Effects of Helminthosporium maydis Race T Toxin on Electron Transport in Susceptible Corn Mitochondria and Prevention of Toxin Actions by Dicyclohexylcarbodiimide

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ABSTRACT

The effect of Helminthosporium maydis race T toxin on electron transport in susceptible cytoplasmic male-sterile Texas corn (Zea mays L.) mitochondria was investigated, using dichlorophenol indophenol and ferricyanide as electron acceptors. Succinate-dependent electron transport was stimulated by the toxin, consistent with the well described increase in membrane permeability induced by the toxin. Malate-dependent electron transport was inhibited. This inhibition of electron transport increased as a function of time of exposure to the toxin. Mitochondria from normal-fertile (N) corn were not affected by the toxin. Both the inhibition of electron transport and the increase in ion permeability, such as dissipation of membrane potential and Ca2+ gradients, induced by the toxin in T corn was prevented by N,N'-dicyclohexylcarbodiimide, a hydrophobic carbodiimide. A water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, was ineffective in preventing dissipation of membrane potential by the toxin. These results suggest that the various toxin actions are mediated via interaction of the toxin with one target site, most probably a 13 kilodalton polypeptide unique to T mitochondria. N,N'-dicyclohexylcarbodiimide may confer protection by modifying an amino acid residue in a hydrophobic portion of the target site.

Helminthosporium maydis race T toxin specifically targets mitochondria of the Texas (T) line of cytoplasmic male-sterile corn (Zea mays L.), while having no effect on normal-fertile corn or other types of cytoplasmic male-sterile corn. HmT1 toxin sensitivity and cytoplasmic male-sterility are tightly linked and are probably the result of the same recombination event in the mitochondrial DNA (8–10). The molecular mechanism of toxin action is unknown. One primary action of HmT toxin is to increase the permeability of the inner mitochondrial membrane to ions. Supporting this thesis is evidence that HmT toxin: (a) stimulates respiration with succinate and NADH as substrates (6), (b) stimulates mitochondrial ATPase activity (2, 16), (c) dissipates mitochondrial membrane potential (3, 16), and (d) increases membrane permeability to calcium (15) and protons (20). However, these studies provide no clues regarding the site of toxin action or the nature of the toxin target site.

HmT toxin also inhibits malate-driven respiration as measured by oxygen consumption (6). This inhibition may be the result of a direct interaction of the toxin with a target site in the NADH-ubiquinone oxidoreductase (complex I) or due to leakage of a cofactor, NAD. Malate-driven respiration, unlike succinate-driven respiration, requires a soluble cofactor, NAD. NAD has been shown to leak slowly from toxin-treated mitochondria (3, 22). Thus, it is possible that inhibition of malate-dependent respiration is caused by toxin-induced increases in mitochondrial membrane permeability and subsequent NAD leakage. However, in one case, HmT toxin inhibition of malate-dependent electron transport was shown to be separate from the action of the toxin on membrane permeability (6, 30), suggesting that the toxin could possibly interact with two target sites.

To understand the nature and the location of the toxin target site(s), we have investigated the effect of HmT toxin on electron transport activities by functionally dissecting the electron transport chain using ferricyanide and DCPIP as electron acceptors. The rationale was that assaying for acceptor reduction would be a more direct measurement of electron transport from complex I or II than assaying for O2 reduction. We also studied the effect of DCCD on toxin-induced increase in membrane permeability and inhibition of electron transport to determine if the different activities are the result of toxin interaction with one or more target sites. Bouthyette et al. (4) reported that DCCD prevents toxin-induced absorbance changes attributed to mitochondrial swelling. We found that malate-dependent ferricyanide and DCPIP reduction is inhibited by HmT toxin and that DCCD protected against the toxin effects on three different activities (electron transport, Ca2+ gradients, and membrane potential). A preliminary report of some of these findings has been made (17). After our studies were completed, Dewey et al. (10) reported that the 13 kD polypeptide, unique to T mitochondria, bound
DCCD. Taken together, these studies provide evidence that the 13 kDa is the toxin target site.

