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Host-Specific Effects of Toxin from the Rough Lemon Pathotype of *Alternaria alternata* on Mitochondria[1]

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ABSTRACT

Host-specific toxin from the rough lemon (*Citrus jambhiri* Lush) pathotype of *Alternaria alternata* (ACR toxin) was tested for effects on mitochondria isolated from several citrus species. The toxin caused uncoupling of oxidative phosphorylation and changes in membrane potential in mitochondria from leaves of the susceptible host (rough lemon); the effects differed from those of carbonylcyanide-*m*-chlorophenylhydrazone, a typical protonophore. ACR toxin also inhibited malate oxidation, apparently because of lack of NAD^+ in the matrix. In contrast, the toxin had no effect on mitochondria from citrus species (Dancy tangerine and Emperor mandarin [*Citrus reticulata* Blanco], and grapefruit [*Citrus paradisi* Macf.]) that are not hosts of the fungus. The effects of the toxin on mitochondria from rough lemon are similar to the effects of a host-specific toxin from *Helminthosporium maydis* (HMT) on mitochondria from T-cytoplasm maize. Both ACR and HMT toxins are highly selective for the respective host plants. HMT toxin and methomyl had no effect (toxic or protective) on the activity of ACR toxin against mitochondria from rough lemon.

Several citrus-infecting forms of *Alternaria alternata* (Fr.) Keissler (formerly, *A. citri* Ellis & Pierce) are opportunistic pathogens that cause leaf and fruit spots on many citrus types and cultivars. However, certain pathotypes of the fungus are selectively virulent on specific citrus types or cultivars and are relatively harmless on all others. One of these specialized pathotypes causes brown leaf and fruit spots on rough lemon (*Citrus jambhiri* Lush) but has no obvious effect on Dancy tangerine and Emperor mandarin (*Citrus reticulata* Blanco), grapefruit (*Citrus paradisi* Macf.), and other citrus species; this pathotype produces toxic compounds with the same selectivity as the fungus itself (19). Toxins from the rough lemon pathogen (ACR[3] toxins) were characterized independently by Nakatsuka *et al.* (22) and by Gardner *et al.* (12). Another pathotype from citrus species in Australia (25) and the United States (28) is selectively pathogenic on Dancy tangerine and Emperor mandarin but not on rough lemon (19). Again, the tangerine pathotype produces a toxin that is highly active only against the host plants. A toxin from the tangerine pathotype was characterized in 1986 by Kono *et al.* (21). However, it is not clear that the toxin described by Kono *et al.* (21) is identical with that described by Kohmoto *et al.* (18).

A mitochondrial site of action for ACR toxin was first indicated in ultrastructural studies by Kohmoto *et al.* (17), using toxin I (the major form) on citrus leaves. Several other lines of evidence supported the tentative conclusion that the toxin selectively affects mitochondria in leaves of susceptible lemon (16). ACR toxin I had the following effects on mitochondria isolated from rough lemon leaves: (a) toxin stimulated the rate of respiration with NADH as the substrate, an effect similar to that of ordinary uncouplers of oxidation and phosphorylation; and (b) toxin inhibited malate oxidation (16). These effects appeared to be similar to those of HMT toxin (from *Helminthosporium maydis* race T) on mitochondria from T-cytoplasm maize (3, 4, 13, 14, 24).

We report herein further studies on the effects of ACR toxin on mitochondria isolated from citrus leaves and compare these effects with those of HMT toxin on mitochondria from maize. Abstracts describing some of this work were published (1, 2).

MATERIALS AND METHODS

Plant Materials

Four kinds of citrus were used in these experiments: rough lemon (*Citrus jambhiri* Lush), Dancy tangerine, Emperor mandarin (*Citrus reticulata* Blanco) and grapefruit (*Citrus paradisi* Macf.). Plants were grown during March to October in a greenhouse at temperatures >18°C; during the winter (November to February), the greenhouse temperature was 28°C from 7:00 AM to 5:00 PM and at 18°C from 5:00 PM to 7:00 AM. Light was supplemented with fluorescent and mercury tubes (about 30,000 lux) from 7:00 AM to 5:00 PM during winter. The very young leaves on growing shoots were used for isolation of mitochondria and for leaf necrosis assays. Leaves from a maize (*Zea mays* L.) hybrid with Texas male-sterile cytoplasm (A295 × W64A, [T-cytoplasm]), which was kindly provided by Dr. R. P. Scheffer of Michigan State University, were used in the assay for HMT toxin. Seeds were

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[3] Abbreviations: ACR toxin, the host-specific toxin produced by *Alternaria alternata* rough lemon pathotype; HMT toxin, the host-specific toxin produced by *Helminthosporium maydis* race T; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; SHAM, salicylhydroxamate.

germinated in the dark for 3 to 4 d at 27°C on wet paper in a moist chamber. Germinated seeds were then placed in soil in pots and grown for 2 to 3 weeks in the greenhouse.

