Respiration of Gametangia of the Aquatic Phycomycete

**Allomyces macrogyrus**

INHIBITION BY CYANIDE OR ANTIMYCIN AND SALICYLHYDROXAMIC ACID OR PROPYL GALLATE

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**ABSTRACT**

Gametangia of the aquatic phycomycete **Allomyces macrogyrus** have a cyanide- and antimycin A-insensitive respiration, which is sensitive to salicylhydroxamic acid (alternative respiration). Propyl gallate is also an inhibitor of this alternative pathway, and propyl gallate is more efficient than hydroxamic acid. Gametangial respiration is insensitive to propyl gallate, but propyl gallate sensitivity is gradually established when the gametangia are treated with cyanide. Carbonyl cyanide m-chlorophenyl hydrazone stimulates the cyanide-sensitive respiration and engages the alternative sensitive respiration. Sodium azide inhibits both the alternative and the cyanide-sensitive respiration, but the cyanide-sensitive respiration is inhibited 10 times more efficiently than the alternative respiration. Rotenone inhibits the total respiration and the propyl gallate-insensitive respiration by 33% and the cyanide-insensitive respiration by 43%.

The kinetic results reported here are discussed with respect to the models of de Troostembergh and Nyns (1977 Arch Int Physiol Biochem 85:404-406; 1978 Eur J Biochem 53:423-432) and of Bahr and Bonner (1973 J Biol Chem 248:3446-3450) for the partitioning of electrons between cyanide-insensitive and propyl gallate-insensitive respiration. The results reported here do not agree with the model of de Troostembergh and Nyns.

**Materials and Methods**

**Maintenance of Culture.** The culture of **Allomyces macrogyrus** used in this study is strain Burma 3-35 (35°C) (14). The strain has a haploid gametophytic and a diploid sporophytic generation (14) when it is grown at 35°C. The culture was maintained on YpsS agar plates at 35°C (6).

**Oxygen Consumption Measurements.** The O₂ uptake rate was measured with a Clark-type O₂ electrode (Rank Brothers) (4), maintained at 30°C. The O₂ uptake rate is expressed as μmol O₂ (g protein × min)⁻¹⁻¹.

The cultures were grown in 9-cm Petri dishes with 50 ml of YpsS agar and were incubated for 7 d at 35°C. The gametangia were harvested with a scalpel just after removal from the incubator. The gametangia were suspended in 12 ml Machlis’ DS (5) and filtered through a nylon filter (pore size, 200 μm) to separate the gametangia from hyphal fragments. The gametangial suspension was pelleted at 1500g for 1 min. The pellet was resuspended in 3 ml of DS and transferred to the reaction vessel of the O₂

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**Abbreviations:** SHAM, salicylhydroxamate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; DS, dilute salts solution.
electrode. DS \times 10 \text{ (DS; 10 times normal concentration)} was used for the azide experiments to ensure a constant pH (7.2).

The gametangial suspension was aerated for 5 min before the measurement was started to ensure that the temperature and the O_2 uptake rate were constant.

**Protein Measurements.** The gametangial suspension was removed from the reaction vessel and frozen immediately. The sample was thawed before the protein measurement, and 1 ml 10 N NaOH solution was added to the gametangial suspension. The suspension was neutralized (pH between 4 and 10) with 5 N HCl, after the gametangia had lysed (2.5 h) in the highly alkaline solution. The neutralized suspension was diluted with distilled H_2O to give a final volume of 8 ml. The protein measurements were carried out with the Bio-Rad protein assay (standard procedure) (3).

**Stock Solutions.** Inhibitor and uncoupler stock solutions included: 0.3 M KCN freshly prepared, neutralized before use, 180 \mu M antimycin A (Sigma) dissolved in absolute ethanol, standardized spectrophotometrically \( f_{\text{max}} = 4.8 \text{ at } 320 \text{ nm} \), 60 mM SHAM (Sigma) dissolved in absolute ethanol, 3 mM propyl gallate (Eastman), 30 \mu M rotenone dissolved in absolute ethanol, 300 \mu M CCCP (Sigma) dissolved in absolute ethanol.