MATERIALS AND METHODS

Mitochondrial Isolation

Mitochondria were isolated from the roots of 3 to 4 d old corn (Zea mays L.) seedlings. The lines of corn used in these experiments were W64AT (Texas cytoplasmic male-sterile and sensitive to HmT toxin) and W64AN (normal male-fertile and HmT toxin insensitive). Seeds were surface-sterilized (0.5% v/v sodium hypochlorite) and germinated in boxes between layers of cheesecloth soaked with 0.5 mM CaSO4. Roots were cut into 1 cm pieces and homogenized, with mortar and pestle, in a medium (10 mL/g wet wt of roots) of 250 mM sucrose (sorbitol replaced sucrose in 44Ca2+ and membrane potential experiments), 25 mM Hepes-BTP (pH 7.4), 3 mM EGTA, 0.2% (w/v) BSA (Sigma fraction V, fatty acid free), and 1 mM DTT. The homogenate was filtered through four layers of cheesecloth and centrifuged at 1,500 g for 15 min. Mitochondria were pelleted at 7,600 g (15 min), resuspended in 250 mM sucrose, 2.5 mM Hepes-BTP (pH 7.4), 0.2% BSA, and 1 mM DTT and were repelleted. The washed mitochondria (3–5 mL) were layered on a 0.6 M sucrose cushion (20 mL) buffered with 10 mM Hepes-BTP (pH 7.4) plus 0.1% BSA and were centrifuged for 20 min at 10,000 g (7). The mitochondrial pellet was resuspended in 250 mM sucrose, 2.5 mM Hepes-BTP to a final protein concentration of 1 mg/mL. Protein was measured by the method of Bradford (5) with BSA as a standard.

Measurement of Electron Transport Using Ferricyanide as an Electron Acceptor

The reduction of K3Fe(CN)6 was monitored at 420 nm in a reaction mixture (1 mL) of 220 mM sucrose, 8 mM Hepes-BTP (pH 7.4), 15 mM KC1, 2 mM K3Fe(CN)6, 1 mM KCN, and 100 to 200 μg mitochondrial protein (21). Reduced ferricyanide was quantified with the extinction coefficient (ε420 = 1.03 × 103 M−1 cm−1, 1 cm light path) (26).

Electron transport via complex I (NADH:ubiquinone oxidoreductase) was measured with malate (50 mM BTP-Malate) in the presence of 1 mM BTP-malonate. Mitochondria were preincubated in the reaction mixture, minus malate, for 2 to 4 min. Initial slight changes in absorbance occurred which stabilized after the 2 to 4 min period. The reaction was then started with the addition of malate.

Electron transport via complex II (succinate:ubiquinone oxidoreductase) was measured with 10 mM BTP-succinate as substrate in the presence of 12.5 μM rotenone. For these assays, the mitochondria were preincubated with 10 mM succinate in the reaction mixture, minus ferricyanide, for 10 min at 30°C to activate succinate dehydrogenase (33). The cuvette was brought to room temperature, and the reaction was started with the addition of ferricyanide.

Measurement of Electron Transport Using DCPIP as an Electron Acceptor

Malate-dependent electron transport was measured by following DCPIP reduction at 600 nm (12). The reaction was started by the addition of 50 mM BTP-malate (pH 7) to a reaction mixture (1 mL) of 50 μM DCPIP, 1 mM KCN, 100 μg mitochondrial protein, 210 mM sucrose, 2 mM Hepes-BTP (pH 7.4). DCPIP reduction was calculated with the extinction coefficient (€600 = 19.1 × 103 M−1 cm−1) (12).

Membrane Potential and Calcium Uptake Measurements

Membrane potential was monitored using the lipophilic cation, safranine 0. In a previous study (16), membrane potential had been monitored by following changes in absorbance (511–533 nm) with a dual wavelength spectrophotometer according to the method of Moore and Bonner (25).

Subsequently, we have determined that safranine produces a fluorescence signal (excitation 495 nm, emission 586 nm) which was quenched with the development of a membrane potential in mitochondria upon addition of substrate. Assays were conducted similar to Holden and Sze (16), with the exception that the safranine/protein ratio was lowered to 7 to 13 nmol/mg protein. Optimization of the ratio was determined empirically for each batch of mitochondria and up to 80% quench of the fluorescence was achieved. 44Ca2+ transport was measured by a filtration procedure previously described (15).