Preparation of Toxins

A highly virulent isolate of *Alternaria alternata* rough lemon pathotype (AC-325) was kindly provided by Dr. J.O. Whiteside, University of Florida, Lake Alfred, FL. The isolate was maintained on potato dextrose agar slants in test tubes. Small pieces of the mycelial mats from cultures were transferred to 500 mL incubation bottles, each containing 200 mL of a modified Richards' medium. The medium contained in g per L: glucose, 25; KNO_3, 10; KH_2PO_4, 5; $MgSO_4$ 2.5; $FeCl_3$, 0.02; and $ZnSO_4$, 0.005. Cultures grown at 25°C for 25 d were filtered, and the culture fluid was used to purify toxin as described previously (22). Purified ACR toxin I was toxic to susceptible leaves at a concentration of 10 ng·mL^{-1}, as shown by the leaf necrosis assay (22). HMT toxin was kindly provided by Dr. J.M. Daly, University of Nebraska, Lincoln, NE, and by Dr. R.P. Scheffer, Michigan State University, East Lansing, MI.

Isolation of Mitochondria

Active mitochondria from young citrus leaves were isolated by the method described previously (15). All procedures were performed as rapidly as possible at 4°C. Young green leaves (approximately 10 g, 1–2 cm long) were rinsed with distilled water and chilled for 30 min at 4°C. The leaf tissue was placed in 100 mL homogenization medium (25 mM Hepes-Tris [pH 7.5], 3 mM EDTA-Tris, 1 mM DTT, 1% [w/v] BSA, 250 mM sorbitol, 1% [w/v] PVP-40, and 1% [w/v] PEG-4000) along with 30 g Amberlite XAD-7. Leaves were chopped into fine pieces with razor blades, and the homogenate was filtered through Miracloth and stored on ice. The remaining tissue was resuspended in 200 mL homogenization medium with 10 g Amberlite XAD-7, macerated with a Virtis 45 homogenizer for 8 s at full speed, and filtered through Miracloth. The filtrates were combined, passed through a column (2.5 cm diameter) packed with 15 g Amberlite XAD-7 and washed with 50 mL homogenization medium. The column eluate was centrifuged at 4,300*g* for 3 min and the supernatant centrifuged again at 13,000*g* for 6 min. The pellet was resuspended in 30 mL of resuspension medium (250 mM sorbitol, 2.5 mM Hepes-Tris [pH 7.2], 1 mM DTT, and 0.5% [w/v] BSA) and centrifuged at 4,300*g* for 3 min. The supernatant was centrifuged at 13,000*g* for 6 min to yield the final mitochondrial pellet which was suspended in resuspension medium and kept on ice until use.

Mitochondrial Activity

Respiratory activities and proton movements were measured by a Gilson Oxygraph (model 5/6) equipped with Clark-type oxygen and pH electrodes at 25°C, in a closed vessel containing 1.5 mL of reaction medium (0.4 M sucrose, 20 mM Hepes-Tris [pH 7.2], 10 mM KCl, 4 mM KH_2PO_4, 2.5 mM $MgCl_2$, and 1 mg·mL^{-1} BSA) as described previously (15). Each mL contained approximately 0.6 to 0.8 mg protein (washed mitochondria). The vessel was closed by a lid fitted with an inlet for addition of reagents, and the suspension was kept homogeneous by a magnetic stirrer during the measurement of respiratory activity. Equilibrium oxygen concentration was calculated to be 258 μM at 25°C (6), and respiration rates were calculated from polarographic traces (6). Protein was determined by Coomassie brilliant blue binding with BSA as the standard (9). All experiments were repeated three or more times with comparable results.

Leaf Necrosis Assay for Toxicity

Young leaves of rough lemon and T-cytoplasm corn were scratched at the center of the lower surface with the sharp tip of scissors. A 30 μL drop containing ACR toxin, HMT toxin, methomyl, or mixtures of these solutions was placed on the wounded site. The treated leaves were incubated in a moist chamber in the dark at 27°C; necrotic areas of the leaves were measured 24 and 48 h after treatment.

Chemicals

Methomyl (analytic standard, 99%+ purity) was kindly supplied by E. I. du Pont de Nemours & Co. All other chemicals were from Sigma Chemical Co.

