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Immunological Identification of the Alternative Oxidase of *Neurospora crassa* Mitochondria

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***Neurospora crassa* mitochondria use a branched electron transport system in which one branch is a conventional cytochrome system and the other is an alternative cyanide-resistant, hydroxamic acid-sensitive oxidase that is induced when the cytochrome system is impaired. We used a monoclonal antibody to the alternative oxidase of the higher plant *Sauromatum guttatum* to identify a similar set of related polypeptides (M_r, 36,500 and 37,000) that was associated with the alternative oxidase activity of *N. crassa* mitochondria. These polypeptides were not present constitutively in the mitochondria of a wild-type *N. crassa* strain, but were produced in high amounts under conditions that induced alternative oxidase activity. Under the same conditions, mutants in the *aod-1* gene, with one exception, produced apparently inactive alternative oxidase polypeptides, whereas mutants in the *aod-2* gene failed to produce these polypeptides. The latter findings support the hypothesis that *aod-1* is a structural gene for the alternative oxidase and that the *aod-2* gene encodes a component that is required for induction of alternative oxidase activity. Finally, our results indicate that the alternative oxidase is highly conserved, even between plant and fungal species.**

The mitochondria of many fungi and higher plants have a branched electron transport system with a normal cytochrome chain and an alternative cyanide-resistant, hydroxamic acid-sensitive pathway (6). Electron transport in these systems branches at ubiquinone, and the alternative pathway is not linked to oxidative phosphorylation (15). The function of the alternative oxidase is unknown, except for some species of thermogenic plants. Most of these species, usually in the family *Araceae*, use the production of heat, through uncoupled respiration, to volatilize insect attractants, thus facilitating pollination (14). For most plants and fungi, the physiological role of the alternative oxidase is obscure. One hypothesis is that it serves as a method to rid the cell of excess or "luxury" carbohydrates (7). In this view, the alternative pathway would allow the tricarboxylic acid cycle to continue operation by lowering the ATP concentration, thus allowing production of carbon skeletons for other metabolic processes (15). Another possibility is that the alternative oxidase defends the fungus or plant against respiratory inhibitors that are produced by competing organisms in the environment (12).

In the fungus *Neurospora crassa*, little or no alternative oxidase is present constitutively, but it is induced in high amounts by inhibitors of mitochondrial protein synthesis and electron transport (1, 10, 11). It is also induced in cytochrome-deficient mutants, such as the extranuclear [*poky*] mutant (10, 11). Bertrand et al. (1) have characterized 27 antimycin-sensitive mutants of *N. crassa* which are unable to induce alternative oxidase activity. These mutants fall into two complementation groups, *aod-1* and *aod-2*. In response to different inducing conditions, the wild-type strain and 19 of 20 *aod-1* mutants were found to accumulate a polypeptide that was tentatively identified as being associated with the alternative oxidase. The remaining *aod-1* mutant and all four *aod-2* mutants that were tested did not accumulate this polypeptide. Based on these findings, Bertrand et al. (1) hypothesized that *aod-1* is the structural gene for the alternative oxidase and that the *aod-2* gene encodes a component that is required for induction of alternative oxidase activity.

The alternative oxidase has been partially purified from several higher plants (2, 4), but there is little information about its identity in any organism. Elthon and McIntosh (5) have raised polyclonal antibodies to the solubilized and partially purified alternative oxidase from the aroid *Sauromatum guttatum*. These antibodies immunoprecipitated alternative oxidase and have been used in immunoblotting experiments to identify three cross-reacting polypeptides with molecular weights of 37,000, 36,000, and 35,000. Recently, monoclonal antibodies have been made which react with these three polypeptides and which also inhibit the alternative oxidase activity from *S. guttatum* (T. E. Elthon, R. L. Nickels, and L. McIntosh, Plant Physiol., in press). These antibodies have also been shown to bind to a similar series of related proteins from a number of higher plants and algae (5; Elthon et al., in press; L. McIntosh and R. L. Nickels, unpublished data). Here, we demonstrate that a monoclonal antibody to the alternative oxidase of *S. guttatum* binds to a similar set of related proteins associated with the alternative oxidase activity of *N. crassa* mitochondria. The antibody was used to further characterize *N. crassa* mutants deficient in the alternative oxidase pathway.

