Contribution of a Cyanide-insensitive Alternate Respiratory System to Increases in Formamide Hydro-lyase Activity and to Growth in *Stemphylium loti* in Vitro

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ABSTRACT

*Stemphylium loti*, a pathogen of a cyanogenic plant, possesses a cyanide-insensitive alternate respiratory pathway. In the absence of cytochrome inhibitors, the alternate system had only a minor role in respiration. When *S. loti* was grown in medium amended with antimycin to block the cytochrome chain, the alternate system accounted for the total oxygen consumption associated with respiration.

The contribution of the alternate respiratory system to increases in formamide hydro-lyase (FHL) activity and to growth in *S. loti* in vitro was assessed. FHL, induced by cyanide, converts cyanide to nontoxic formamide and is partially responsible for the tolerance of *S. loti* to high concentrations of cyanide in vitro. When the cytochromes were blocked and the cytochrome-insensitive respiratory pathway accounted for 100% of the oxygen uptake associated with respiration, FHL activity, but not changes in dry weight, was positively correlated with activity of the alternate pathway. As the alternate pathway activity decreased with increasing concentrations of salicyldroxyacetic acid, the level of FHL activity correspondingly decreased. The alternate respiratory system may provide for increases in FHL activity but not for growth. *S. loti* appears to have two mechanisms for cyanide tolerance in vitro: cyanide-insensitive respiration and FHL activity. The initial activity of FHL for detoxification of cyanide may depend on the alternate respiratory pathway when the cytochromes of the electron transport chain are blocked.

Hydrogen cyanide is released from cyanogenic glucosides in birdsfoot trefoil (*Lotus corniculatus* L.) upon infection by *Stemphylium loti* (16). This fungus grows in the presence of relatively high concentrations of cyanide in vitro (3) and is more tolerant to cyanide than are other fungi (16). Fry and Millar (3-5) reported that cyanide tolerance in *S. loti* is induced upon exposure to cyanide and is due to the production of the enzyme formamide hydro-lyase which converts cyanide to nontoxic formamide.

We questioned how *S. loti* derives energy for FHL synthesis if the electron transport chain is blocked by cyanide. Fry and Millar (3, 4) had obtained preliminary evidence of cyanide-insensitive respiration in this organism. Therefore, we consid-

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3 Abbreviations: FHL: formamide hydro-lyase; SHAM:salicyldroxyacetic acid.

er the possibility that *S. loti* possesses a cyanide-insensitive respiratory system that provides energy for synthesis of this detoxifying enzyme and for limited growth. An alternate system exists in many plants, animals, fungi, and algae (8). Reports suggest that ATP formation is associated with operation of the alternate respiratory system (21-23).

If a cyanide-insensitive respiratory system provides energy for FHL synthesis, then *S. loti* may have at least two cyanide tolerance mechanisms which may be important during pathogenesis on a cyanogenic plant. Cyanide, released from the host, may exist in quantities sufficient to block the Cyt in fungal cells. An alternate respiratory system might provide energy for growth and for increases in FHL activity to detoxify cyanide. Consequently, the Cyt pathway could provide energy for growth and for continuing FHL activity to detoxify additional cyanide released as the fungus ramifies through host tissue.

Cyanide-insensitive respiration is generally associated with an unidentified alternate terminal oxidase (ATO) (8) which reportedly connects with the normal electron transport chain at ubiquinone (24). This alternate respiratory pathway coexists with the normal respiratory chain in mitochondria (8). Studies of the normal and alternate respiratory systems have shown the following: (a) antimycin, cyanide, azide, and carbon monoxide, through inhibition of certain Cyt in the normal electron transport chain, inhibit the normal respiratory system but not the alternate pathway (9, 14, 25); (b) salicyldroxyacetic acid specifically inhibits the alternate pathway but not the normal pathway (20); and (c) the addition of Cyt inhibitors to a growth medium promotes the appearance of the alternate respiratory pathway in microorganisms such that 100% of the respiration is insensitive to cyanide but is sensitive to SHAM (6, 10, 12).

Our objectives were to determine if *S. loti* possesses a cyanide-insensitive alternate respiratory pathway and to assess the contribution of such an alternate pathway to growth and to increases in FHL activity. A preliminary report of this work has been published (17).

