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Alterations of the Respiratory System of *Neurospora crassa* by the mi-1 Mutation

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The respiratory mechanism of the cytochrome-defective mi-1 mutant of *Neurospora crassa* was partially characterized and compared with that of the wild type. Both systems of electron transport were linked to oxidative phosphorylation and showed similar characteristics with respect to substrates and components. The system operating in the cytochrome-deficient mi-1 mutant showed no characteristic α absorption bands of cytochromes *a* or *b* and was very unstable in vitro. The possibility that this system may be an altered form of the normal cytochrome electron transport chain which occurs in the wild type is discussed.

The ascomycete *Neurospora crassa* has a mitochondrial cytochrome system consisting of cytochromes *a*, *b*, and *c* (3). Thus, the respiratory system of *Neurospora* is similar to those of mammalian systems, but there are significant differences. Cyanide and azide, poisons which completely inactivate cytochrome oxidase activity in mammalian systems, can inhibit respiration in *Neurospora* only by about 80% (7). In *Neurospora*, a grossly defective cytochrome system resulting from mutation is not always lethal since there exist many mutants with abnormal cytochrome systems (7, 12). Haskins et al. (7) described the deficiencies of cytochromes *a* and *b* in the respiratory mutant mi-1 (poky) and the accumulation of cytochrome *c*, up to a 16-fold excess in this strain. However, the phenotype associated with the maternally inherited mi-1 gene is subject to variation during growth. Thus, the deficiencies of cytochromes *a* and *b* and the accumulation of cytochrome *c* is at its extreme in young cultures, and as a culture ages cytochromes *a* and *b* eventually appear and the excess of cytochrome *c* is greatly reduced (7). Corresponding to the deficiency of cytochrome *a*, Haskins et al. (7) found that young cultures of mi-1 had no cytochrome *c* oxidase activity. They also found that these cultures were deficient in succinate oxidase activity but had normal levels of succinic dehydrogenase activity, corresponding to a deficiency of cytochrome *b*. As with the cytochromes, the oxidase activities appeared and increased during the growth of a culture. The succinic dehydrogenase activity remained near the wild type level throughout growth. Tissieres et al. (13) showed that oxygen uptake of both intact mycelium and cell-free extracts from young cultures of mi-1 was insensitive to cyanide and azide, but that sensitivity to these poisons increased as a culture aged.

These results led to a postulation of an alternate electron transport pathway independent of cytochrome oxidase to account for respiration in cytochrome-deficient strains. The experiments presented here partially characterize the respiratory system of the mi-1 strain and demonstrate its similarity to the normal cytochrome respiratory system of wild type strains.

MATERIALS AND METHODS

Strains of Neurospora. Two strains of *N. crassa* were used in this investigation. The strain 25a described by Beadle and Tatum (1) was used as a standard wild type. The mi-1 strain used was obtained as a single ascospore from a cross of po 3627-2a (protoperithecial parent) with the wild-type strain 4A as the conidial parent. The origin of the mi-1 parent was given by Mitchell and Mitchell (10), and that of the wild-type parent was given by Beadle and Tatum (1).

Preparative procedures. The procedures used for preparing conidia, growing mycelia, and isolating mitochondria have been described previously (4). Cytochrome *c* was removed from mitochondria for reconstitution experiments by washing mitochondrial suspensions with water and dilute salt solutions. Suspensions with an initial mitochondrial protein concentration of 30.0 mg/ml were washed with 10 volumes of distilled water for 10 min, followed by 10 volumes of 0.15 M KCl for 110 min, and finally with 0.15 M KCl for 12 hr.

Assay procedures. Mitochondrial suspensions were

diluted with 200 to 500 volumes of distilled water to determine the protein content. Samples of 1.0 ml were used to assay for protein by the method of Lowry et al. (8), with bovine serum albumin (BSA) as a standard. Appropriate corrections were made for the BSA in the medium in which the mitochondria were suspended. The cytochrome content of mitochondria was determined from reduced versus oxidized difference spectra obtained by the method of Williams (14). Spectra were recorded by scanning the wavelength range from 650 to 480 nm on a Cary model 15 recording spectrophotometer equipped with 0 to 0.1 absorbance slide wire. Oxidation of substrates coupled to the reduction of molecular oxygen and oxidative phosphorylation were measured as described previously (4).

