Characterization of a Split Respiratory Pathway in the Wheat “Take-all” Fungus, *Gaemunnomyces graminis var. tritici*(*

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This article describes the first detailed analysis of mitochondrial electron transfer and oxidative phosphorylation in the pathogenic filamentous fungus, *Gaemunnomyces graminis var. tritici*. While oxygen consumption was cyanide insensitive, inhibition occurred following treatment with complex III inhibitors and the alternative oxidase inhibitor, salicylhydroxamic acid (SHAM). Similarly, maintenance of a ΔΨ across the mitochondrial inner membrane was unaffected by cyanide but sensitive to antimycin A and SHAM when succinate was added as the respiratory substrate. As a result, ATP synthesis through complex V was demonstrated to be sensitive to these two inhibitors but not to cyanide. Analysis of the cytochrome content of mitochondria indicated the presence of those cytochromes normally associated with electron transport in eukaryotic mitochondria together with a third, b-type heme, exhibiting a dithionite-reduced absorbance maxima at 560 nm and not associated with complex III. Antibodies raised to plant alternative oxidase detected the presence of both the monomeric and dimeric forms of this oxidase. Overall, this study demonstrates that a novel respiratory chain utilizing the terminal oxidases, cytochrome c oxidase and alternative oxidase, are present and constitutively active in electron transfer in *G. graminis tritici*. These results are discussed in relation to current understanding of fungal electron transfer and to the possible contribution of alternative redox centers in ATP synthesis.

Under aerobic conditions, respiration of carbon metabolites in animal, plant, and fungal cells occurs in a tightly regulated manner to produce carbon dioxide and water. Transfer of the electron pairs associated with the respiration of carbon metabolites is indirect and complex, involving the reduction of the coenzymes NAD+ and FAD at two sites within glycolysis and the citric acid cycle. The electrons (associated with NADH and FADEL) are subsequently transferred, via at least four distinct sites, into the electron-transport chain, where a series of reduction and oxidation events occur in a sequential manner at approximately 10 different redox centers. In the classical scheme, these redox centers are composed of a series of cytochromes and iron-sulfur complexes. About half the energy generated is lost as heat. The remaining energy generated by the electron flow is utilized in the translocation of hydrogen ions from the mitochondrial matrix to the intermembrane mitochondrial membrane space. The free energy is thus stored in the proton gradient (proton motive force) and is subsequently used to drive the synthesis of ATP.

While in mammalian systems the components and sequence of events associated with electron transport appear tightly conserved, those of plants and fungi appear more complex and diverge in nature, often involving alternative redox centers and pathways. Significant research has been conducted in the area of plant respiration, leading to the characterization of these alternative systems, but research into fungal respiration has been limited, and this is especially true for the pathogenic filamentous fungi. With the development and recent launch of a new antifungal chemistry (the methoxyacrylates) renewed interest is developing in this field (1, 2). Associated with the respiratory chain in most higher plants, some fungi and protozoa are the alternative oxidase, which is reduced by electrons from the ubiquinol pool (the only conserved element of all characterized respiratory chains). While biochemically consistent with a terminal oxidase, as it reduces O2 to water, this oxidase is distinguished by its insensitivity to cyanide, azide, and carbon monoxide. Plant alternative oxidase has been the focus of interest since it was first described (3) but real progress was hampered until Elthon *et al.* (4) raised antibodies to the alternative oxidase of *Saurornatum guttatum* resulting in the eventual isolation of its cDNA clone (5). Subsequent analyses of plant alternative oxidases have shown that the protein has a molecular mass of between 32 and 40 kDa and exists as a membrane bound protein dimer. Each monomer consists of two membrane spanning a-helices with hydrophilic domains flanking the membrane anchoring helix which then extend into the matrix (6). Monomers are separated by a sulfhydryl-disulfide system, which plays a key regulatory role in the activity of the oxidase, with increased activity associated with the reduced state (–SH-HS−). Regulation of the activity has also been linked with the induction of gene expression (7), the redox state of the ubiquinol pool (8), levels of a-keto acids (9), and intermediates of the tricarboxylic acid cycle (10, 11).