HmT Toxin

Purified HmT toxin and corn seeds were generous gifts of Dr. J. M. Daly, University of Nebraska, Lincoln. HmT toxin was solubilized in DMSO. Small aliquots (5–10 μL) of toxin stocks were added to reaction mixtures to keep the final DMSO concentration to 1% (v/v) or less. Water-insoluble inhibitors were solubilized in ethanol or DMSO and handled similarly.

RESULTS

Electron Transport Measured by Ferricyanide and DCPIP Reduction

Mitochondrial electron transport inhibitors were used to confirm the site of reduction of ferricyanide in corn mitochondria (see electron transport chain schematic, Fig. 1). Ferricyanide is thought to be impermeant to the inner membrane and to accept electrons from Cyt c in intact mitochondria (21). Succinate-dependent ferricyanide reduction in corn mitochondria was sensitive to malonate and TTFA (Fig. 2A), both of which have sites of action in complex II (19, 32). Antimycin A also inhibited succinate-dependent ferricyanide reduction (Table I) as would be expected if Cyt c is the electron donor to ferricyanide. However, ferricyanide was reduced by succinate if Triton X-100 was added to antimycin A-inhibited mitochondria (data not shown), indicating that ferricyanide can be reduced before Cyt c in permeabilized mitochondria. Thus, antimycin A sensitivity is an indicator of inner mitochondrial membrane intactness (11, 21).

Malate-dependent ferricyanide reduction was sensitive to antimycin A (Table I) and rotenone (Fig. 2B). Rotenone caused nearly total inhibition of malate-dependent ferricyanide reduction. Under the conditions of our assay, little or no
Figure 1. A model of the mitochondrial inner membrane electron transport showing sites of action for electron transport inhibitors. Complex I is defined as NADH:ubiquinone oxidoreductase sensitive to rotenone. Plant mitochondria have, in addition, two separable NADH dehydrogenase activities that are rotenone-insensitive. One site has access to cytoplasmic (or exogenously supplied) NADH and the other is located on the matrix side of the inner membrane as is the rotenone-inhibitable site (24). The other inner membrane complexes are defined as follows: complex II, succinate:ubiquinone oxidoreductase; complex III, ubiquinone:Cyt c reductase; complex IV, Cyt c oxidase.

electron transport occurred via the rotenone-insensitive bypass of the NADH:ubiquinone oxidoreductase (Complex I) (24).

Unlike ferricyanide, DCPIP probably accepted electrons at more than one site on the electron transport chain. Malate-dependent DCPIP reduction was only slightly sensitive to rotenone (Fig. 2B) and antimycin A (Table I). Antimycin A insensitivity is consistent with the report of Flavell (12). In contrast, Alexandre and Lehninger (1) reported that DCPIP reduction by NAD-linked substrates in rat liver mitochondria was sensitive to rotenone. Since malate-dependent ferricyanide reduction appeared to be exclusively via the rotenone-sensitive NADH:ubiquinone oxidoreductase (Fig. 2B), we interpret our results with DCPIP to mean that in corn mitochondria DCPIP can accept electrons before, as well as after, the rotenone-inhibited site in complex I.

HmT Toxin Stimulated Succinate-Dependent and Inhibited Malate-Dependent Electron Transport in Susceptible T Mitochondria

HmT toxin-treated T corn mitochondria stimulated succinate-dependent ferricyanide reduction over the rates measured in the absence of toxin (Table II). Significant stimulation was detected even at low toxin concentrations (2.5 ng/mL). Observations that HmT toxin increased O2 consumption in cms T mitochondria (under low KCl conditions) have led to suggestions that the toxin has uncoupling activity in susceptible mitochondria (6). This would be the direct result of the toxin's ability to increase membrane permeability to ions (3, 15, 16). Similarly, toxin-induced increases in O2 consumption have been reported when exogenously supplied NADH was metabolized via the external dehydrogenase (2, 12, 14, 30).