**Statistical Treatment of the Inhibitor Titrations.** The inhibitor constants for the different inhibitors were found by a nonlinear regression fit of the equation, \( R_i = 1/(1 + i/K_i) \) (where \( i \) is the inhibitor concentration, \( K_i \) is the inhibitor constant, and \( R_i \) is the calculated respiration rate at the inhibitor concentration \( i \)). The fit was found by the use of the least squares method on: \( R^{\text{obs}} - 1/(1 + i/K_i) \) (where \( R^{\text{obs}} \) is the observed respiration rate at the inhibitor concentration \( i \)).

**RESULTS**

The respiration of the gametangia without inhibitors or with cyanide or SHAM was constant for the first 15 min after the measurements were started. All measurements were therefore made within 15 min. Respiration of gametangia was found to vary from 26 to 59 \mu mol O_2 (g protein \cdot min)^{-1} with a mean of 39 \mu mol O_2 (g protein \cdot min)^{-1} and with a standard deviation of 10 \mu mol O_2 (g protein \cdot min)^{-1}.

Figure 1 shows that 1 mM cyanide inhibits more than half of the respiration, and that most of the cyanide-insensitive respiration is sensitive to SHAM. The cyanide and SHAM-insensitive respiration (residual respiration) was insensitive to 3 mM NaN_3. The amount of residual respiration varies between 0 and 25% (usually about 5%) of the initial respiration. The residual respiration is assumed to be constant (19) and is therefore subtracted from the respiration in the following calculations, diagrams, and mentioned inhibitor efficiencies. The amount of alternative (cyanide-insensitive) respiration varies between 15 and 35% of the total respiration, but the spread can be reduced to a standard variation of 3% if gametangia from identically grown plates were measured on the same day. Therefore, all measurements for an experiment were made on the same day. SHAM alone has no effect on respiration (Fig. 1B). When cyanide is added after SHAM, the same inhibition is observed as when cyanide and SHAM were added in the reverse order.

A titration of coupled and CCCP-uncoupled respiration with cyanide is illustrated in Figure 2A; note that the alternative respiration was blocked with propyl gallate. The inhibition at different cyanide concentrations was measured with several preparations, as it was more reproducible than by the addition of successively higher concentrations of cyanide to the same preparation. The results (corrected for the residual respiration) are shown in Figure 2C. The \( K_i \) for cyanide is found to be 2.4 \times 10^{-5} M for coupled respiration and 1.1 \times 10^{-5} M for uncoupled respiration.

All the cyanide-insensitive and SHAM-sensitive respiration was propyl gallate-sensitive (Fig. 3A), and none of the cyanide- and SHAM-insensitive respiration was sensitive to propyl gallate (Fig. 3B). And since propyl gallate also had the same effect as SHAM when it was added before cyanide (Fig. 3C), we consider propyl gallate to be an inhibitor of the alternative respiratory pathway. Propyl gallate is a much more efficient inhibitor than SHAM, with a \( K_i \) value of 5.0 \times 10^{-4} M. The titration curve with propyl gallate is shown in Figure 4.

Antimycin A and cyanide have similar effects on O_2 uptake. All the cyanide-insensitive respiration is insensitive to antimycin A (Fig. 5A) and all the antimycin A-insensitive respiration is insensitive to cyanide (Fig. 5B).

NaN_3 was found to be an inhibitor of both the cyanide-insensitive and the cyanide-sensitive respiration but with different inhibitor constants. NaN_3 inhibits 90% of the cyanide-insensitive but propyl gallate-sensitive respiration at a concentration of 2 \times 10^{-2} M with an inhibition constant of 1.6 \times 10^{-3} M, compared with 1.7 \times 10^{-4} M for the cyanide-sensitive respiration (measured by titration after the addition of propyl gallate). Figure 6B shows the titration curve with NaN_3 alone, it demonstrates that 70 to 80% of the respiration is inhibited by small concentrations and that most of the remaining respiration is inhibited by higher concentrations. The respiration can be inhibited by 95%
FIG. 2. Effect of various concentrations of cyanide on propyl gallate-inhibited coupled and uncoupled respiration. The cyanide titration of the coupled respiration was carried out as shown in A. a, Respiration after the addition of 0.01 mM propyl gallate; b, respiration after the addition of xM cyanide, where x is a concentration between 0.01 and 0.1 mM; and c, respiration after the Cyt-mediated respiration has been blocked with 1 mM cyanide. The cyanide inhibition of the coupled propyl gallate-insensitive, cyanide-sensitive respiration \((b - c) \times 100/(a - c)\) is plotted in C (●) as a function of the cyanide concentration. The best possible fit of these measurements to the ideal equation for noncompetitive inhibition \(R = 1/(1 + i/K_i)\), where \(i\) is the inhibitor concentration and \(K_i\) is the inhibitor constant, is shown as an unbroken line. The cyanide titration of the uncoupled respiration was carried out as shown in B. a, Respiration after the addition of 0.01 mM propyl gallate; b, respiration after uncoupling with 1 μM CCCP, respiration after the addition of xM cyanide, where x is a concentration between \(5 \times 10^{-6}\) and \(5 \times 10^{-5}\) M; and d, respiration after the Cyt-mediated respiration has been blocked with 1 mM cyanide. The cyanide inhibition of propyl gallate-insensitive, CCCP-uncoupled cyanide-sensitive respiration \((c - d) \times 100/(b - d)\) is plotted in C (■). The best possible fit of these measurements to the equation \(R = 1/(1 + i/K_i)\) is drawn as a dashed line.