The alternative oxidase is of considerable interest in plants due to its nonphosphorylating nature. Although evidence in support of possible roles exist, a precise function remains unclear. Early reports by Bahr and Bonner (12) indicated that the alternative oxidase could be activated following the inhibition or saturation of the cytochrome pathway and, therefore, lead to the suggestion that the alternative oxidase in plants provided an “energy overflow” capability. Subsequent work has chal-

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lenged this, on the basis that the alternative pathway may compete directly with the main pathway for electrons. A refined function has consequently been proposed whereby the alternative oxidase in plant mitochondria may provide a route for the maintenance of the high rate of electron transfer required under conditions of cold stress or during the operation of the photosynthetic cycle in photosynthesizing tissues.

In fungi, and particularly in filamentous fungal species, the presence, function, and regulation of the alternative oxidase remains controversial. A recent report, based on whole-cell sensitivity studies to the alternative oxidase inhibitor, salicylhydroxamic acid (SHAM), inferred that the alternative oxidase may be more extensively distributed among pathogenic fungal species than had previously been reported (13), but remains to be conclusively demonstrated.

Induction of the alternative pathway in filamentous fungi following inhibition of cytochrome b by methoxyacrylates and antimycin A, again defined by the development of pathogen sensitivity to SHAM, varies in extent dependent on which cytochrome b inhibitor was employed. This may be explained by the different target sites of the inhibitors within cytochrome b and the resultant electron “leakiness” observed in relation to electron flow through either the Q cycle (14), or the alternative cycle hypothesis of Matsuno-Yagi and Hatefi (15). A common observation for all pathogenic fungi utilizing the alternative respiratory pathway was a fitness penalty, presumably due to the decreased ATP generating capacity of this pathway (2).

Induction and regulation of the plant alternative oxidase by compounds such as salicylic acid has been well documented. However, induction of the fungal alternative oxidase appears more limited, and has only been demonstrated to occur following inhibition of the respiratory chain (16), and as a stimulatory response to the presence of nucleotides AMP, ADP, dAMP, more limited, and has only been demonstrated to occur following inhibition of the respiratory chain (16), and as a stimulatory response to the presence of nucleotides AMP, ADP, dAMP, dTMP, and dTTP (17).

In the present study, we present evidence that in the phytopathogenic fungus, Gaeumannomyces graminis var. tritici, the cytochrome and alternative oxidase respiratory pathways are constitutively expressed and active. In contrast to the plant alternative respiratory pathway, the alternative oxidase of G. graminis tritici provides a mechanism for the generation and maintenance of a proton motive force (and hence ATP biosynthesis). Since its reliance on the alternative oxidase apparently carries no discernible fitness penalty, we anticipate that G. graminis tritici provides a model system for future studies to probe the function, regulation, and electron partitioning between cytochrome and alternative pathways in filamentous fungi.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals, unless otherwise stated, were purchased from Sigma, Poole, UK, and were of the highest purity. Carboxin was supplied by Uniroyal Chemicals, Evesham, UK. Stock solutions of di- nitrophenol (54 mM), antimycin A (19 mM based on an average formula weight 527.6 as purchased as a mixture of antimycin A1, A2, A3, and A4), oligomycin (13 mM, based on an average formula weight 790.4 as purchased as a mixture of oligomycins A, B, and C1) and carbinox (0.01 mM) were prepared in ethanol. Potassium cyanide was dissolved in dH2O (0.5 mM) and SHAM (0.2 mM) was dissolved in dimethyl sulfoxide. All inhibitors were prepared immediately prior to use and the solvent concentration never exceeded 1% (v/v) in any assay. Rhodamine 123 and the luciferin: luciferase ATP determination kit were supplied by Molecular Probes, Inc., Europe. 

**Strains**—G. graminis tritici strains UK22A-1 and DK22A were isolated from the field in 1995 and strain T7 was a gift from Dr. Paul Bowyer, IACR-Long Ashton Research Station. The strain MT3 was isolated at Long Ashton. All cultures were maintained at 18 °C on Czapek Dox Agar (Oxoid) supplemented with n-biotin (0.2 ppm) and thiamine (0.2 ppm). Cultures for analysis were prepared routinely as described. Five, 5-mm agar plugs removed from the outer zone of 2-week-old cultures were inoculated onto 20 ml of Czapek Dox liquid medium (Oxoid) supplemented with n-biotin (0.2 ppm) and thiamine (0.2 ppm) and allowed to grow again. After 3 days at 18 °C, mycelia were disrupted with a hand-held, all glass, homogenizer (30 ml), and the resulting cellular suspension transferred to either 50 or 100 ml of Czapek Dox liquid medium in 100- or 200-ml conical flasks and maintained at 100 rpm, at 18 °C for 72 h.