RESULTS

Selective Effect of ACR Toxin on Respiration of Citrus Mitochondria

Host-specific action of ACR toxin was examined with mitochondria from four different species of citrus. The mitochondria from rough lemon leaves (susceptible) were affected by ACR toxin at 1 μg·mL^{-1}. Toxin induced a significant increase in O_2 uptake with exogenous NADH as the substrate (Fig. 1 and Table I). This stimulation resembled that caused by ordinary uncouplers such as CCCP or 2,4-dinitrophenol. In contrast to NADH oxidation, malate oxidation was almost completely inhibited by the toxin at 1 μg·mL^{-1}. When an additional supply of exogenous NADH was added to the reaction medium, the inhibition of O_2 uptake in the toxin-treated malate oxidation system was canceled, but respiratory control in the mitochondria disappeared (Fig. 1 and Table I). In contrast, the mitochondria from leaves of resistant citrus, such as Dancy tangerine and Emperor mandarin (Table I) and grapefruit (data not shown), were not affected by toxin at 1 μg·mL^{-1}; oxidation and phosphorylation by these mitochondria were well-coupled after exposure to toxin (Table I).

Effect of ACR Toxin on NADH Oxidation by Rough Lemon Mitochondria

The effect of toxin on electron transport steps in NADH-oxidation was examined by using electron transport inhibitors whose sites of action in mitochondria are known. Antimycin A (2 μM) and NaN_3 (1 mM) completely stopped toxin- or CCCP-enhanced respiration immediately after treatment (Fig. 2). SHAM (1 mM) had no significant effect on the toxin-induced increase in respiration when NADH was the substrate (Fig. 2). Oligomycin at 1 μM immediately decreased the rate

of NADH oxidation by 60 to 70% (Fig. 3). However, additional toxin (1 $\mu g \cdot mL^{-1}$) or CCCP (10 μM), added after oligomycin treatment, caused a two-fold increase in respiratory rate (Fig. 3). Rotenone was applied to mitochondria 120 s after toxin or CCCP treatment to inhibit the function of complex I in the electron transport chain. Under these conditions, toxin and CCCP caused a similar increase in the rate of O_2 consumption. Toxin-enhanced respiration was decreased by 23% following addition of rotenone (25 μM); CCCP-enhanced respiration was not decreased by this concentration of rotenone (Fig. 4). Oxidative phosphorylation and proton movement were measured simultaneously on isolated mitochondria from susceptible rough lemon and resistant Emperor mandarin in order to examine proton movement across the mitochondrial membranes of toxin-treated tissues. Toxin (1 $\mu g \cdot mL^{-1}$) was added to the NADH-dependent respiration system at state 3 in cycle 2; this obstructed proton movement and increased oxygen uptake in susceptible, but not in resistant mitochondria (Fig. 5).

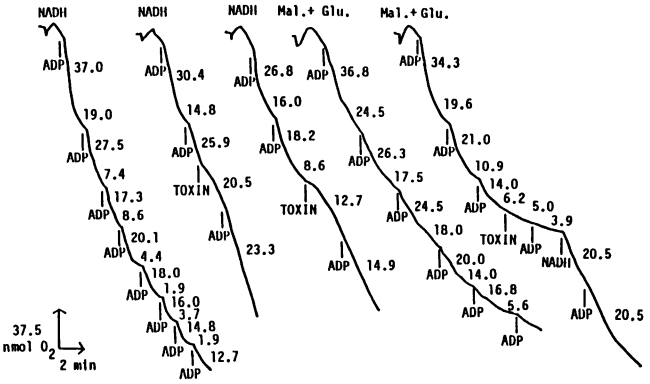

Figure 1. Dual effects of ACR toxin (1 $\mu g \cdot mL^{-1}$) on NADH (in state 3 or state 4) and malate oxidation by mitochondria from rough lemon. Numbers along the traces indicate oxygen uptake in nmol O_2 $min^{-1}mg^{-1}$ protein. Final concentrations of substrates and additions: NADH, 1 mM; malate (Mal.), 10 mM; glutamate (Glu.), 10 mM; and ADP, 100 μM.

Effect of Action of ACR Toxin on Malate Oxidation by Rough Lemon Mitochondria

ACR toxin at 1 $\mu g \cdot mL^{-1}$ completely inhibited malate oxidation by susceptible rough lemon mitochondria but had no effect on mitochondria from resistant Dancy tangerine or Emperor mandarin (Fig. 1 and Table I). Addition of ADP following toxin treatment did not affect the rate of respiration. However, addition of exogenous NADH to toxin-inhibited mitochondria resulted in a significant increase in O_2 uptake. This NADH-stimulated respiration was also inhibited completely by addition of antimycin A or NaN_3 (Fig. 6). Oxygen uptake was completely stopped in rough lemon when toxin was added after CCCP treatment (Fig. 7). Moreover, addition of 0.5 mM NAD^+ restored mitochondrial respiration which had been inhibited by toxin (Fig. 7); NAD^+ alone had no effect on malate oxidation by mitochondria from rough lemon (data not shown). The restoring effect of NAD^+ was not affected by pre-treatment with the uncoupler, CCCP (Fig. 7). Further addition of exogenous NADH after NAD^+ was supplied gave a significant increase in respiratory rate (Fig. 7).

