type strain oxidized 350 nmol of ferrocytochrome *c* per min per mg of mitochondrial protein. This specific activity was approximately three-fifths of that found in the logarithmic-phase cells of the wild-type strain. This demonstration of cytochrome *c* oxidase activity in the dormant spores of the wild-type strain, coupled with the detection of the full complement of respiratory cytochromes, leads us to the conclusion that the cytochrome components necessary for the function of the cyanide-sensitive pathway are assembled and probably functional in the mitochondria of the dormant spores. That this respiratory system of the dormant spores is probably functional at the outset of incubation is supported by the kinetics of the oxygen uptake by the wild-type spores (Fig. 1); there was no lag in the acceleration in the rates of oxygen uptake at the start of the germination sequence, an occurrence that would be expected if the components of the standard electron transport pathway otherwise had to be assembled during the early stages of spore germination.

TABLE 1. *Cytochrome c oxidase activity in whole mitochondria isolated from dormant spores*

Strain	Sp act[a]	% of wild type
74A (wild type)	350 ± 16	100
mi-3	207 ± 27	59
cya-4-23	82 ± 9	23
cya-5-34	118 ± 15	34

[a] Nanomoles of ferrocytochrome *c* oxidized per minute per milligram of protein.

Cytochrome *c* oxidase catalytic activity measurements also were performed with mitochondria isolated from the dormant spores of the mutant strains. We considered it likely that at least small quantities of cytochrome *c* oxidase were present in these cells since the oxygen consumption during the early stages of spore germination was cyanide sensitive. The results, as shown in Table 1, supported that expectation. Cytochrome *c* oxidase activity was found in the dormant spores of all of the mutant strains, with *mi-3*, *cya-4-23*, and *cya-5-34* displaying 59, 23, and 34% of the specific activity of the wild-type strain, respectively. Since cytochrome *a* is an integral part of the functional cytochrome *c* oxidase enzyme, we concluded that cytochrome *a* was present in the dormant spores, but in such low amounts as to be spectrophotometrically undetectable. It can also be concluded that, like the wild-type strain, the dormant spores of the three mutant strains contain all of the cytochrome components required for the function of the standard, cytochrome-mediated electron transport pathway.

The experiments described above indicate that cytochrome *c* oxidase must be preserved in the mitochondria of the dormant spores of *N. crassa*. Through use of an enzyme-specific antiserum and electrophoresis of the immunoprecipitate, we also established that the mitochondria of dormant spores of the wild-type strain (radiolabeled during sporulation) contained the seven-subunit complex of the complete enzyme, as would be expected (data not shown). Because the dormant spores contained cytochrome *c* oxidase that had been assembled in the respiratory membrane to function during the earliest stages of germination, we also wished to determine when, during spore germination, a new cytochrome *c* oxidase was synthesized as a replacement for the preserved enzyme. The germination sequence of the wild-type spores was divided into four 60-min periods, and the spores were labeled with radioactive leucine during each of the periods. At the conclusion of each labeling period, the spores were collected rapidly and chilled, the mitochondria were prepared, and the radiolabeled cytochrome *c* oxidase was isolated by immunoprecipitation with an antiserum to the purified enzyme (8).

Gel electrophoresis (in dodecyl sulfate) of these cytochrome *c* oxidase immunoprecipitates (Fig. 5) showed that during the first 60-min period of germination, only low quantities of the mitochondrial subunits (polypeptides 1 through 3) of the enzyme were synthesized; trace quantities of some cytoplasmic subunits (polypeptides 4 through 7) might also have been synthesized. During the second and succeeding labeling periods, however, all subunits of the enzyme were synthesized at accelerating rates. Because the antiserum used in this experiment should recognize both free and assembled enzyme subunits (8), it is likely that these results accurately reflect the synthesis of the enzyme subunits as well as their assembly into a membrane complex. The results of this experiment demonstrate that, despite the presence of a preformed and functional cytochrome *c* oxidase in the dormant spores, these cells initiated the synthesis of a new enzyme within 60 min after the onset of germination. These newly synthesized subunits were not required for respiratory activity in germinating spores; inhibition of their synthesis with chloramphenicol or cycloheximide (data not shown) did not abolish cyanide-sensitive respiration in these cells.

DISCUSSION

Upon initiation of germination, fungal spores typically exhibit rapid increases in rates of respiration as they convert from a dormant, physiologically quiescent state to a state characterized by high rates of metabolic activity. In this study we sought to characterize the respiratory activity in germinating conidiospores of *N. crassa* to learn which electron transport pathway was responsible for this early oxygen consumption and to establish whether the components of the electron transport pathway, such as the cytochromes and cytochrome *c* oxidase, were preserved in the dormant spores. We attempted to determine whether the activity of this cytochrome electron transport pathway was always required for *N. crassa* spore germination by examining the respiratory activity of germinating spores of three strains reported to be deficient in cytochrome *c* oxidase.