MATERIALS AND METHODS

*S. loti* Graham, obtained from J. H. Graham (USDA, Beltsville, Md.), was cultured routinely at 20 to 23°C on V-8 juice agar medium (18) under fluorescent lights. Virulence was maintained by regularly inoculating trefoil and reisolating the fungus. Potassium cyanide was dissolved in 0.05 M sodium phosphate buffer (pH 7); salicyldroxyacetic acid (SHAM) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in acetone; and antimycin (Sigma Chemical Co., St. Louis, Mo.) in 95% ethanol. Reagents were prepared fresh for each experiment.

Growth Conditions. Conidia were harvested from 6- to 13-day-old cultures in phosphate buffer (0.05 M, pH 7, with 34 μl of Tween 20/l of buffer) and were filtered through three layers
of sterile cheesecloth to remove mycelial fragments. Conidia (10") in buffer were added to 25 ml of medium in 125-ml Erlenmeyer flasks. The growth medium was prepared by adding the following g/l of distilled H2O: NH4NO3, 3 g; KH2PO4, 1 g; MgSO4·7 H2O, 0.5 g; NaCl, 0.1 g; CaCl2·2H2O, 0.13 g; glucose, 15 g; trace element solution, 1 ml. Trace element solution was prepared according to Beadle and Tatum (2) but with the addition of KI to provide 0.01 mg of I/l of medium. The medium was adjusted to pH 6. Seeded flasks were incubated at 21 to 29°C on a horizontal-stroke-action shaker (86 strokes/min) for 12 to 15 hr.

**Measurement of Growth, O2 Consumption, and FHL Activity.** Growth was defined as an increase in dry wt. Mycelium from each flask was filtered through a 4.25-cm Büchner funnel with glass-fiber filter paper and was rinsed twice with 10 ml of distilled H2O. Papers with mycelium were dried in an 80°C oven for 24 hr, placed in a desiccator at room temperature for 6 hr, and weighed. Each treatment at each sample time had two or three replications. Filter papers through which medium alone had been filtered were treated similarly.

Oxygen consumption was measured with a Gilson oxigraph model KM (Gilson Medical Electronics, Middleton, Wis.) equipped with a Clark O2 electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) and a 1.7-ml reaction chamber maintained at 25°C. Mycelium, harvested from flasks by filtration through a 4.25-cm Büchner funnel with Whatman No. 1 filter paper, was suspended in oxygenated growth medium at 24 to 25°C. Clumps of mycelium were gently dispersed by a mortar and pestle. After each measurement, mycelium was removed from the cell, filtered, rinsed, dried, and weighed. Between measurements the reaction chamber was rinsed several times with ethanol and distilled H2O. Only one O2 consumption measurement was made for each treatment because of the time required for each measurement and the number of treatments per experiment. To determine the effect on O2 consumption of additions of HCN or SHAM, the initial uptake rate was established, then HCN (0.9 mM final concentration) or SHAM (2.7 mM final concentration) was added separately to the reaction chamber with a μl syringe. Aliquots of HCN or SHAM were added until no further inhibition of O2 consumption occurred. O2 consumption rates were expressed as nmol of O2 consumed/mg dry wt/min. The percentage of CN-insensitive (= SHAM-sensitive) O2 consumption was calculated as the O2 consumption that was sensitive to SHAM as a proportion of the total percentage of O2 consumption inhibited by both HCN and SHAM.

For assay of FHL activity, mycelium was filtered through a 4.25-cm Büchner funnel with Whatman No. 1 filter paper, rinsed twice with 10 ml of 0.05 M phosphate buffer at pH 7, and frozen for 12 to 24 hr. Frozen mycelium was suspended in 10 ml of 0.05 M tris-HCl (pH 8) and fragmented in a hand-operated glass homogenizer. The assay mixture containing 0.5 ml of mycelial homogenate and 0.5 ml of 0.1 mM KCN in 0.05 M tris-HCl (pH 8) was incubated at 25°C for 2 to 2.5 hr. Assay mixtures prepared identically but assayed immediately after adding KCN were used as zero time controls. Boiled enzyme controls gave no information beyond zero time controls and thus were not routinely used. FHL activity was measured by determining the amount of formamide produced/hr at 25°C. Formamide concentration was measured colorimetrically according to the method of Fry and Millar (5). FHL activity was expressed as μmol of formamide produced/hr mg dry wt. Dry weight data were taken from growth measurements. Each treatment had two replications.