**Toxicity Assay**—Sensitivity to respiratory and oxidative phosphorylation inhibitors was determined in 10 ml of liquid Czapek Dox medium maintained at 100 rpm at 18 °C, containing approximately 5 mg wet weight mycelia. Inhibition of growth was evaluated after 72 and 96 h, by dry cell weights. All assays were conducted in triplicate.

**Measures of Oxygen Consumption in Whole Cells**—Oxygen consumption by 10 mg ml−1 wet mycelia was measured at 18 °C with a Clark-type oxygen electrode in 2 ml of buffer 1 (50 mM potassium phosphate (pH 7.4)). Inhibitors were added at the concentrations indicated with subsequent oxygen consumption recorded over 15 min unless otherwise indicated.

**Spectrophotometric Measurements of Mitochondrial Cytochromes**—The mitochondrial fraction was prepared as follows. Three-day-old mycelia were washed three times in 20 ml of citrate phosphate buffer containing 50 mM citrate, 50 mM disodium phosphate (pH 5.6), 1.2 mM sorbitol, and 20 mM 2-mercaptoethanol. Protoplasts were generated by wall digestion for 50–75 min with 3 mg/ml Novozyme 234 (Interspec Products, Inc., Foster City, CA) at 18 °C. Protoplast formation was monitored microscopically and once 90% of mycelia were protoplasted the Novozyme was removed by three washes with Buffer II (30 mM Tris-HCl, 15 mM sucrose, 5 mM KCl, 1 mM K2HPO4, 0.5 mM MgCl2, and 0.2 mM Na3EDTA (pH 7.4)) at 2,000 × g for 8 min at 0 °C. Protoplast disruption was performed at 0 °C in a Bead-Beater with 40 × g of acid-washed glass beads (150–212 μm diameter), 30 ml of protoplasts in buffer II, and 250 μl of fungal protease inhibitor mixture (Sigma P 8215). Glass beads and large cellular debris were removed by filtering the homogenate through Mira cloth (0.007 μm) and large cellular particulates were removed by centrifugation at 6,000 × g for 10 min at 4 °C. The mitochondrial fraction was obtained following centrifugation at 20,000 × g for 25 min at 4 °C. The resultant mitochondrial pellet was resuspended in Buffer III (1 ml: 0.1 mM KH2PO4, 0.1 mM KH2PO4, 20% (v/v) glycerol, 1 mM EDTA, and 1 mM reduced glutathione). Ten μl of this suspension was removed for the determination of protein concentration and the remainder immediately frozen in liquid nitrogen. All mitochondrial samples were stored at −80 °C.

**Mitochondrial Alternative Respiration**—Respiration by 100 µg of isolated mitochondrial protein using either a Unicam UV2 100 UV/Vis Spectrometer v4.15 or a spectrometer developed “in-house” by Prof. Peter Rich at the Glynn Laboratory, UCL (Gower Street, London, United Kingdom). Prior to analysis, mitochondria were washed in 2 ml of buffer III (pH 7.4) + 0.6 M KCl to remove any pigment adsorbed to the mitochondrial membrane during preparation.

**Detection of Alternative Oxidase**—Resolution of proteins by SDS-polyacrylamide gel electrophoresis was essentially as described by Laemmli (18) with equal amounts of mitochondrial protein from *Arum maculatum* and *G. graminis tritici* strain T7 (approximately 20 μg) electrophoresed on a 10% SDS-polyacrylamide electrophoresis gel.