Secondary substrates are known to be required for oxidation of α-ketoglutarate and pyruvate by mitochondria from several plants (7, 10). Therefore, the following additives were tested as secondary substrates for mitochondria from rough lemon: AMP, 3.75 mM; ATP, 100 μM; flavine-adenine dinucleotide, 250 μM; glutamate, 10 mM; and thiamine pyrophosphate, 250 μM. Malate (1 mM) and glutamate (1 mM) were incubated as 'sparkers.' These chemicals were used either singly or in different combinations, with α-ketoglutarate (10 mM) or pyruvate (10 mM) as a common substrate. Results showed that

Table I. *Effects of ACR Toxin on NADH and Malate Oxidations by Mitochondria from Susceptible and Resistant Citrus Leaves*

Plant	Substrate[a]	Cycle 1[b]		Cycle 2[b]		Toxin[c]	Change in O_2-Uptake Rate	Cycle 3[b]		Cycle 4[b]	
		RCR	ADP/O	RCR	ADP/O			RCR	ADP/O	RCR	ADP/O
						$\mu g \cdot mL^{-1}$	%				
Rough lemon	NADH	1.95	1.33	3.72	1.54	0	0	2.01	2.86	4.57	2.11
	NADH	1.68	1.38	2.12	1.48	1	+81[d]	—[e]	—	—	—
	Malate	1.50	1.85	1.50	2.58	0	0	1.36	2.77	1.43	3.88
	Malate	1.75	0.91	1.75	1.60	1	−45[d]	2.26	2.26	—	—
Dancy tangerine	NADH	1.58	1.14	1.92	1.38	0	0	2.07	1.82	2.63	1.90
	NADH	1.65	1.21	2.87	1.60	1	0	2.11	1.90	2.26	6.67
	Malate	1.88	1.25	1.79	1.61	0	0	2.09	1.76	2.52	2.58
	Malate	1.50	1.90	1.64	1.74	1	0	1.64	1.74	1.45	1.48
Emperor mandarin	NADH	1.91	1.25	1.75	2.50	0	0	2.10	1.74	2.32	1.90
	NADH	1.51	2.00	2.70	1.67	1	0	2.20	1.38	2.70	1.54
	Malate	1.80	1.61	1.55	1.34	0	0	2.21	1.44	2.38	1.55
	Malate	1.43	2.35	2.54	2.22	1	0	2.59	2.22	2.72	3.64

[a] Concentrations of substrates and additions are given in Figure 1. [b] Respiratory control rate (RCR) and ADP/O rate were determined from values of state 3 and state 4 transition in O_2 uptake. [c] ACR toxin (1 $\mu g \cdot mL^{-1}$) was added in state 4 or cycle 2 on NADH oxidation, and in state 4 of cycle 3 on malate oxidation. [d] Rate of change in oxygen uptake after toxin supply was indicated by the average of 5 experiments. [e] RCR and ADP/O values were not determined, because there was no response after addition of ADP.

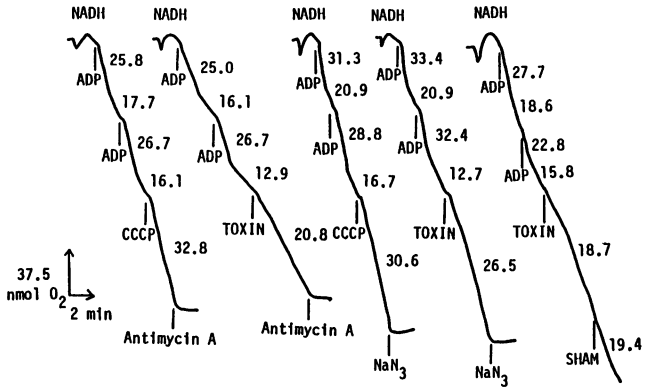

Figure 2. Effects of inhibitors (antimycin A, NaN_3, and SHAM) on toxin- and CCCP-stimulated respiration (NADH-dependent) by mitochondria from rough lemon. Numbers along the traces indicate oxygen uptake in nmol O_2 $min^{-1}mg^{-1}$ protein. Final concentrations: NADH, 1 μM; ADP, 100 μM; antimycin A, 2 μM; NaN_3, 1 mM; SHAM, 1 mM; ACR toxin, 1 $\mu g \cdot mL^{-1}$; and CCCP, 10 μM.