When malate was the substrate, a decrease in acceptor reduction rather than a stimulation was observed. Acceptor reduction by susceptible T mitochondria was inhibited by low concentrations of toxin (10 ng/mL or 13 nM with ferricyanide as acceptor). Malate-dependent DCPIP reduction was rather insensitive to rotenone (Fig. 1), yet the toxin inhibited DCPIP reduction nearly as effectively as ferricyanide reduction. One interpretation of these results is that the site of toxin action that results in inhibition of malate-dependent electron transport must lie at or prior to the rotenone-inhibited site. In contrast to the effect on T mitochondria, HmT toxin had essentially no effect on malate-dependent acceptor reduction by resistant N corn mitochondria (Table II).

HmT toxin inhibition of malate-dependent electron transport in T mitochondria increased as a function of the length of time that susceptible mitochondria were exposed to the toxin. A low toxin concentration of 2.5 ng/mL had no immediate detectable effect, but significant inhibition (75%) of
Malate-dependent electron transport was completely inhibited within a minute when susceptible mitochondria were treated with 33 nm (25 ng/mL) HmT toxin (Fig. 3B). The initial absorbance change seen in the first minute after toxin addition (Fig. 3B) could be interpreted as evidence of uncoupling or toxin-induced mitochondrial swelling, as previously reported (20, 23). A similar transient absorbance change could be induced by treating susceptible mitochondria with the toxin in the absence of substrate (Fig. 3C). Because this absorbance change (Fig. 3C) is independent of electron transport activity, we interpret the initial change in Figure 3, B and C, as caused by swelling. Mitochondria treated with gramicidin (a channel-forming ionophore which increases membrane permeability to H+ and monovalent cations) showed initial uncoupling followed by a continually decreasing rate of ferricyanide reduction (Fig. 3D). Therefore, the toxin effects on electron transport can be partially mimicked by gramicidin, but the time required for gramicidin-induced inhibition was much greater than for inhibition induced by HmT toxin.

Table I. Effect of Antimycin A on the Reduction of Electron Acceptors

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Substrate</th>
<th>Electron Transport Rate</th>
<th>Inhibition</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>mmol/min · mg</td>
<td>%</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>Succinate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>289 (120–534)</td>
<td>14 (0–48)</td>
<td>95</td>
</tr>
<tr>
<td>Antimycin A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>149 (78–184)</td>
<td>15 (5–29)</td>
<td>90</td>
</tr>
<tr>
<td>Control</td>
<td>62 (56–73)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antimycin A</td>
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DCPIP reduction was measured when the same concentration of toxin was preincubated with mitochondria for 10 min. The relationship between the extent of inhibition and duration of exposure time would account for the quantitative difference between inhibition of ferricyanide and DCPIP reduction in T mitochondria by HmT toxin (Table II), as ferricyanide assays were conducted over a longer time period than the DCPIP assays (10 versus 1 min).

Table II. HmT Toxin Stimulates Succinate-Dependent and Inhibits Malate-Dependent Electron Transport in Susceptible, but not Resistant, Mitochondria

Mitochondrial protein (100 μg) was preincubated with HmT toxin (2 min—succinate, 5 min—malate). FeCN reduction by T mitochondria was conducted for 10 min and lost linearity with time. Rates reflect averaged total reduction for 10 min. FeCN reduction by N corn was conducted for 5 min and the rate was linear. DCPIP reduction by T and N mitochondria was conducted for 1 min and also was linear. Control rates (in mmol/mg min) were as follows: succinate FeCN-T = 588, malate FeCN-T = 214, FeCN-N = 340, DCPIP-T = 66, DCPIP-N = 37.