**Electroblotting to Nitrocellulose Membrane**—Electrophoresed proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, 0.45 μm pore size) according to the method of Towbin et al. (19). The transfer buffer contained 12.6 mM Tris buffer (pH 7.5), 192 mM glycine, and 0.02 M Na3EDTA (pH 7.4) at 2,000 × g for 8 min at 0 °C. Proteolysis digestion for 50–75 min with 3 mg/ml Novozyme 234 (Interspec Products, Inc., Foster City, CA) at 18 °C. Protoplast formation was monitored microscopically and once 90% of mycelia were protoplasted the Novozyme was removed by three washes with Buffer II (30 mM Tris-HCl, 15 mM sucrose, 5 mM KCl, 1 mM K2HPO4, 0.5 mM MgCl2, and 0.2 mM Na3EDTA (pH 7.4)) at 2,000 × g for 8 min at 0 °C. Protoplast disruption was performed at 0 °C in a Bead-Beater with 40 × g of acid-washed glass beads (150–212 μm diameter), 30 ml of protoplasts in buffer II, and 250 μl of fungal protease inhibitor mixture (Sigma P 8215). Glass beads and large cellular debris were removed by filtering the homogenate through Mira cloth (0.007 μm) and large cellular particulates were removed by centrifugation at 6,000 × g for 10 min at 4 °C. The mitochondrial fraction was obtained following centrifugation at 20,000 × g for 25 min at 4 °C. The resultant mitochondrial pellet was resuspended in Buffer III (1 ml: 0.1 mM KH2PO4, 0.1 mM KH2PO4, 20% (v/v) glycerol, 1 mM EDTA, and 1 mM reduced glutathione). Ten μl of this suspension was removed for the determination of protein concentration and the remainder immediately frozen in liquid nitrogen. All mitochondrial samples were stored at −80 °C.

**Western Blotting**—Filters were initially incubated with 3% (w/v) bovine serum albumin and 2% (w/v) milk powder in phosphate-buffered saline (145 mM NaCl (pH 7.2), 12.5 mM Na2HPO4, and 2.5 mM NaH2PO4) in order to block unbound protein reactive sites. The filters were then washed in 0.6% (w/v) milk powder, 0.1% (v/v) Tween 20 buffer in phosphate-buffered saline. Treated filters were incubated with the monoclonal antibody to the alternative oxidase antibody (4) at a dilution of 1:1000 in 3% (w/v) bovine serum albumin and phosphate-buffered saline buffer. The filters were washed as described above, prior to incubation with a secondary anti-mouse antibody linked to horseradish-peroxidase enzyme and used at a concentration of 1 in 1000 as the primary antibody. Filters were washed in 0.1% (v/v) Tween 20 and bound antibody was detected using an
enhanced chemiluminescence kit (Amersham International plc, Aylesbury, Bucks, United Kingdom).

Inhibitor Effects on the Proton Motive Force across the Inner Mitochondrial Membrane—Isolated mitochondrial fraction (300 μg) was loaded with 1 μM potassium dithionite, rhodamine 123, in 1 μl of buffer II (pH 7.4) and maintained at 4 °C prior to analysis. The effect of the various inhibitors on rhodamine 123 fluorescence was monitored with a Zeiss Axioshot microscope equipped for epifluorescence with excitation filter BP546, dichroic mirror FT580, and barrier filter LP590.

ATP Quantitation—Cultures of G. graminis tritici strains in 100 ml of liquid Czapek Dox medium were maintained at 18 °C for the duration of the assays. At each time point, 15 ml of culture was removed, rapidly filtered through Miracloth, and washed with 50 ml of deionized water. The mycelia were immediately frozen in liquid nitrogen to prevent further ATP formation or hydrolysis and stored at −80 °C prior to analysis. ATP was extracted from mycelia by grinding a known biomass under liquid nitrogen, in a pestle and mortar until a fine powder was obtained. The debris was resuspended in 500 μl of deionized water (2 °C) and immediately frozen in liquid nitrogen. A 10-μl aliquot was removed for protein estimation. Isolation of ATP was achieved by vortex mixing the mycelial debris in phenol:chloroform (1:1) for 30 s and centrifuging in a precooled Biofuge 17RS centrifuge (at 4 °C) at 10,000 × g for 10 min to obtain a clear aqueous phase. The aqueous phase was removed and stored at −80 °C prior to analysis. ATP levels remained constant over 7 days when stored under these conditions.

The ATP concentration of the aqueous phases were determined by the quantity of light produced from a Luciferin-Luciferase assay in a Perkin-Elmer LS50B luminometer with a Total Emission Accessory fitted. Conversion of light intensity to ATP levels was by reference to a standard curve generated from known ATP concentrations which had been prepared following this protocol.

Protein Estimations—Protein levels were measured using the BCA protein assay reagent system (Pierce) adapted for a 96-well microtiter plate assay. Absorbance values (560 nm) were converted to micrograms of protein ml⁻¹ with reference to a bovine serum albumin standard curve.