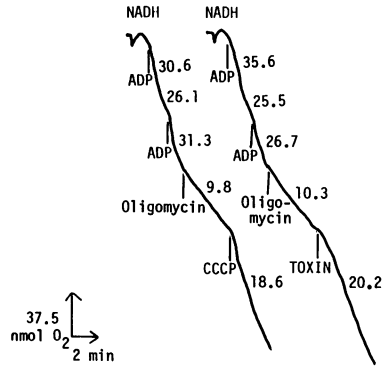

Figure 3. Uncoupling effect of ACR toxin (1 $\mu g \cdot mL^{-1}$) on H^+-ATPase-inhibited respiration by mitochondria from rough lemon. Numbers along the traces indicate oxygen uptake in nmol O_2 $min^{-1}mg^{-1}$ protein. Final concentrations of additions: NADH, 1 mM; ADP, 100 μM; oligomycin, 1 μM; and CCCP, 10 μM.

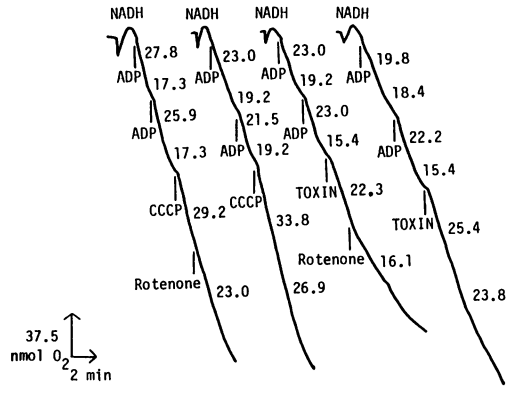

Figure 4. Effect of rotenone (25 μM) on NADH-dependent respiration by mitochondria from rough lemon, previously stimulated by ACR toxin (1 $\mu g \cdot mL^{-1}$) or CCCP (10 μM). Numbers along the traces indicate oxygen uptake in nmol O_2 $min^{-1}mg^{-1}$ protein. Final concentrations of additions were NADH, 1 mM and ADP, 100 μM.

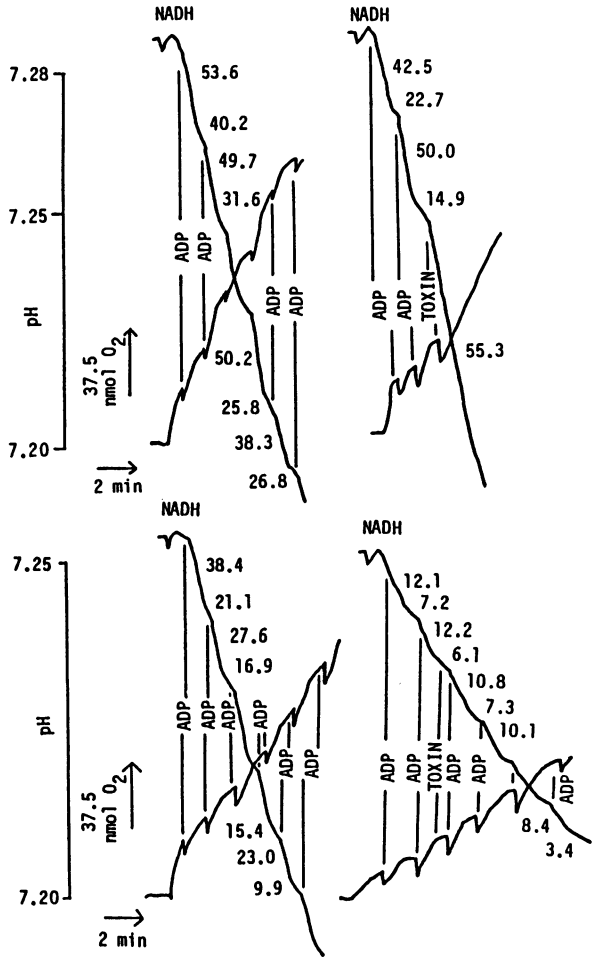

Figure 5. Effect of ACR toxin (1 $\mu g \cdot mL^{-1}$) on proton gradient and concomitant substrate oxidation by mitochondria from susceptible (top two traces) or resistant (lower two traces) leaves, using NADH as substrate. Control traces are shown on left; toxin-treated results are on right. Numbers along the traces indicate oxygen uptake in nmol O_2 $min^{-1}mg^{-1}$ protein. Proton movement is indicated, along with O_2-uptake. Final concentrations of additions were NADH, 1 mM, and ADP, 100 μM.

no combination gave increased respiration. With each combination, there was a very low oxidation of the substrates, and no respiratory control was observed (data not shown).

Mitochondria from several plants are known to oxidize proline (5, 29); however, mitochondria from rough lemon did not oxidize proline (10 mM) with or without glutamate (10 mM). Addition of proline (10 mM) did not stimulate malate oxidation by mitochondria that were inhibited by toxin (data not shown).