DCPIP Prevented HmT Toxin Actions

DCCD has been shown to prevent toxin-induced swelling of susceptible T corn mitochondria (4). We have used DCCD to determine whether three toxin-induced effects (on Ca2⁺ gradients [15], membrane potential [16], and electron trans-
HmT toxin (5 ng/mL) or the calcium ionophore, A23187, dissipated Ca\(^{2+}\) gradients in susceptible mitochondria (Fig. 4A). Generation of a Ca\(^{2+}\) gradient by malate in the absence of DCCD reflected the formation of a membrane potential (negative inside) (15, 16). Addition of DCCD did not alter the Ca\(^{2+}\) gradient nor did subsequent addition of HmT toxin result in dissipation of the gradient (Fig. 4B). These results clearly indicate that DCCD protected against toxin-induced increase in Ca\(^{2+}\) permeability in susceptible mitochondria. We have previously shown that HmT toxin effectively dissipated the membrane potential in susceptible mitochondria (16) (Fig. 5). We examined the effect of DCCD on membrane potential, as measured by quenching of safranine fluorescence, in two ways. DCCD was added before (Fig. 5, b and c) or after (Fig. 5d) succinate-dependent formation of a membrane potential. Although the magnitude of the substrate-generated potential was decreased by DCCD, HmT toxin was much less effective in dissipating the potential in DCCD-treated T mitochondria (cf. Fig. 5, b and c to 5a). Thus, DCCD protected against toxin-induced dissipation of membrane potential in susceptible mitochondria. When DCCD was added after formation of the membrane potential, the rate of potential dissipation by HmT toxin was retarded (Fig. 5d). EDAC, a water-soluble carbodiimide, did not protect against HmT toxin even at much higher concentrations (100 {\mu}M) (Fig. 5e). The effectiveness of DCCD, a hydrophobic carbodiimide, as compared to EDAC suggests that the toxin target site is located in a hydrophobic membrane domain.

DCCD also protected against toxin action on electron transport. HmT toxin at 25 ng/mL completely inhibited malate-dependent electron transport as measured by ferricyanide reduction (Fig. 6A). In the presence of DCCD alone (80 nmol/mg protein), the rate of malate-dependent ferricyanide reduction was partially reduced (48%, Fig. 6B) as compared to control (Fig. 6D). This is very similar to what has been shown by Honkakoski and Hassinen (18) for rat liver mitochondria (150 = 90 nmol/mg protein). The electron transport values of mitochondria treated with DCCD followed by toxin were similar to those of mitochondria treated with DCCD alone (Fig. 6C).

**DISCUSSION**

DCCD prevention of HmT toxin effects on (a) calcium gradients, (b) membrane potential, and (c) electron transport strongly suggests that all of the actions of the toxin are linked...
and may be the result of the interaction of the toxin with either a single target or several similar targets. Although DCCD is capable of modifying a number of mitochondrial proteins, it seems unlikely that the toxin would have more than one target site that is susceptible to DCCD. Also supporting the concept of a single target site is the finding that the concentration range and time frame for inhibition of electron transport by the toxin were the same as for dissipation of calcium gradients (15) and membrane potential (16).

The effectiveness of DCCD, as compared to EDAC, implies that the target molecule is a hydrophobic membrane protein. The F_1F_0-ATPase proteolipid and Cyt c oxidase subunit III of mitochondria have been shown to react with DCCD but not with EDAC, and DCCD modification specifically interferes with proton translocation (27, 34). These proteins share a common feature of a segment of hydrophobic amino acids containing a glutamic or aspartic acid residue which is modified by DCCD. It is highly probable that the target site of the toxin is a 13 kD protein of mitochondrial membranes (9), which has been shown to bind DCCD (10). Mitochondria from cms T corn, but not normal-fertile corn, synthesize a 13 kD protein (13). A unique sequence of DNA has been found in the genome of cms T corn mitochondria which contains an open reading frame sufficient to encode a 13 kD protein (8). Antibodies made to a synthetic peptide corresponding to a segment of the postulated amino acid sequence of the open reading frame were used to demonstrate expression of the 13 kD protein in mitochondrial membranes (9) and in bacteria expressing this gene (10). These findings constitute strong evidence that the 13 kD protein functions as the toxin target. According to the hydropathy profile of the protein, this putative toxin target has a large hydrophobic segment containing an aspartic acid residue (9), a likely candidate for DCCD modification. One feasible model is that one or several 13 kD polypeptides form an ion channel after binding of the toxin. DCCD modification of the 13 kD protein prevents the binding of the toxin and maintains an ion-impermeant structure (or closed channel).