RESULTS

Cytochrome content of mitochondria. The mitochondria from very young cultures of mi-1 contained a large quantity of cytochrome *c*, but no spectroscopically detectable cytochromes *a* or *b*. During growth, the amount of mitochondrial cytochrome *c* relative to total mitochondrial protein decreased as the culture aged. The α absorption band of cytochrome *b* did not begin to appear until later stages of growth, about 30 hr after inoculation. In older cultures, the amount of cytochrome *b* relative to total mitochondrial protein increased. The α absorption band of cytochrome *a* did not appear until about 60 hr after inoculation. Wild type mitochondria contained a complete cytochrome system during all stages of growth.

Characteristics of the respiratory systems. Respiratory systems were compared by use of mitochondria isolated from wild type and from young cultures of mi-1 which contained no α absorption bands of cytochromes *a* or *b*.

When mitochondria isolated from young cultures of mi-1 were incubated at 4 C in the homogenizing medium described by Hall and Greenawalt (6), the specific activity of succinate oxidation rapidly decreased with time. The kinetics of inactivation was a function of the concentration of mitochondria in the incubating suspension, as shown in Fig. 1. Inactivation was not nearly as rapid in suspensions with a high concentration of mitochondria.

The inactivation of the succinate oxidase system could be inhibited by strongly buffering the isolation medium, as shown in Fig. 2. The optimal *p*H for stabilizing the system was about 7.5. There was very little variation in stability over the osmotic pressure range from 0.2 to 0.5 M sucrose, but 0.25 M sucrose gave optimal stability. Rapid degeneration of succinate oxidase activity was not observed in wild-type mitochondria.

Both respiratory systems could be inhibited by

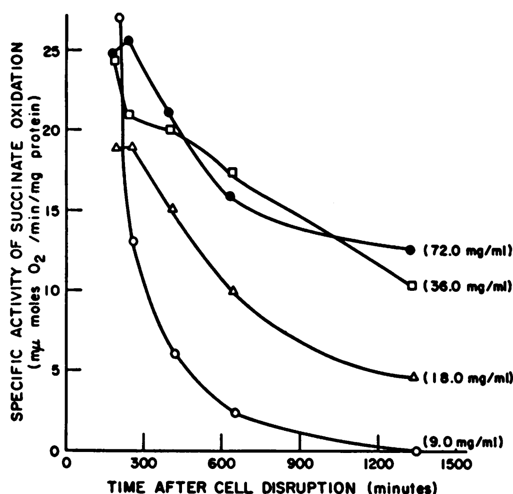

FIG. 1. *Degeneration of succinate oxidase activity for various concentrations of mitochondria suspended in the medium of Hall and Greenawalt (6). Reactions were run in a volume of 3.0 ml starting with 0.6 μmole of dissolved oxygen and 5.0 μmoles of succinate. Mitochondria were prepared from a 17-hr culture of mi-1. Buffering was obtained with 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride or 0.05 M potassium phosphate.*

removal of cytochrome *c*, and reconstituted, at least partially, by adding back horseheart cytochrome *c* (Table 1). Almost all of the original activity could be reconstituted in the wild type system. Succinate oxidase activity of the mi-1 system was increased significantly by external horseheart cytochrome *c*, but not to the same extent as was the wild type system.

The kinetics of oxygen consumption linked to succinate oxidation was measured in the absence and in the presence of catalase. Catalase did not alter the kinetics of either system, indicating that the terminal oxidation step of both the mi-1 and the wild type respiratory systems involves the reduction of molecular oxygen to water rather than to peroxide.

The mi-1 respiratory system is able to couple phosphate esterification to the oxidation of both reduced nicotinamide adenine dinucleotide (NADH) and succinate. The P/O ratios obtained for succinate and NADH oxidation by both mi-1 and wild type mitochondria are shown in Table 2. Wild type had higher P/O ratios for both substrates than did mi-1. Oxidative phosphorylation was uncoupled in both systems by dinitrophenol.