FIG. 3. Oxygen electrode recordings of gametangia, illustrating the effect of SHAM, propyl gallate, and CN. At the point indicated by an arrow, sufficient inhibitor was added to give the specified final concentration. The values given on the O₂ electrode recordings are O₂ uptake in μmol O₂ (g protein · min)⁻¹. Curve A, propyl gallate after KCN and then SHAM. Curve B, SHAM after KCN and then propyl gallate. Curve C, KCN after propyl gallate.
concentration arrows, the (g protein gallate. Respiration after the Cyt-mediated respiration has been blocked with 1 mM cyanide; b, respiration after the addition of \( x \) mM propyl gallate; and c, residual respiration. The amount of cyanide-insensitive respiration, propyl gallate-sensitive respiration is plotted as a function of the propyl gallate concentration in B.

Fig. 4. Response of cyanide-insensitive, SHAM-sensitive respiration to varying concentrations of propyl gallate. A, Titration procedure. a, Respiration after the Cyt-mediated respiration has been blocked with 1 mM cyanide; b, respiration after the addition of \( x \) mM propyl gallate; and c, residual respiration. The amount of cyanide-insensitive respiration, propyl gallate-sensitive respiration is plotted as a function of the propyl gallate concentration in B.

with \( 1 \times 10^{-2} \text{ M NaN}_3 \).

The fraction of the alternative respiration which is operating is designated \( \rho \) by Bahr and Bonner (1) and by Theologis and Lattes (19). To find the relation between this fraction and the Cyt-mediated respiration, a cyanide titration was carried out as illustrated in Figure 7A. The respiration was inhibited with a low concentration of cyanide (0.02 to 0.2 mM), followed by a total inhibition of the alternative pathway by \( 1 \times 10^{-3} \text{ M} \) propyl gallate. Figure 7B shows that propyl gallate-sensitive respiration is dependent on the cyanide-sensitive respiration. The operating fraction of the alternative respiration (\( \rho \)) was plotted against the Cyt-mediated respiration. It is seen that the alternative path remains inactive (\( \rho = 0 \)) until respiration is inhibited to about 45% (corresponding to \( 2 \times 10^{-3} \text{ M} \) cyanide). The alternative pathway becomes gradually more effective as respiration becomes further inhibited by cyanide; it does not attain its maximal activity before the Cyt pathway is totally inhibited by cyanide. It seems that \( \rho \) is increasing linearly from 0 to 1.

The respiration of the gametangia was increased when the uncoupler CCCP was added. This increase was largest when 1 \( \mu \text{M} \) CCCP was added. The enhanced respiration is constant only in the first 5 to 10 min. All measurements on CCCP-stimulated respiration were therefore carried out within the first 5 min after 1 \( \mu \text{M} \) CCCP was added. The respiratory enhancement was 53% (mean of 18 measurements; standard deviation, 8%) on the uninhibited respiration compared with 30% (mean of 12 measurements; standard deviation, 12%) on the propyl gallate-inhibited respiration. This indicates that a CCCP engagement of the alternative path was further confirmed by a cyanide titration of the CCCP-stimulated respiration. The propyl gallate-sensitive respiration was measured at each cyanide concentration and was found to be constant and about 19% of the initial respiration (18 measurements; standard deviation, 5%). This means that the alternative path is fully engaged, i.e., \( \rho = 1 \).