**Presentation of Data.** Each experiment was repeated at least once. Although numerical values varied from experiment to experiment, trends and effects were similar in comparable experiments. Data from single experiments are presented in graphs which accurately represent the effects observed in two or more similar experiments.

**RESULTS**

**Growth and O2 Consumption Rates of S. loti Cultures Grown in Absence of Respiratory Inhibitors.** To determine the normal growth and O2 consumption rates of S. loti cultures, flasks were seeded as described above and at 0, 12, 24, and 36 hr, samples for determining dry wt. O2 consumption rates, and percentage CN-insensitive O2 consumption were taken (Fig. 1). Subsequent experiments were conducted with mycelium in the exponential phase of growth (12-36 hr). During this period, O2 consumption rates were near maximum and percentage CN-insensitive O2 consumption was fairly constant.

**Antimycin and SHAM Concentrations.** To assess the effect of the alternate respiratory system on growth and FHL activity, 12- to 15-hr-old mycelium was incubated with antimycin to obtain 100% CN-insensitive respiration. Increasing amounts of SHAM then were added to the antimycin-treated mycelium to determine the effect of increasing degrees of inhibition of the alternate system on dry wt and FHL activity. Results from preliminary experiments with a range of antimycin concentrations indicated that incubation with 1 μM antimycin was adequate to obtain high O2 consumption rates and 100% CN-insensitive O2 consumption. Growth was markedly inhibited at all concentrations of antimycin tested. Ethanol at a final concentration of 0.6 to 0.7% was used as a solvent control. Experiments were conducted with a final concentration in the growth medium of 0.25 to 1 mM SHAM. Acetone at a final concentration of 0.9 to 1.1% was used as a solvent control. The concentrations of antimycin and SHAM used in these experiments were within the range used in other systems (8).

**Effect of HCN and SHAM on O2 Consumption of S. loti in the Absence or Presence of Antimycin.** Generally, 90 to 100% of O2 consumption was inhibited by HCN and SHAM-added to the oxigraph reaction chamber (Fig. 2). At saturating HCN and SHAM concentrations, O2 consumption frequently did not decrease to zero. Other cellular processes not affected by these
antimycin (Duncan's method) and the effect of HCN on FHL. Samples were taken at 0.05-min intervals and were centrifuged at 15,000× for 1 min. Antimycin (1 μM) or SHAM (2.7 mM) was added to the medium, and the O2 consumption by the mycelium was measured by the oxygraph technique. The activity of the alternate respiratory pathway was measured in a series of experiments: (1) the effect of SHAM on the O2 consumption by the mycelium grown in the absence of antimycin; (2) the effect of SHAM on the O2 consumption by the mycelium grown in the presence of antimycin; (3) the effect of SHAM on the O2 consumption by the mycelium grown in the absence of antimycin, but with added HCN.

**DISCUSSION**

*S. loti* possesses a cyanide-insensitive alternate respiratory pathway. Growth in a medium amended with a Cyt inhibitor leads to O2 consumption which is fully resistant to cyanide and sensitive to SHAM. These results indicate a high level of activity of the alternate system and are similar to reports of CN-insensitive respiration in *Neurospora crassa* (12, 15) and in *Candida albicans* (11) for which antimycin and SHAM have been used similarly to illustrate that the CN insensitivity is associated with an alternate respiratory system.

Although our objective was not to determine critically whether the alternate pathway is constitutive, induced, or activated (8), it appears that *S. loti* may have a constitutive alternate pathway which, under normal conditions, has only a minor role in electron transport. But when the Cyt are inhibited during a period of growth, the alternate system can account for the total electron flux (1). Bahr and Bonner (1), working with higher plant mitochondria, suggest that when the two pathways exist together in the mitochondria, the rate in the main pathway is maximum at all times, that the rate in the alternate pathway is lower in the absence than in the presence of cyanide, and that consequently only part of the alternate pathway is used in the absence of inhibition of the main respiratory chain. The nature of the regulation of electron flow through the alternate pathway is not known (8). The question of activation, modulation, and induction of the alternate system remains controversial (7, 8).