Earlier work on the respiratory activity of spores of *B. theobromae* showed that these spores required activity of the cyanide-sensitive respiratory pathway for spore germination and that the mitochondria of the dormant spores possessed an incomplete respiratory membrane, lacking both cytochrome *c* oxidase and the oligomycin-sensitive ATPase (5, 7–9, 26). Both of these enzyme activities were elaborated rapidly during germination, however, and it was found that two different methods existed for the assembly of the enzyme complexes into the respiratory membrane early in spore germination. ATPase was found to become functional during early spore germination as a result of the de novo synthesis of its subunits via translation of an mRNA that had been preserved in the dormant spores (25, 26). The assembly of cytochrome *c* oxidase during spore germination, on the other hand, involved a mobilization of preserved, compartmentalized subunits to form an active enzyme; the four cytoplasmic subunits, preserved in the cytoplasm during dormancy, were found to move into the mitochondria during the early stages of spore germination and complex with the three mitochondrial subunits, which were preserved in the mitochondria during dormancy, and with heme *a* to form the functional enzyme (8).

Results from the germination and oxygen uptake studies in the presence of the respiratory inhibitors showed that, like *B. theobromae*, wild-type *N. crassa* also requires a functional, cyanide-sensitive electron transport pathway for spore germination; the SHAM-sensitive alternate oxidase did not function significantly in these germinating spores. The findings of this study, as well as other reports (10, 12, 14), therefore contradict the view that the alternate oxidase is primarily responsible for the oxygen consumption observed during early spore germination of *Neurospora* (18).

In *B. theobromae*, cytoplasmic protein synthesis is required for spore germination and the development of respiratory activity, but mitochondrial protein synthesis is not required (5). Our experiments show that in *N. crassa* also mitochondrial protein synthesis is not required for the reactivation of respiratory activity during spore germination. However, unlike *B. theobromae* spores, in *N. crassa* cytoplasmic protein synthesis may not be strictly required for the initial development of respiratory activity, since small but significant amounts of oxygen consumption are observed in the presence of cycloheximide. These results suggest, therefore, that the products of both mitochondrial and cytoplasmic protein synthesis required for a functional respiratory membrane are preserved in the dormant spores of *N. crassa* and that resumption of respiratory activity is not immediately dependent upon the function of either protein synthesis system.

Support for this conclusion comes from our finding that the dormant spores of wild-type *N. crassa* contain all of the cytochrome species necessary for activity of the standard electron transport pathway. Furthermore, the subunit polypeptides of cytochrome *c* oxidase were