S. guttatum Schott (voodoo lilies) were maintained and mitochondria were isolated as described previously (4, 5). *N. crassa* cultures were maintained and cells were grown in liquid culture essentially as described previously (3, 9). Wild-type *N. crassa* 74*A* and *aod-1* and *aod-2* mutants were grown in liquid culture for 15 h at 25°C or in liquid cultures containing chloramphenicol (4 mg/ml) for 18 h at 25°C. The cytochrome-deficient [*poky*] mutant was grown for 24 h at

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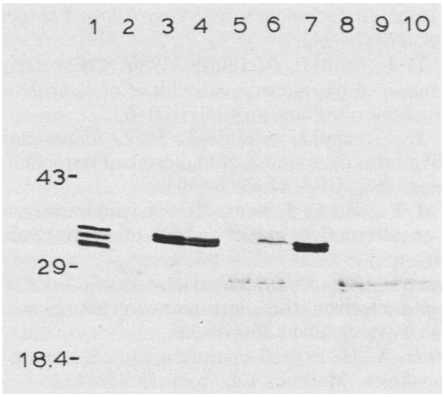

FIG. 1. Immunoblot analysis of total mitochondrial proteins from *S. guttatum* and *N. crassa*. Lane 1, *S. guttatum* (30 μg of total mitochondrial protein); lane 2, wild-type *N. crassa* 74*A* (300 μg of protein); lane 3, wild-type strain 74*A* grown in the presence of chloramphenicol (23 μg of protein); lane 4, [*poky*] 13-6*A* (24 μg of protein); lane 5, 7064 (*aod-2-4 nic-1 al-2 a*) (300 μg of protein); lane 6, 7064 grown in the presence of chloramphenicol (300 μg of protein); lane 7, 7301 (*aod-1-1 qa-2 aro-9 a*) grown in the presence of chloramphenicol (11 μg of protein); lane 8, 7301 (301 μg of protein); lane 9, 7202 (*aod-1-4 A*; previously designated *ANT-1*) (300 μg of protein); lane 10, 7202 grown in the presence of chloramphenicol (300 μg of protein). Numbers to the left of the gel are molecular weights, in thousands.

25°C. Measurements of whole-cell respiration were carried out with a Clarke oxygen electrode (model 53; Yellow Springs Instrument Co., Yellow Springs, Ohio) as described previously (10). The inhibitors used to characterize *N. crassa* cell respiration were sodium cyanide (1 mM) and salicyl hydroxamic acid (780 mM). Mitochondria were isolated from *N. crassa* by a modified flotation gradient procedure (8), except that the mitochondrial band that was isolated from sucrose gradients was diluted slowly with 0.15 M sucrose–0.05 mM EDTA–10 mM Tricine (pH 7.5) until a final concentration of 0.3 M sucrose was obtained. Mitochondria were then pelleted by centrifugation, as described previously (8). Mitochondrial proteins were analyzed by electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel containing a 10 to 17.5% gradient of polyacrylamide and immunoblotted as described previously (4, 5). Immunoblots were probed with a monoclonal antibody (AOA) that was specific for the mitochondrial alternative oxidase of *S. guttatum* (Elthon et al., in press).

As indicated above, little or no alternative oxidase activity is present in wild-type *N. crassa* strains, which have fully functional mitochondria, but it can be induced readily by a number of treatments that impair the cytochrome system (1, 10). In the present study, we used chloramphenicol, an inhibitor of mitochondrial protein synthesis, to induce alternative oxidase activity. Figure 1 shows an immunoblot of a sodium dodecyl sulfate-polyacrylamide gel of whole mitochondrial proteins from wild-type *N. crassa* 74*A* and alternative oxidase-deficient mutants in the presence or absence of chloramphenicol. Data are also shown in Fig. 1 for mitochondria from the cytochrome-deficient mutant [*poky*], which has high constitutive levels of alternative oxidase (10, 11). The blots were probed with a monoclonal antibody, AOA (Elthon et al., in press), which was raised to the alternative oxidase of *S. guttatum*. A detailed characterization of the *aod* mutants with respect to respiration and cytochrome content has been described previously (1). For each strain in the present study, the presence or absence of alternative oxidase activity was confirmed by respiration measurements on whole cells (data not shown).