Henry et al. (7) have suggested that an increase of alternate respiratory activity involves new protein synthesis. We have not shown whether the increased activity of the alternate system when *S. loti* is grown in the presence of antimycin is due to increased activity of a constitutive pathway or whether the elements of the pathway are synthesized.

Our approach was to assess the effect of increasing SHAM concentrations on growth and FHL activity when the main respiratory system was inhibited by antimycin. Antimycin treatment led to O2 consumption which was totally insensitive to cyanide but sensitive to SHAM.

Growth was not positively correlated with activity of the alternate system. If there were small differences in growth, the technique used was not sensitive enough to detect them. These results are compatible with those of Slayman et al. (21) who report a sharp drop in cellular ATP and cessation of growth of *N. crassa* in antimycin-amended medium. They suggest that when the Cyt system is blocked, ATP levels cannot be maintained to support growth. Although antimycin may have deleter-
Fig. 3. Effect of growth, per cent CN-insensitive \( \text{O}_2 \) consumption, \( \text{O}_2 \) consumption rates, and FHL activity of the simultaneous addition of antimycin (1 \( \mu \text{M} \)) and acetone (0.9–1.1%) or SHAM (0.25, 0.5, 1 mM). Acetone or SHAM at the same concentrations was added to a control series (0.6–0.7% ethanol). The effect of addition of HCN to the growth medium (to induce FHL) on these parameters is shown. Samples were taken 24 hr after addition of antimycin and SHAM (12 hr after addition of HCN). A: Growth (change in mg dry wt/flask since addition of antimycin and SHAM; each point represents the mean of three measurements). B: Per cent CN-insensitive \( \text{O}_2 \) consumption (each point represents one measurement). C: \( \text{O}_2 \) consumption rates (initial rates before addition of HCN or SHAM to reaction chamber; each point represents one measurement). D: FHL activity (each point represents the mean of two measurements). E: Correlation of \( \text{O}_2 \) consumption rate with FHL activity. Data points, best-fit line, equation for best-fit line, correlation coefficient \( r \), and significance of \( r \) are given. Key to lines on graphs: (\( \Delta \)--\( \Delta \)): antimycin; (\( \bullet \)--\( \bullet \)): antimycin + HCN (from another graph).
ious effects other than on the Cyt chain and thus reduce growth in other ways. Rieske (19) suggests that except for an effect on hepatic aldehyde oxidase, the inhibitory action of antimony A is confined to enzymes of the electron transport chains of respiration or photosynthesis.

In mycelium incubated with cyanide, FHL activity was positively correlated with activity of the alternate pathway as measured by CN-insensitive O₂ consumption when the Cyt pathway was blocked. The progressive degree of inhibition of the alternate pathway effected by increasing concentrations of SHAM was correlated with a progressive decline in FHL activity. When the SHAM concentration was increased to block completely the alternate system in antimony-treated mycelium, there was no FHL activity (unpublished data).

FHL apparently can be synthesized via the normal system, which was indicated by the level of FHL activity obtained in controls when the alternate pathway was blocked and only the normal respiratory system was operating. That SHAM was not directly inhibitory to FHL production was shown in the lack of inhibition of FHL activity with increasing SHAM in controls. Higher levels of FHL activity in antimony-treated than in control mycelium were due partially to the calculation of activity on a dry wt basis. Lower weights in antimony treatments caused the specific activity values to be higher even though the actual amount of formamide might have been comparable or less per flask than in controls.

If the alternate pathway is necessary for FHL activity in Cyt-blocked mycelium, then S. loti would appear to have a sequence of cyanide tolerance mechanisms in vitro: a cyanide-resistant respiratory system that serves to provide energy for increases in activity of a cyanide-detoxifying enzyme. If these mechanisms function in vivo, we suggest that the following sequence of events could occur during pathogenesis of S. loti on birdsfoot trefoil. Cyanide, released from the host, could block the Cyt pathway in pathogen cells. Subsequently, the alternate respiratory pathway could be induced or activated. Growth would not occur but the organism could produce FHL to detoxify the cyanide. Then the Cyt pathway would function to provide for growth and for increases in activity of FHL to detoxify any cyanide subsequently released as the fungus invaded more host cells. It remains to be determined whether either or both of these tolerance mechanisms are required for S. loti to be a pathogen of a cyanogenic plant.

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LITERATURE CITED

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