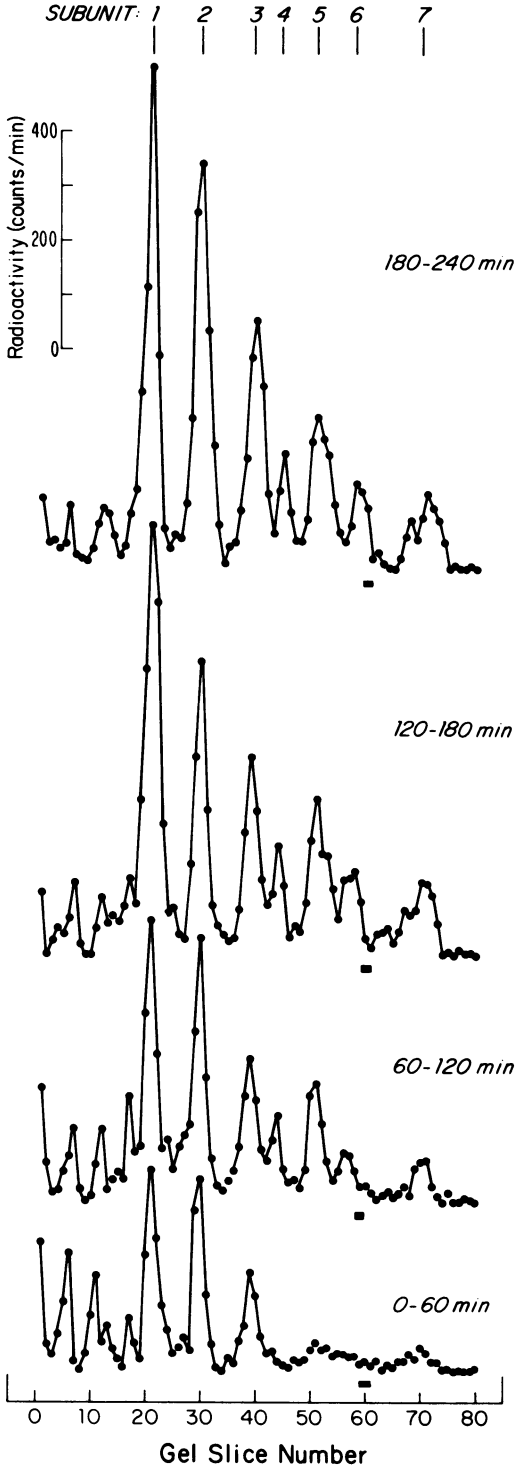

FIG. 5. *Polyacrylamide gel electrophoretic comparison of radiolabeled peptide subunits of cytochrome c oxidase obtained by immunoprecipitation from mitochondria (1 mg of protein in each sample) of spores labeled during four 60-min periods of germination with [^{3}H]leucine and collected at the end of each labeling interval. Migration is from left to right, and the closed block indicates the position of cytochrome c (tracking dye) at the termination of electrophoresis. The same radioactivity scale applies to all four analyses. Background counts have been subtracted from the data in this figure; each sample was counted for at least 4 min.*

found to be assembled and catalytically active in the dormant spores, and other work in our laboratory has shown that the dormant spores of *N. crassa* also possess a catalytically active oligomycin-sensitive ATPase (26). It seems probable, therefore, that all of the components required for cyanide-sensitive respiratory activity are preserved in the dormant spores in the functional form and the activity of these preserved components must be responsible for the low but accelerating rates of cyanide-sensitive oxygen uptake observed immediately after the spores are suspended in the incubation medium.

The cytochrome *c* oxidase preserved in the dormant spores clearly was functional during the earliest stages of germination, and it seemed likely that de novo synthesis of the enzyme subunits would not begin until relatively late in the germination sequence. Nevertheless, these spores initiated the synthesis of the mitochondrial subunit peptides of the enzyme within the first 60 min of germination, and all seven subunits were synthesized at accelerating rates after 60 min. These newly synthesized subunits were not required for the early respiration of spore germination, but instead they contributed to the assembly of a new respiratory membrane which was organized during germination as a successor to the respiratory membrane preserved in the dormant spores. This pattern of synthesis of the subunits of cytochrome *c* oxidase resembles that of *B. theobromae* spores, where a distinct temporal separation exists between the initial synthesis and assembly of the mitochondrial subunits and, subsequently, the cytoplasmic subunits of the enzyme (8).

We examined the respiration during the germination of spores of three *N. crassa* mutant strains which have been characterized as cytochrome *c* oxidase deficient to test whether the function of the cytochrome electron transport system is always necessary for germination. Earlier reports had shown that respiration of these mutant strains was primarily dependent upon the function of their alternate electron transport pathway during logarithmic-phase growth due to a deficiency in cytochrome *c* oxidase which could be traced directly to the complete or near-complete absence of one or more of the polypep-

tide subunits of the enzyme (1, 3, 4, 17). It was hypothesized previously that the mutations in these strains involve regulatory genes rather than genes for the structural components of cytochrome *c* oxidase (3, 4, 17). Therefore, it seemed possible to us that (i) spores of these strains could contain cytochrome *c* oxidase because of a developmental phase-specific synthesis during sporulation and (perhaps) a phase-specific requirement for this enzyme during germination, or that (ii) since the enzyme was not synthesized and was not functional in the logarithmic-phase cells, the enzyme would not be present in the dormant and germinating spores and would not be required for germination. Instead, we found that spore germination in these mutant strains, like the wild-type strain, was dependent upon the function of the cytochrome electron transport pathway. The results of the respiratory inhibitor experiments clearly showed that SHAM, the inhibitor of the alternate respiratory pathway, had only a small effect on both the germination and oxygen uptake rates of the spores of the mutant strains, but cyanide, the inhibitor of the standard cytochrome pathway, caused a nearly complete inhibition of both germination and oxygen consumption. In addition, all of the cytochrome species required for the function of the standard cytochrome pathway were found to be present in the dormant spores of the mutant strains; cytochromes *b* and *c* were detected through difference spectrophotometry, whereas the presence of cytochrome *a* was indicated by the detection of catalytically active cytochrome *c* oxidase in the dormant spores.

Our results show that spore germination in *N. crassa* is dependent upon the function of the standard, cytochrome-mediated electron transport pathway. All of the enzyme components of the standard pathway appear to be assembled and preserved in the mitochondria of the dormant spores for immediate function upon initiation of spore incubation without requisite steps of synthesis or assembly early in germination.

ACKNOWLEDGMENT

This research was supported by Public Health Service grant GM-19398 from the National Institutes of Health.

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