Both respiratory systems were sensitive to azide, though the wild-type system was more sensitive than the mi-1 system at very low azide concentrations (Fig. 3). At an azide concentration of 10^{-3} M, which completely inactivates

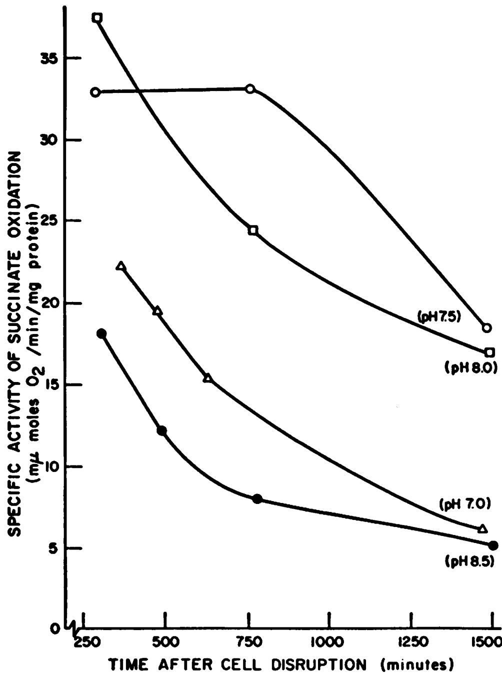

FIG. 2. *Degeneration of succinate oxidase activity for mitochondrial suspensions of 20.0 mg/ml at various pH values. Reactions were run in a volume of 3.0 ml, starting with 0.6 μmole of dissolved oxygen and 5.0 μmoles of succinate. Mitochondria were prepared from a 16-hr culture of mi-1.*

TABLE 1. *Reconstitution of mi-1 and wild type mitochondrial oxidase systems with horseheart cytochrome c*[a]

System	Rate of oxygen consumption (nmoles of oxygen/min)	Percentage of control rate
Control, mi-1	141	100
Washed mi-1	9	6
Washed mi-1, cytochrome *c*	38	27
Wild type control	66	100
Washed wild type	29	44
Washed wild type, cytochrome *c*	64	97

[a] Reactions were run in a volume of 3.0 ml containing 0.6 μmole of dissolved oxygen, with the use of 0.1 ml of mitochondrial suspension and 5.0 μmoles of succinate. Rates were determined initially and then again after the addition of 1 mg of horseheart cytochrome *c*. The mitochondria used in the experiment were prepared from a 15-hr culture of mi-1 and from a 24-hr culture of wild type.

TABLE 2. *Oxidative phosphorylation by mi-1 and by wild type mitochondria*[a]

Substrate	Uncoupler	P/O ratios	
		mi-1 mitochondria	Wild type mitochondria
Succinate	None	0.2	0.4
Succinate	Dinitrophenol	0.0	0.0
NADH	None	0.35	0.5
NADH	Dinitrophenol	0.1	0.0

[a] Reactions were run in a volume of 3.0 ml containing 0.6 μmole of dissolved oxygen and with the use of 5.0 μmoles of substrate. When used, the dinitrophenol concentration was 0.003 M. Mitochondria were prepared from an 18-hr culture of mi-1 and from a 12-hr culture of wild type.

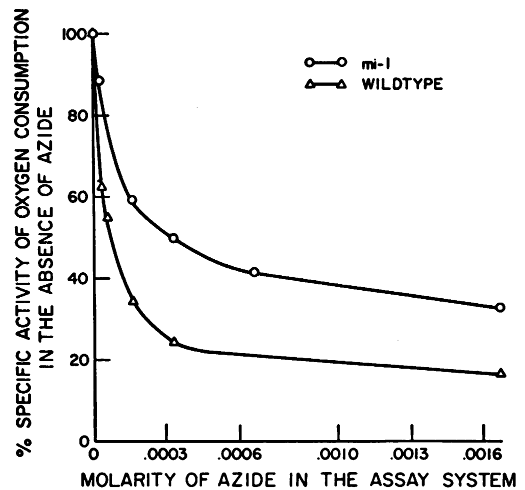

FIG. 3. *Sensitivity of succinate oxidation to azide. Reactions were run in a volume of 3.0 ml containing 0.6 μmoles of dissolved oxygen, and with the use of 5.0 μmoles of succinate. Mitochondria were prepared from a 17-hr culture of mi-1 and from a 24-hr culture of wild type.*

mammalian cytochrome oxidase activity, the wild type succinate oxidase system was inhibited by about 80%, and the mi-1 succinate oxidase system was inhibited by about 60%.