RESULTS

Toxicty Assays—To determine the active involvement of the cytochrome and alternative respiratory pathways, in G. graminis tritici strains T7, UK22A-1, DK22A, and MT3, sensitivities to cyanide, antimycin A, carboxin, SHAM, and oligomycin B were determined. Minimum inhibitory concentration (MIC) required for 100% growth inhibition (MICs) for each inhibitor were obtained and are presented in Table I.

The MICs for cyanide (an inhibitor of cytochrome aa₃) against each G. graminis tritici strain was 10 μM. Antimycin A, an inhibitor of electron flow from heme b₅₉₅ to the Qi site of complex III showed strong activity against all strains, with MIC values between 10 and 19 μM. Both the succinate dehydrogenase inhibitor, carboxin, and SHAM, an inhibitor of the alternative oxidase, showed activity against all four strains. Similarly oligomycin B, an inhibitor of the Fo subunit of the F₁F₀-ATPase was also active against these strains. Overall, these effects are consistent with the presence of G. graminis tritici of both an active cytochrome and an alternative respiratory pathway, and with ATP generation being associated with the F₁F₀-ATPase.

Oxygen Consumption Assays—To establish that the inhibitor activities observed in the toxicity assays were indicative of inhibition of the components of the respiratory chain, oxygen consumption was measured in whole cells. It was also anticipated that the data would provide information as to the possible site of action of the alternative respiratory pathway following inhibition of the primary cytochrome pathway. Inhibitor doses were selected with reference to previous studies with other fungi (2, 13, 20) and reflect concentrations in excess to that required for 100% inhibition of their respective target sites.

Treatment with 1 mM KCN resulted in no discernible reduction in the rate of O₂ consumption over the assay period in any G. graminis tritici strain (Table II), indicating either a target site alteration or that KCN was prevented from gaining access to cytochrome aa₃. In contrast, treatment with 20 μM antimycin A reduced O₂ consumption by between 36 and 48% compared with the control consumption rate in the G. graminis tritici strains. This apparently contradictory finding was reproducible in all G. graminis tritici strains as shown by the O₂ consumption rate changes in Table II, and confirms the involvement of cytochrome b in the respiratory pathway of G. graminis tritici.

Similar to antimycin A, oligomycin reduced O₂ consumption by between 38 and 48%, which is consistent with the effect that an inhibitor of ATP biosynthesis would exert on electron transfer over the assay period. Treatment of strains T7 and DK22A with the alternative oxidase inhibitor, SHAM, resulted in a reduction in O₂ consumption by 50%. Oxygen consumption was also reduced in strains UK22A-1 and MT3, although to a lesser extent (28 and 34%, respectively). The specificity of SHAM for the alternative oxidase at these doses implies that inhibition may be attributed to inhibition of the alternative oxidase itself. As cyanide-sensitive electron transfer was not detected in these assays, this provides strong evidence for the presence of an active alternative oxidase in G. graminis tritici. Complete inhibition of oxygen consumption was only recorded following inhibition by both antimycin A and SHAM.

Treatment of whole cells with the complex II (succinate dehydrogenase) inhibitor, carboxin, at the MIC dose (0.01 mM) for G. graminis tritici resulted in a small decrease in O₂ consumption over the initial 2 min of the assay (7 and 8% in T7 and UK22A-1, respectively). This level of inhibition increased over a 30-min period to 62 and 48%, respectively.

Characterization of the Respiratory Components of G. graminis tritici—To determine whether the inhibitory effects of antimycin A and KCN resulted from inhibition of the cytochrome components of the respiratory chain, we sought evidence for the presence of their respective target cytochromes. Sodium dithionite reduced spectra were obtained by scanning intact mitochondria of T7, DK22A, UK22A-1, and MT3 from 400 to 650 nm at room temperature. A representative reduced versus oxidized difference spectrum is reproduced in Fig. 1A. The pronounced absorbance peak, with maxima at 531 and 560–562 nm, corresponded to cytochrome b. The proportion of this attributed to the b-hemes of complex III was determined from the succinate reduced, antimycin A-treated absorbance spectra (Fig. 1B). As the mitochondrial content of cytochrome b₁ (antimycin A sensitive) is equivalent to that of cytochrome b₁, the proportion of the total cytochrome b reduced absorbance peak derived from the hemes of the b₁ complex can be estimated as the sum of these two absorbances, and was therefore equivalent to 40%. The remainder of the absorbance was attributed to, as yet uncharacterized, b-heme. Peaks corresponding to cytochromes c₁, c₁, and aa₃ with absorbance maxima at 550 nm (+521 nm), 554 nm (+523), and 603 nm, respectively, are indicated.