The location of the 13 kD protein within the mitochondrial membrane is unclear. Two possibilities are: (a) the 13 kD is associated with one of the electron transport complexes or the H^+ATPase, and (b) the 13 kD is a unique mitochondrial protein, independent of the respiratory complexes. It is unlikely that the toxin receptor is either a modified F_1F_0-ATPase proteolipid or cytochrome oxidase subunit III, both of which bind DCCD, for the following reasons. First, Boughyette et al. (4) showed that DCCD at 50 to 100 nmol/mg protein had essentially no effect on ATPase activity in T mitochondria when activity was measured immediately following DCCD addition. This is both quantitatively equivalent and within the same time frame as our experiments, yet DCCD at 40 to 80 nmol/mg was able to completely inhibit the toxin effect on membrane potential (5), Ca^{2+} efflux (4), and electron transport (6). Second, the molecular mass of the H^+ATPase proteolipid is 8 kD and subunit III is 30 kD, while the toxin target is 13 kD (34). Finally, a large proportion of DNA sequence of the open reading frame that encodes the 13 kD protein is homologous with a flanking region of the 26S ribosomal gene (8). The 13 kD may be associated with other sites in the respiratory chain. For example, DCCD interferes with proton pumping by NADH:ubiquinone oxidoreductase in rat liver mitochondria (18).

One way to approach the problem of the location of the toxin target is to determine the effect of the toxin on electron transport by functionally dissecting the electron transport chain. Our results with ferricyanide and DCPIP as electron acceptors showed high toxin specificity and potency and were similar to previous studies that measured toxin effects on O_2 consumption (3, 6, 14, 23, 31). The rate of succinate-dependent electron transport, as measured by acceptor reduction, was increased by the toxin, while the rate of malate-driven electron transport was decreased. Toxin inhibition of malate-dependent ferricyanide and DCPIP reduction was essentially the same, indicating that inhibition is probably a very early event in the electron transport process. If HmT toxin increases membrane permeability to ions (H^+, Ca^{2+}) alone, it would be expected that a stimulation of electron transport would be seen with malate as well as succinate. Inhibition of malate-dependent electron transport by HmT toxin may result from one or both of the following possibilities: (a) the toxin might directly inhibit electron transport by interaction with a target site specifically associated with or near the NADH:ubiquinone oxidoreductase (complex I), and this interaction also induces an increase in membrane permeability, and (b) the toxin induces leakage of ions and soluble cofactors, such as NAD (directly or indirectly), by interaction with a site independent of the respiratory complexes. It is unlikely that malate entry was limiting as the exogenous concentration was high (50 mM) in the assays and succinate- and ATP-dependent activities were not substrate limited (Table II, this paper; and ref. 16).

NAD, a required cofactor for malate-dependent respiration, has been shown to leak from toxin-treated mitochondria (3, 22). NAD is accumulated in actively respiring mitochondria via an NAD carrier protein, and NAD leaks from the mitochondria also via this carrier in the absence of an electrochemical gradient (28, 29). Taken together these suggest that inhibition of malate-dependent electron transport is a secondary effect of toxin-induced collapse of the proton electrochemical gradient and subsequent NAD leakage. The observation that gramicidin, a channel-forming ionophore which dissipates electrochemical gradients, decreased malate-dependent elec-
electron transport would support this interpretation (Fig. 3). Also important are reports that malate-dependent electron transport activity can be recovered if NAD is added back to toxin-treated mitochondria (3, 22, 31).

There is reasonable evidence supporting a primary leakage hypothesis, but a direct effect on the toxin on the NADH:ubiquinone complex is not ruled out until some problems and inconsistencies can be resolved: (a) the time course of NAD leakage is too slow (3) to account for the rapidity of electron transport inhibition (Fig. 3 and refs. 3, 14, 23); (b) HmT toxin inhibition of electron transport occurred faster than gramicidin-induced inhibition; and (c) the recovery of electron transport in toxin-inhibited mitochondria by exogenous NAD may occur via a different pathway, through a rotenone-insensitive NADH:ubiquinone oxidoreductase bypass (preliminary data) as has been shown for other plant species (24). In theory, this pathway could circumvent a toxin-inhibited site. If NAD leakage could be prevented, then it would be possible to test whether the toxin has a direct effect on electron transport and, if so, to identify the specific location for the target site in respiratory complexes of the inner mitochondrial membrane of cms T corn.

LITERATURE CITED