In *S. guttatum*, the AOA monoclonal antibody reacted specifically with a series of three polypeptides of 37, 36, and 35 kilodaltons (kDa) (Fig. 1, lane 1), which correlated with alternative oxidase activity (5; Elthon et al., in press). A set of two *N. crassa* polypeptides of 37 and 36.5 kDa were barely visible in wild-type 74*A* under standard growth conditions (lane 2), but were present at a high concentration in wild-type 74*A* that were grown in the presence of chloramphenicol to induce alternative oxidase (lane 3). These polypeptides were also present in the cytochrome-deficient mutant [*poky*] (lane 4). The putative structural gene mutant *aod-1* (7301), which showed no induction of alternative oxidase activity in response to chloramphenicol (1), synthesized apparently inactive protein under these conditions (lanes 8 and 7). The *aod-2* mutant 7064 showed very little induction of these proteins in response to chloramphenicol (lanes 5 and 6). In addition, the *aod-2* mutant appeared to specifically lack the 36.5-kDa band, while it was grossly deficient in the 37-kDa band. Finally, we confirmed that the single *aod-1* mutant (7202; previously designated ANT-1 [1]), which was believed to be unable to synthesize the alternative oxidase polypeptide, did not produce the 36.5- or 37-kDa polypeptides in the absence or presence of chloramphenicol (lanes 9 and 10, respectively).

In the immunoblot shown in Fig. 1, gel loads were 10-fold higher for strains which lacked the polypeptides at 37 and 36.5 kDa (lanes 2, 5, 6, 8, 9, and 10) than they were for strains producing these polypeptides (lanes 3, 4, and 7). At these higher gel loads, very faint bands could be seen at several other molecular weights (lanes 2, 5, 6, 8, 9, and 10). While there is presently no explanation for these bands, similar results have been obtained with plant mitochondria that exhibit very low alternative oxidase activities (L. McIntosh and R. L. Nickels, unpublished data).

The results presented here demonstrate that a monoclonal antibody to the *S. guttatum* alternative oxidase specifically recognizes polypeptides of 36.5 and 37 kDa that are correlated with alternative oxidase activity in *N. crassa* mitochondria. Since the antibody was prepared to a gel-purified, 36-kDa polypeptide from an *S. guttatum* alternative oxidase preparation and since it specifically inhibited alternative oxidase activity (Elthon et al., in press), our results suggest that the 36.5-kDa polypeptide, the 37-kDa polypeptide, or both correspond to components of the alternative oxidase of *N. crassa* mitochondria. Other experiments showed that a previously described polyclonal antibody to partially purified *S. guttatum* alternative oxidase (5) also recognizes the *N. crassa* polypeptides (data not shown). The alternative oxidase appears to be remarkably conserved between *N. crassa* and higher plants, as judged by the molecular weights and the ability to react with antibodies raised against the plant oxidase.

The finding that the monoclonal antibody binds to two closely spaced polypeptide bands in *N. crassa* mitochondria is similar to the situation in higher plants, in which the same antibody binds to three closely spaced bands (5; Elthon et al., in press). Since the *N. crassa* alternative oxidase component appears to be encoded by a single gene, *aod-1*, the most likely interpretation is that there is a single polypeptide which is modified to give the multiple bands that were detected in the immunoblots.

Our results show further that induction of the alternative oxidase in *N. crassa* results from the synthesis of alternative oxidase polypeptides and not from the activation of preex-

isting polypeptides. Previous results for *S. guttatum* and *N. crassa* have demonstrated that induction could be blocked by inhibitors of transcription and translation (1, 13). Considered together, these findings suggest that induction of the alternative oxidase in *N. crassa* results from transcriptional activation of the nuclear gene(s) encoding the alternative oxidase.