The immunoblot of the alternative oxidase protein in mitochondria isolated from T7 gave two distinct signals in the

### Table I

| Inhibitor       | Strain MIC*       |
|-----------------|-------------------|
|                 | T7    | DK22A | UK22A-1 | MT3   |
| Antimycin A     | 19    | 10    | 10      | 19    |
| (μM)            |       |       |         |       |
| Cyanide (μM)    | 10    | 10    | 10      | 10    |
| Carboxin (μM)   | 10    | 10    | 10      | 10    |
| SHAM (μM)       | 200   | 300   | 300     | 300   |
| Oligomycin B    | 7     | 7     | 7       | 7     |
| (μM)            |       |       |         |       |

* MICs were determined in triplicate with variation between determinants of <1%.
absence of a reductant (Fig. 2). These corresponded to the monomeric (oxidized form of approximately 37 kDa) and dimeric (reduced form of approximately 74 kDa) forms of the protein being present (4).

Results from these experiments implicated the involvement of both cytochromes and alternative oxidase in mitochondrial respiration. To probe the specific activity of the two respiratory pathways further, and to analyze their respective contributions to both electron transport and ATP generation in *G. graminis tritici*, the maintenance of the membrane potential (Δψ) and ATP generation were monitored.

The primary function of electron transport remains the generation and maintenance of a proton motive force across the inner mitochondrial membrane, which is then coupled to ATP synthesis at the F1F0-ATPase (complex V). In coupled mitochondria, the maintenance of Δψ may be used as a marker to evaluate the functional state of the respiratory chain. A procedure for monitoring this in *G. graminis tritici* was developed employing the potentiometric dye rhodamine 123.

Penetration of whole cells and isolated mitochondria by rhodamine 123 is rapid and, similarly to previous studies, accumulation had no discernible detrimental effects on cellular or mitochondrial function over the assay period (21). As the intensity of fluorescence is related to Δψ, fluctuations in the proton motive force may be monitored. Fig. 3 depicts the fluorescence of rhodamine-loaded mitochondria of T7 in the presence and absence of inhibitory doses of antimycin A, KCN, SHAM, and oligomycin B. Inhibitory effects observed were consistent with the effects on whole cell O2 consumption. Treatment with antimycin A and SHAM resulted in a rapid loss of Δψ, as reflected by the loss of rhodamine fluorescence. No such loss was observed following

### Table II

| Inhibitor                | Strain | O2 consumption (µM O2/min/g wet cell) | Decrease | O2 consumption (µM O2/min/g wet cell) | Decrease | O2 consumption (µM O2/min/g wet cell) | Decrease | O2 consumption (µM O2/min/g wet cell) | Decrease |
|--------------------------|--------|--------------------------------------|----------|--------------------------------------|----------|--------------------------------------|----------|--------------------------------------|----------|
| None                     | T7     | 0.375                                |          | 0.300                                |          | 0.430                                |          | 0.500                                |          |
| KCN (1 mM)               | DK22A  | 0.375                                | 0        | 0.300                                | 0        | 0.430                                | 0        | 0.500                                | 0        |
| SHAM (300 µM)            |        | 0.188                                | 50       | 0.150                                | 50       | 0.310                                | 28       | 0.330                                | 34       |
| Oligomycin B (7 µM)      | UK22A-1| 0.173                                | 46       | 0.160                                | 48       | 0.270                                | 38       | 0.300                                | 40       |
| Carboxin (10 µM)         |        | 0.200                                | 8        | 0.165                                | 45       | 0.275                                | 36       | 0.290                                | 42       |
| Antimycin A (20 µM)      | MT3    | 0.180                                | 48       | 0.165                                | 45       | 0.275                                | 36       | 0.290                                | 42       |
| Antimycin A (20 µM) + SHAM |        | 0.000                                | 100      | 0.000                                | 100      | 0.000                                | 100      | 0.000                                | 100      |

* Initial inhibitory effect of carboxin; oxygen consumption decreased by 62% in T7 and 48% in UK22A-1 after 30 min.
treatment with either KCN or oligomycin B over a comparable time period. These results indicate that both an alternative oxidase and at least cytochrome b of the cytochrome pathway are active in the generation and maintenance of the proton gradient in *G. graminis tritici* and that inhibition of cytochrome oxidase imparts no discernible inhibitory effect on the maintenance of this ion gradient.