Comparative Effects of HMT Toxin, Methomyl, and ACR Toxin on Tissues and Mitochondria from Rough Lemon and T-Cytoplasm Maize

HMT toxin and methomyl have similar effects on respiration of mitochondria from T-cytoplasm maize. ACR toxin has comparable effects on respiration of mitochondria from rough lemon. Therefore, toxicities of HMT toxin and meth-

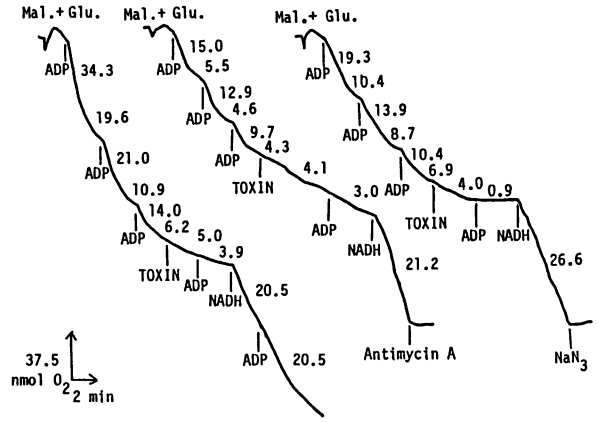

Figure 6. Inhibitory effect of ACR toxin (1 $\mu g \cdot mL^{-1}$) on malate-dependent respiration by mitochondria from rough lemon. Numbers along the traces indicate oxygen uptake in nmol O_2 $min^{-1}mg^{-1}$ protein. Final concentrations of additions: malate (Mal.), 10 mM; glutamate (Glu.) 10 mM; ADP, 100 μM; NADH, 1 mM; antimycin A, 2 μM; and NaN_3, 1 mM.

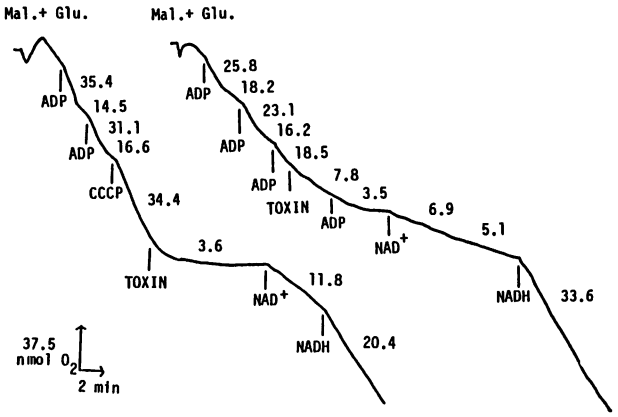

Figure 7. Effect of NAD^+ on ACR toxin-inhibited oxidation of malate by mitochondria from rough lemon. Numbers along the traces indicate oxygen uptake in nmol O_2 $min^{-1}mg^{-1}$ protein. Final concentrations of additions: malate (Mal.), 10 mM; glutamate (Glu.), 10 mM; ADP, 100 μM; CCCP, 10 μM; ACR toxin, 1 $\mu g \cdot mL^{-1}$; NAD^+, 0.5 mM; and NADH, 1 mM.

omyl to leaves of rough lemon, and of ACR toxin to leaves of T-cytoplasm maize were examined by the leaf necrosis assay.

A 30 μL drop of HMT toxin solution (5 $\mu g \cdot mL^{-1}$) or methomyl (50 mM) was placed on wounded sites of rough lemon leaves. ACR toxin solution was used as a control. Leaves were incubated for 24 or 48 h at 27°C. There was no necrosis in rough lemon leaves following treatment with HMT toxin or methomyl, whereas ACR toxin induced necrosis (Table II). On the other hand, ACR toxin (5 $\mu g \cdot mL^{-1}$) did not affect the leaves of T-cytoplasm maize (Table II). Simultaneous treatment with HMT toxin or methomyl plus ACR toxin gave no protective or enhancing effects on the induction of necrosis in rough lemon leaves (Table II).

HMT toxin (1 $\mu g \cdot mL^{-1}$) was added to mitochondria from rough lemon during cycle 2 of NADH oxidation and cycle 3

Table II. *Comparative Effects of HMT Toxin, Methomyl, and ACR Toxin on Leaves of Rough Lemon and T-Cytoplasm Maize*

Treatment[a]	Necrosis[b]	
	24 h	48 h
	mm^2/100 mm^2 leaf	
Rough lemon		
ACR toxin (1 $\mu g \cdot mL^{-1}$)	100	100
HMT toxin (5 $\mu g \cdot mL^{-1}$)	0	0
Methomyl (50 mM)	0	0
ACR toxin (1 $\mu g \cdot mL^{-1}$) + HMT toxin (5 $\mu g \cdot mL^{-1}$)	100	100
ACR toxin (1 $\mu g \cdot mL^{-1}$) + methomyl (50 mM)	100	100
T-cytoplasm corn		
ACR toxin (5 $\mu g \cdot mL^{-1}$)	0	0

[a] Thirty μL of each solution was placed on the wounded leaves of rough lemon or T-cytoplasm corn. Leaves were incubated in the dark at 27°C. [b] The necrotic area in a leaf section that was 100 mm^2 in area.