Finally, our results are consistent with the previous interpretation that the mutants in the putative structural gene *aod-1* synthesize inactive alternative oxidase polypeptides, whereas *aod-2* mutants are unable to induce the synthesis of these polypeptides. The *aod-2* gene may encode a component that regulates induction of the alternative oxidase at either the transcriptional or the posttranscriptional level, or it may encode a membrane protein or subunit of alternative oxidase that is required for stable accumulation of the *aod-1* polypeptide. The antibodies described here should be useful tools in further studies of the alternative oxidases from *N. crassa* and other fungi and for cloning of the plant and fungal genes, which is currently in progress in our laboratories.

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LITERATURE CITED

1. **Bertrand, H., C. A. Argan, and N. A. Szakacs.** 1983. Genetic control of the biogenesis of cyanide insensitive respiration in *Neurospora crassa*, p. 495–507. *In* R. J. Schweyen, K. Wolf, and F. Kaudewitz (ed.), Mitochondria 1983. Walter de Gruyter & Co., Berlin.
2. **Bonner, W. D., S. D. Clarke, and P. R. Rich.** 1986. Partial purification and characterization of the quinol oxidase activity of *Arum maculatum* mitochondria. Plant Physiol. **80**:838–842.
3. **Davis, R. H., and F. J. de Serres.** 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17A**:79–143.
4. **Elthon, T. E., and L. McIntosh.** 1986. Characterization and solubilization of the alternative oxidase of *Sauromatum guttatum* mitochondria. Plant Physiol. **82**:1–6.
5. **Elthon, T. E., and L. McIntosh.** 1987. Identification of the alternative terminal oxidase of higher plant mitochondria. Proc. Natl. Acad. Sci. USA **84**:8399–8403.
6. **Henry, M. F., and E. J. Nyns.** 1975. Cyanide-insensitive respiration: an alternative mitochondrial pathway. Sub-cell. Biochem. **4**:1–65.
7. **Lambers, H.** 1982. Cyanide-resistant respiration: a nonphosphorylating electron transport pathway acting as an energy overflow. Physiol. Plant **55**:478–485.
8. **Lambowitz, A. M.** 1979. Preparation and analysis of mitochondrial ribosomes. Methods Enzymol. **59**:421–433.
9. **Lambowitz, A. M., R. J. LaPolla, and R. A. Collins.** 1979. Mitochondrial ribosome assembly in *Neurospora*. Two-dimensional gel electrophoretic analysis of mitochondrial ribosomal proteins. J. Cell Biol. **82**:17–31.
10. **Lambowitz, A. M., and C. W. Slayman.** 1971. Cyanide-resistant respiration in *Neurospora crassa*. J. Bacteriol. **108**:1087–1096.
11. **Lambowitz, A. M., E. W. Smith, and C. W. Slayman.** 1972. Electron transport in *Neurospora* mitochondria; studies on wild type and *poky*. J. Biol. Chem. **247**:4850–4858.
12. **Lambowitz, A. M., and D. Zannoni.** 1978. Cyanide-insensitive respiration in *Neurospora*; genetic and biophysical approaches, p. 283–291. *In* G. Ducet and C. Lance (ed.), Plant mitochondria. Elsevier/North-Holland Biomedical Press, Amsterdam.
13. **McIntosh, L., and B. J. D. Meeuse.** 1978. Control of the development of cyanide-resistant respiration in *Sauromatum guttatum* (Araceae), p. 339–345. *In* G. Ducet and C. Lance (ed.), Plant mitochondria. Elsevier/North-Holland Biomedical Press, Amsterdam.
14. **Meeuse, B. J. D.** 1975. Thermogenic respiration in aroids. Annu. Rev. Plant Physiol. **26**:117–126.
15. **Palmer, J. M.** 1976. The organization and regulation of electron transport in plant mitochondria. Annu. Rev. Plant Physiol. **27**:133–157.