Quantitation of ATP in whole cells following treatment of *G. graminis tritici* strain T7 with the inhibitors KCN, antimycin A, SHAM, oligomycin B, and SHAM + Antimycin A were examined over a 15-min time period (Fig. 4). Under these assay conditions, and in accordance with previous reports, ATP levels in non-inhibited cells were constant (Fig. 4). Under identical conditions the effects of inhibitors on cellular ATP concentration were evaluated. Oligomycin (7 μM) was included as a control to demonstrate the effect of the ATP synthase, rather than inhibition of electron transport. The results obtained also provide a standard for interpreting the inhibitory effects of the respiratory inhibitors.

Treatment with 20 mM KCN resulted in a rapid reduction in cellular ATP over the initial 6 min of the assay. However, ATP levels then rose from 20% of the basal ATP level to 79% by 15 min, indicating the re-establishment of ATP generation. Antimycin A (20 μM) treatment resulted in a 74% reduction in ATP compared with the basal level. Unlike treatment with KCN, no reversal of this inhibitory effect on ATP concentration was observed during the time course of this experiment. The effect of combined treatment with both SHAM (0.2 mM) and antimycin A (20 μM) was the reduction in cellular ATP concentration by 77% after 15 min, which was comparable with the 80% reduction in ATP levels observed following oligomycin treatment of T7.

**DISCUSSION**

The present study was conducted in order to evaluate the components of the electron transport chains in the filamentous fungus, *G. graminis tritici*, and their contributions to cellular respiration and ATP synthesis.

Analysis of the biochemical effects of respiratory chain inhibitors, both in whole cells and in isolated mitochondria, describe several novel features associated with the regulation of fungal respiration and the contribution that the alternative pathway makes to mitochondrial ATP generation. Treatment with the cytochrome b inhibitor antimycin A suppressed whole cell oxygen consumption rates, promoted the rapid disruption of the ΔΨ (and hence proton motive force) associated with the inner mitochondrial membrane within 1.5 min following treatment, and significantly decreased cellular levels of ATP. However, cyanide treatment had no effect on oxygen consumption in whole cells or on the ΔΨ and only a brief transitory inhibitory effect on ATP generation was seen. This indicated that a second, cyanide-insensitive terminal oxidase was present in *G. graminis tritici*, which has a proton pumping capability associated with its capacity to reduce oxygen.

Spectral analysis of the cytochrome content of *G. graminis tritici* indicated the presence of the standard cytochrome configuration, but that levels of cytochrome c, c1, and aa3 were significantly lower than that of cytochrome b, as predicted from the dithionite reduced minus oxidized spectra. The relative proportion of b-heme (identified from this spectral trace) associated with the bc1 complex was determined by analysis of the

**Fig. 3.** The effects of respiratory chain inhibitors and oligomycin on the generation and maintenance of ΔΨ across the inner mitochondrial membrane in intact T7 mitochondria as indicated by changes in the intensity of fluorescence of rhodamine. Rhodamine 123 (1 μM) was loaded into 50 μg of mitochondria over 5 min at 4 °C. Subsequent to the addition of the inhibitors the substrates β-hydroxybutyrate (20 mM) and ADP (1.5 mM) were added to ensure maximal rates of electron transport. The effects of inhibitors on fluorescence were monitored over 10 min at room temperature, with the 5-min samples shown here. A represents non-inhibited; B, cyanide (10 mM); C, antimycin A (20 μM); D, SHAM (0.3 mM); and E, oligomycin B (7 μM) treated T7 mitochondria. M, mitochondria and V, vacuoles.
reduced minus oxidized spectra of antimycin A (Fig. 1B) and myxothiazol (data not shown) treated mitochondria (reducing substrate was succinate). The level of $b$-heme associated with the $bc_1$ complex was estimated to be approximately 40% of the total $b$-heme in mitochondria and was, therefore, as expected, comparable to the levels of $aa_3$ and $c_1$. Characterization of the non-$bc_1$ associated $b$-heme, with an absorbance maximum at 560 nm, is the focus of further research.