Rotenone is an inhibitor of the internal NADH dehydrogenase complex (8, 10). When rotenone was added in a concentration of \( 2 \times 10^{-7} \text{ M} \) (Fig. 8A), respiration was immediately inhibited by 33\% \pm 5\% (mean of four measurements). Higher concentrations of rotenone did not inhibit the respiration further. Cyanide was able to inhibit the rotenone-insensitive respiration (Fig. 8A) by
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80.3 ± 3.3% (mean of four measurements). Rotenone-insensitive respiration was insensitive to propyl gallate (Fig. 8B). Rotenone inhibits the cyanide-insensitive respiration (Fig. 8C) by 43.4 ± 7.5% (mean of four measurements) and the propyl gallate-insensitive respiration (Fig. 8D) by 33% (one measurement). Two experiments were made where CCCP was added after rotenone. The CCCP stimulation was then 0 and 12%, respectively.

DISCUSSION

A. macrogyrus gametangia have two respiratory pathways, both a cyanide-sensitive and a cyanide- and antimycin A-insensitive, propyl gallate- and SHAM-sensitive respiratory pathway. This is the first report of such an alternative pathway among the uniflagellate Phycomycetes. This report, among many others, supports the suggestion that a cyanide-insensitive respiration is quite common among the fungi (9).

Residual respiration is observed when both the cyanide-mediated and the alternative pathways are blocked with cyanide and SHAM or propyl gallate. The residual respiration is insensitive to 3 mM NaN₃, and is therefore not homologous to the cyanide-, antimycin-, and hydroxamic acid-insensitive but azide-sensitive respiration found in Schizosaccharomyces pombe by Goffeau (7). The azide titration curve (Fig. 7) indicates that azide has both a high and a low affinity inhibition site, and that the alternative pathway branches from the main respiratory pathway between the two azide inhibition sites.
de Troostembergh and Nyns (22, 23) have developed a model for the partitioning of electrons between the Cyt-mediated and the alternative pathway, assuming that the electrons are randomly distributed between the two pathways. The actual electron flux through the pathways can therefore be calculated from the maximum capacities of the two branches and the maximum capacity on the substrate side of the branchpoint. These capacities can be found from the uninhibited (total) respiration $v_d$, the respiration after the Cyt-mediated pathway has been inhibited $v_{lab}$, and the respiration after the alternative respiration has been inhibited $v_{rpm}$. The formulas are given by de Troostembergh and Nyns (22, 23). Their model predicts that both pathways are always active, so that the addition of inhibitors of one of them will channel some of the electrons through the other path (supposing that it is not saturated), but the other path will never accept all the electrons, so addition of an inhibitor will always result in a decrease in respiration.

In the experiments reported here, it was found that the two inhibitors of the alternative pathway used (propyl gallate and SHAM) were without any perceivable inhibitory effect on the total respiration. Complete insensitivity to inhibitors of the alternative pathway is in disagreement with the model of de Troostembergh and Nyns. But it is possible that the alternative respiratory pathway contributes to the total respiration, inasmuch as a decline in respiration of 1 or 2% could remain undetected by the methods used.

A fit of the de Troostembergh and Nyns model can be made to our results, and the model is best fitted to our results if one assumes that the capacity of the Cyt-mediated pathway is much higher than the capacity of the alternative pathway, and by assuming that the activity of the electron carriers on the substrate side of the branchpoint is lower than the capacity of the Cyt-mediated pathway. But even using these assumptions, it is difficult to explain the results represented in Figure 7. It is seen that propyl gallate is without inhibitory effect ($p = 0$) even when the Cyt-mediated respiration has been inhibited 45% by cyanide. Using the de Troostembergh and Nyns model fit and the formulas given by de Troostembergh and Nyns (22, 23), it can be calculated that the alternative pathway should be functional at least 25% of its maximal capacity ($p > 0.25$) when 45% of the respiration is inhibited by cyanide. Because an inhibitory effect of propyl gallate is not observed at this point, we conclude that the results represented in Figure 7 are in disagreement with the model of de Troostembergh and Nyns.