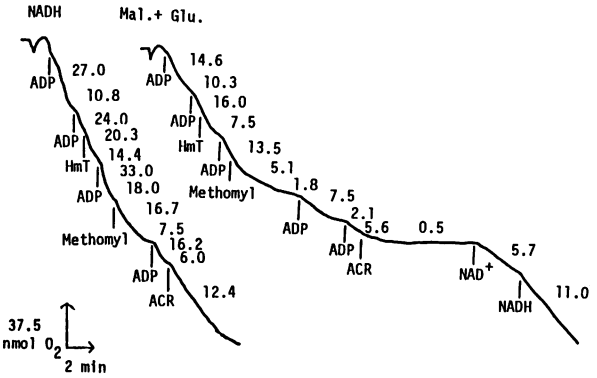

Figure 8. Effect of HMT toxin (1 $\mu g \cdot mL^{-1}$) and methomyl (5 mM) on the oxidation of NADH and malate by mitochondria from rough lemon. Numbers along the traces indicate oxygen uptake in nmol O_2 $min^{-1}mg^{-1}$ protein. Final concentrations of additions: NADH, 1 mM; malate (Mal.), 10 mM; glutamate (Glu.), 10 mM; ADP, 100 μM; ACR toxin, 1 $\mu g \cdot mL^{-1}$; and NAD^+, 0.5 mM.

of malate oxidation. HMT toxin caused no change in activity of mitochondria from rough lemon (Fig. 8). In contrast, HMT toxin (100 $ng \cdot mL^{-1}$) caused a significant inhibition of O_2 uptake by mitochondria from T-cytoplasm maize with malate as the substrate (data not shown). Next, rough lemon mitochondria were exposed to HMT toxin (1 $\mu g \cdot mL^{-1}$) or methomyl (5 mM), followed by addition of ACR toxin (1 $\mu g \cdot mL^{-1}$). Malate or NADH was used as the substrate. Neither HMT toxin nor methomyl had an effect on activity of ACR toxin on uptake of O_2 by the mitochondria (Fig. 8). Finally, the restorative effect on NAD^+ on ACR toxin-inhibited malate oxidation was not affected by pretreatments with HMT toxin or methomyl (Fig. 8).

DISCUSSION

Previous ultrastructural and physiological studies indicated that the initial site of action by ACR toxin may be in the mitochondrion (16, 17). Further studies on mitochondrial

effects were hindered because intact mitochondria were very difficult to isolate from citrus leaves. This problem was solved with an improved isolation procedure using Amberlite XAD-7 (15). ACR toxin at 1 $\mu g \cdot mL^{-1}$ had a marked effect on respiration by mitochondria from susceptible rough lemon; there was no effect on respiration of mitochondria from resistant citrus (Dancy tangerine, Emperor mandarin, and grapefruit) (Fig. 1 and Table I). This selective action of ACR toxin on isolated mitochondria confirms our previous suggestion that mitochondria are an initial action site of ACR toxin in host cells (16, 17). ACR toxin stimulated NADH oxidation by about 80% in susceptible mitochondria, with apparent uncoupling of oxidation and phosphorylation (Fig. 1). Toxin-stimulated respiration in mitochondria was inhibited by both antimycin A and NaN_3, known inhibitors of complexes III and IV of the mitochondrial electron transport chain (Fig. 2). These inhibitory actions by antimycin A and NaN_3 suggested that, even in the presence of ACR toxin, external NADH can be oxidized by a NADH dehydrogenase and electrons are transferred via complex III and complex IV to oxygen. SHAM, an inhibitor of the cyanide-resistant pathway (26), had no effect on toxin-stimulated oxidation (Fig. 2). Although cyanide-resistant respiration was not ruled out in this study, the results suggest that the cyanide-resistant pathway may not be involved in toxin-stimulated respiration; complexes III and IV appear to work in external NADH oxidation in the presence of ACR toxin.

Oxidative phosphorylation is another process whereby energy released from the oxidation-reduction reactions in the electron transport chain is used for the synthesis of ATP (8). Oligomycin, a known inhibitor of H^+-ATPase in the mitochondrial membrane, decreased oxygen uptake in NADH oxidation by mitochondria (Fig. 3). ACR toxin canceled the inhibition in NADH oxidation that was induced by oligomycin; the increase in oxygen consumption suggests an uncoupling action (Fig. 3). Thus, it appears that the action of ACR toxin may not be a direct inhibition of the proton pump but rather an uncoupling of the oxidation-reduction step in oxidative phosphorylation. ACR toxin also affected the proton motive force (Fig. 5), which is known as the obligatory link between respiration and ATP synthesis (8). These effects of ACR toxin appear to be very similar to those of common uncouplers such as CCCP and 2,4-dinitrophenol. However, there was a difference, as shown by the use of rotenone, an inhibitor of complex I. There was a 23% decrease in the respiration rate when rotenone was added after ACR toxin treatment. In contrast, rotenone caused no decrease in the stimulation of respiration caused by CCCP (Fig. 4).