Respiration in *G. graminis tritici* Is a Function of Both the Cytochrome and Alternative Pathways—Inhibitor and spectral data confirmed the presence of an active cytochrome electron transport chain in *G. graminis tritici*, similar in composition and function to that of other eukaryotes. This electron transport chain contained complex I (mitochondria were sensitive to rotenone, data not shown) and able to utilize $b$-hydroxybutyrate as a reducing substrate. Complex II was also present as succinate was a utilizable substrate in isolated mitochondria and toxicity assays with whole cells demonstrated sensitivity to curfoxin. The presence of complexes III and IV were demonstrated spectrophotometrically with inhibitor stud-
ies (antimycin A, myxothiazol, and cyanide), and CO photolysis of CO bound aa₃ (data not shown).

In contrast to earlier studies with filamentous fungi (13), our study shows that the alternative respiratory pathway of *G. graminis tritici* is constitutively expressed, as SHAM treatment resulted in a decrease in oxygen consumption in whole cells, without prior incubation with either cyanide or antimycin A. More significantly, the anti-Alt Ox antibody readily detected both the monomeric and dimeric forms of the alternative oxidase from protein prepared from T7 mitochondria, demonstrating the presence of both the active and inactive forms of this terminal oxidase.

**Contributions of the Cytochrome and Alternative Electron Transport Chains on ATP Generation**—The function of the alternative oxidase has been the subject of considerable discussion in relation to a possible ATP generating system. In fungi, where the presence of the active form has been demonstrated, no functional roles have been ascribed. The present study has demonstrated that the alternative oxidase is required by actively growing *G. graminis tritici* for respiration and ATP formation, because it provides an essential function in the generation and maintenance of the Δψ associated with ATP synthesis. Indeed, the inhibitory effects of SHAM on mitochondrial function (*O₂* consumption, proton accumulation at the cytosolic side of the inner mitochondrial membrane, and ATP generation) were similar both in relation to the time of onset of the inhibitor effects following treatment and in the magnitude of their effects to those observed following antimycin A treatment. Maximal inhibitory rates of *O₂* consumption and ATP synthesis were only achieved following treatment with both compounds, indicating that both pathways are essential for ATP synthesis and cell growth.

**Site of Electron Partitioning to the Alternative Pathway**—The alternative respiratory pathway of plants withdraws electrons from the ubiquinol pool and complete inhibition of *O₂* consumption only occurs following inhibition of both cytochrome and alternative pathways. In keeping with the nonphosphorylating nature of the plant alternative oxidase, ATP biosynthesis remains largely unaffected by SHAM, due to the presence of all the proton translocating sites within the cytochrome pathway. In *G. graminis tritici*, electron transport appears more complex with the site of electron partitioning between the two pathways less clear. ATP synthesis and *O₂* consumption were inhibited to a comparable level following treatment with either a cytochrome *bc₁* or an alternative oxidase inhibitor. Maximal rates of inhibition of these two processes were only obtained following treatment with both inhibitor classes, indicating the presence of two, inter-related pathways, each with a terminal oxidase and a proton translocatory function capable of generating a proton motive force of sufficient magnitude to drive ATP synthesis. The site of electron partitioning between these two pathways is not clear. Sensitivities to antimycin A and SHAM would support the site of electron partitioning being the same as for plants (namely at the ubiquinone pool), whereas the cyanide insensitivity and the ATP generating capacity of the SHAM-sensitive pathway would indicate that the partitioning event may occur downstream of cytochrome *b*, but prior to cytochrome *aa₃*.

We propose, therefore, from our inhibitor studies on both *O₂* consumption and ATP synthesis that the alternative pathway of electron transport in *G. graminis tritici* involves two components. The first possesses the proton translocatory capacity associated with the SHAM sensitive alternative pathway and provides the proton motive force for ATP synthesis. Given the presence of a second, antimycin A and myxothiazol insensitive *b*-type heme, this cytochrome may form a redox center in the alternative system, and analogous to the *b*-type hemes of the *bc₁* complex, may possess a proton translocatory ability. The terminal oxidase of the alternative respiratory pathway is the alternative oxidase and we therefore propose that respiration in *G. graminis tritici* may follow the scheme depicted in Fig. 5.

The focus of our current research is to characterize the non-*bc₁* associated *b*-type heme and to determine its function. We are also evaluating the effect(s) that such alternative respiratory pathways may have on the antifungal efficacy of inhibitors of the *bc* complex such as the methoxyacrylates and related chemistries.

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