Bahr and Bonner (2) have suggested another model for the partitioning of electrons between the two pathways. They assume that the branchpoint consists of two components in equilibrium. Each of the pathways receives its electrons from one of the separate equilibrated carriers. The $E'_0$ values of the carriers would be such that under conditions where the carrier (A), which is connected to the alternative pathway, is nearly fully oxidized, the carrier (B), which is connected to the Cyt-mediated pathway, would remain nearly fully reduced. The respiratory activity of each of the two pathways should then depend on the degree of reduction of the carrier from which the pathway receives its electrons. This model explains how respiration could be unaffected by inhibition of the alternative respiration even when respiration is inhibited 45% by cyanide, by assuming that the Cyt-mediated pathway still has the capacity to receive enough electrons from carrier B so that carrier A remains nearly fully oxidized.

It was found that CCCP stimulated the respiration about 50% by increasing the propyl gallate-insensitive respiration about 30% and by an engagement of the alternative pathway, which accounts for the remaining 20%.

The engagement of the alternative respiration by CCCP can be explained by the model of Bahr and Bonner if one assumes that CCCP enhances the activity of the substrate side of the branchpoint (e.g., substrate mobilization) more than it enhances the activity in the Cyt-mediated pathway. This will result in a further reduction of the electron carrier B. The alternative pathway would consequently be functional if the reduction of carrier B has been sufficient to give full or partial reduction of the equilibrated carrier A.

Our results with CCCP are in good agreement with the work of Theologis and Latties (19–21) on fresh sweet potatoes, avocado, and banana fruits. Theologis and Latties found that m-chlorobenzhydroxamic acid, an inhibitor of the alternative respiration, was without effect on coupled respiration, but inhibited CCCP-un-coupled respiration with the same, or nearly the same, efficiency as the cyanide-insensitive respiration. This suggests that CCCP induced the alternative respiration.

We have also tried to explain the CCCP results with respect to the model of de Troostembergh and Nyns. The easiest way to explain the observed propyl gallate-sensitive respiration is by supposing that CCCP engages the alternative pathway, which was not found. Another way to explain increased activity of the alternative pathway is by assuming that CCCP enhances the
activity of the electron carriers upstream of the branchpoint $V_{red}$ more than the capacity of the Cyt-mediated pathway $V_{cyt}$ (the same assumption as for the Bahr and Bonner model). The changes of $V_{red}$ and $V_{cyt}$ have to be large enough to account for a change in $\rho$ from close to 0 to nearly 1 (as noted by Laties [13] as it is not possible to reach $\rho = 1$, using the formula given by de Troostembergh and Nyns [22, 23]). Such a change in capacity is much higher than the capacity change which was needed to explain the induction of the alternative pathway by the Bahr and Bonner model.

Among the rotenone experiments, the experiment where rotenone was added after cyanide is particularly interesting. The alternative pathway is not engaged in the uninhibited respiration. This means that the capacity of the Cyt-mediated pathway is lower than the activity upstream from the branchpoint, i.e., the respiration is determined by the activity on the substrate side of the branchpoint. Inhibition with rotenone, which decreases the capacity on this side of the branchpoint to 70% of the original activity, will therefore give a 30% decrease in respiration. The situation is different when rotenone acts on the cyanide-inhibited respiration. Cyanide inhibits the respiration more than half; this means that the capacity of the alternative pathway is lower than the original activity on the substrate side of the branchpoint, i.e., the alternative pathway is rate limiting when the Cyt path is blocked. If rotenone then decreases the capacity upstream from the branchpoint to 70% of the activity before cyanide inhibition, then rotenone inhibition should not give a 30% decrease in respiration, because the capacity upstream from the branchpoint still exceeds the downstream capacity, i.e., the capacity of the alternative path. Using the above-mentioned fit of the de Troostembergh and Nyns model to our results, it can be calculated that rotenone should only inhibit about 10% of the cyanide-inhibited respiration. It was actually found that rotenone inhibits 43% of the cyanide-inhibited respiration, compared with 33% of the total respiration. This is a statistically significant inconsistency with the expected result. We have no explanation for this result. The other experiments where rotenone was used in combination with other inhibitors (cyanide or propyl gallate after rotenone, and rotenone after propyl gallate) were in better agreement with the expected result. There was only a small response when CCCP was added to the rotenone-inhibited respiration, probably because the rotenone inhibition at the first energy conservation site prevents substrate mobilization to increase the electron flux in the terminal pathways.

In summary, we concluded that the kinetic data for A. macrognus respiration are in better agreement with the model of Bahr and Bonner than with the model of de Troostembergh and Nyns.

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