These results indicate that stimulation of respiration by ACR toxin is caused in part by a NADH oxidation via a rotenone-sensitive path, whereas increased respiration induced by CCCP is dependent only on external NADH oxidation via flavoprotein. This difference between the effects of ACR toxin and CCCP on NADH oxidation shows that the mechanisms of action of ACR toxin may be more complex than simple uncoupling and movement of protons across the mitochondrial membrane. Based on these data, we propose that ACR toxin uncouples oxidative phosphorylation and obstructs the development of membrane potentials by opening channels for proton or other ions. The obstruction appears to differ from that caused by a protonophore such as CCCP.

The effects of ACR toxin on malate oxidation by rough lemon mitochondria indicate effects in addition to those shown by NADH oxidation experiments. The toxin significantly inhibited malate oxidation (Fig. 1). Inhibition was detected even when phosphorylation was uncoupled by pretreatment with CCCP (Fig. 7). Further, the re-addition of ADP had no effect on toxin-inhibited malate oxidation (Fig. 1). These data suggest that the inhibition of malate oxidation by ACR toxin may not be related directly to phosphorylation. The effect of inhibitors (antimycin A and NaN_3) (Fig. 6) also indicated that at least complex III and complex IV functions were not inhibited by ACR toxin; addition of external NADH after toxin treatment markedly stimulated respiration, and this was inhibited by antimycin A or NaN_3.

Exogenous NAD^+ can be a cofactor in malate oxidation in the TCA cycle, as shown by data with a complex enzyme system. Douce *et al.* (23, 27) showed that NAD^+ is actively transported and that this transport has a direct influence on malate oxidation by potato tuber mitochondria, which thus differ from mammalian mitochondria. We observed that exogenous NAD^+ restored toxin-inhibited malate oxidation (Fig. 7). The observation suggests that inhibition of oxidation can be due to a toxin-induced decrease of NAD^+ in mitochondria. Further, this reduction of NAD^+ seems to be independent of phosphorylation, because the response to toxin was not affected by pretreatment with CCCP (Fig. 7). Thus, inhibition of respiration by ACR toxin may be based on blockage of malate oxidation because of the absence of NAD^+ in the matrix; this toxin action may be completely independent of the effect on NADH oxidation. We suggest that there may be two different effects of ACR toxin in rough lemon mitochondria.

Gardner *et al.* (11, 12) and Kono *et al.* (20) reported that ACR toxin inhibited proline incorporation by leaf tissues of rough lemon. A 2 to 4 h pretreatment with ACR toxin resulted in 50% inhibition of proline incorporation during a subsequent incubation time of 2 to 4 h (11, 12). Such inhibition did not occur in leaf tissue that was resistant to the fungus and its toxin (11). However, the ultrastructural modifications of mitochondria in leaf cells were observable much earlier, within 1 h after exposure to ACR toxin; about 80% of the mitochondria were affected in toxin-treated tissues within 3 h (17). The resulting shortage of ATP may account for the inhibition of proline incorporation in toxin-treated tissues. Further, exogenous proline had no effect on toxin-inhibited oxidation of malate in mitochondria from rough lemon (data not shown). Thus, it is still not clear that the inhibition of proline incorporation into leaf tissues is a direct or an indirect effect of ACR toxin. Our results suggest that inhibited incorporation of proline is a secondary phenomenon resulting from toxin-induced disruption of mitochondrial metabolism.

The responses of rough lemon mitochondria to ACR toxin appear to be similar to those of mitochondria from T-cytoplasm maize to HMT toxin and to methomyl (3, 4, 13, 14, 24). Two different responses of T-mitochondria to HMT toxin and to methomyl are known: stimulation of NADH oxidation and inhibition of malate oxidation. These common effects of

ACR toxin and HMT toxin on mitochondria from the respective susceptible plants are of interest, because both toxins have a similar polyol moiety (12, 18, 22), and both are long chain compounds. Each toxin retains the selectively toxic effect for mitochondria from the sensitive plant that is the host of the producing fungus. Both HMT toxin and methomyl lacked toxicity to rough lemon and did not interfere with the activity of ACR toxin on susceptible citrus tissues or mitochondria. Conversely, ACR toxin had no effect on T-cytoplasm maize. Thus, ACR and HMT toxins appear to have common features in regard to molecular structure, site of action, and selectivity, although different plant species are affected. Hopefully, future studies will reveal the parts of each molecule that are involved in toxicity to mitochondria and the parts that determine selectivity for host species.

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