DISCUSSION

The rapid degeneration of succinate oxidase activity in mi-1 mitochondria during in vitro aging is important to consider when interpreting previous data on mi-1 mitochondria and respiration. The previously reported finding of an absence of succinate oxidase activity in cytochrome-deficient mi-1 cultures (13) could have been a

result of such in vitro degeneration. The dependence of succinate oxidase inactivation on mitochondrial concentration apparently is not caused by passive diffusion of an essential component, since no diffusible component was found, and at the lowest concentration the inactivation kinetics was not exponential, as would be expected for infinite dilution.

Young cultures of mi-1 must depend solely on a cytochrome *a*-independent respiratory system. Primary substrates of the mi-1 system are NADH and succinate, as with the wild type cytochrome system. This indicates that the same dehydrogenases that are used in the wild type cytochrome system are also used by the mi-1 respiratory system. The partial reconstitution of succinate oxidase in KCl-extracted mi-1 mitochondria after cytochrome *c* addition suggests the participation of cytochrome *c* in the mi-1 respiratory system. Failure to obtain complete reconstitution in the mi-1 system may have been related to the degeneration observed in unbuffered suspensions of mitochondria, or it may have been the result of something in addition to cytochrome *c* being partially removed by the extraction procedure.

The pathway between cytochrome *c* and oxygen in the mi-1 respiratory system is associated with membrane and consists of a terminal oxidase which is less sensitive to azide than is cytochrome *a*. The terminal oxidase uses molecular oxygen as an electron acceptor, and water rather than peroxide is formed. Edwards and Woodward (5) have found a heme *a* protein in mi-1 mitochondria which has cytochrome *c* oxidase activity but without a characteristic cytochrome *a* absorption spectrum. This is probably the terminal oxidase used in the mi-1 respiratory system.

The mi-1 respiratory pathway is linked to an adenosine-5′-diphosphate phosphorylation system, as phosphate esterification dependent on succinate oxidation could be detected. It is most likely the same system that is linked to the cytochrome chain, because elementary particles associated with mi-1 mitochondrial membranes can be demonstrated (9).

The similarities between the wild type and the mi-1 respiratory systems are very striking. In addition to the similarities of components, there are no gross differences between the two systems except absorption spectra and instability in unbuffered solution. One possibility which might account for these facts is that the mi-1 respiratory system is actually an altered form of the normal cytochrome chain, containing all the membrane-bound protein structures but with defective binding of the heme prosthetic groups which give membrane cytochromes their characteristic absorption spectra.

Hence, the appearance of cytochromes *a* and *b* late in the growth cycle of mi-1 may represent a conversion in heme binding instead of de novo protein synthesis. Such a conversion, perhaps involving the substitution of an amino acid side chain for a solvent molecule in the fifth and sixth coordination positions of the heme complex, could also occur in wild type, with the mi-1 mutation affecting the kinetics. It has been shown that the kinetics of conversion of cytochrome *c* from one form to another is drastically altered by the mi-1 mutation (11). By trimethylation of one specific lysine residue, cytochrome *c* is converted from an unbound form to a form which can be associated with membrane (2). In the mi-1 mutant, the appearance of cytochromes *b* and *a* occurs during the time that cytochrome *c* is converted from one form to the other. Furthermore, in wild type the conversion of cytochrome *c* and the appearance of cytochromes *a* and *b* are not delayed. Thus, if the appearance of cytochromes *a* and *b* in older cultures of mi-1 does represent a conversion in the conformation of pre-existing proteins, it may be related to cytochrome *c* conversion.

ACKNOWLEDGMENT

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