Glyceollin: A Site-Specific Inhibitor of Electron Transport in Isolated Soybean Mitochondria

RICHT BOYDSTON, JACK D. PAXTON, AND DAVID E. KOEPPE

Departments of Plant Pathology (J. D. P.) and Agronomy (R. B., D. E. K.) University of Illinois, Urbana, Illinois 61801

ABSTRACT

The glyceollin inhibition of electron transport by isolated soybean and corn mitochondria was similar to that of rotenone, acting at site I between the internal NADH dehydrogenase and coenzyme Q. Coupled state 3 malate oxidation was inhibited by glyceollin and rotenone with apparent K_i values of about 15 and 5 micromolar, respectively. Carbonylcyanide m-chlorophenyl hydrazone uncoupled state 4 malate oxidation was also inhibited by glyceollin and rotenone, but uncoupled succinate and exogenous NADH state 4 oxidation was only slightly inhibited by both compounds. Glyceollin also inhibited ferricyanide reduction with malate as the electron donor, with an apparent K_i of 5.4 micromolar, but failed to inhibit such reduction with succinate or externally added NADH as electron donors. Glyceollin did not inhibit state 4 oxidation of malate, succinate, or exogenous NADH. Glyceollin did not act as a classical uncoupler or as an inhibitor of oxidative phosphorylation.

MATERIALS AND METHODS

Soybean seedlings (Glycine max L., cv Amsoy 71) were germinated and grown in the dark at 28°C in moist vermiculite saturated with 0.1 mM CaCl_2 solution. Corn seedlings (Zea mays L., 222, Crows Seed Co.) were germinated and grown in the dark at 28°C on paper towels saturated with 0.1 mM CaCl_2 solution. Washed mitochondria were isolated from soybean hypocotyls or corn shoots of 4-d-old seedlings by the procedure of Miller et al. (15). Isolation of mitochondria from cotyledon tissue was the same, except that the cotyledons were ground through an Oster Automatic Juicer (model 362; Oster Co., Milwaukee, WI) and filtered through Miracloth (Chicopee Mills, Inc., Milltown, NJ) before the first centrifugation.

Purification of washed mitochondria consisted of resuspending washed mitochondria in 1 ml of standard resuspension medium consisting of 0.3 mM mannitol, 0.1 mM EDTA, and 0.1% BSA at pH 7.2. The resuspended mitochondria were then placed on top of a discontinuous sucrose gradient similar to that used by Douce et al. (1), and consisting of 1.45 mM sucrose (4 ml) layered under 1.2 mM sucrose (10 ml). Tubes were centrifuged for 45 min at 60,000g at 4°C in a Sorvall AH 627 swinging bucket rotor placed in a Sorvall OTD 65 centrifuge.

After centrifugation, mitochondria were located at the boundary of the two sucrose layers and were removed with a curved tip pasteur pipette. The purified mitochondria were then diluted slowly over a period of 20 min with the slow addition of 0.1 mM sucrose containing 10 mM KH_2PO_4 and 0.1% BSA until a sucrose concentration of 0.3 mM (pH 7.2) was achieved. The diluted mitochondrial suspension in 0.3 mM sucrose was then centrifuged at 14,600g for 8 min. After centrifugation, the purified mitochondria were resuspended in the standard 0.3 mM mannitol resuspension medium. Mitochondrial protein was estimated by the method of Lowry et al. (11) using BSA (fraction IV) as the standard.

O_2 uptake experiments were measured polarographically in a 4.0-ml cell (28°C), fitted with a Clark O_2 electrode (Yellow Springs Instrument Co., Yellow Springs, OH).

The mitochondrial reaction medium consisted of 0.3 mM mannitol, 10 mM Tris-HCl (pH 7.2), 5 mM MgCl_2, 10 mM KCl, 4 mM KH_2PO_4, 1 mg/ml BSA, and about 0.7 mg mitochondrial protein/4 ml.

Experiments measuring the reduction of ferricyanide were conducted in a 3-ml glass cell with the standard reaction medium (excluding BSA) containing 20 mM KCl. Mitochondria, followed by additions to give 1 mM KCN and 1 mM K_2Fe(CN)_6, and 1.2 μmol ADP were added prior to the initiation of the reaction by addition of substrate. The reduction of ferricyanide was monitored at 405 nm on a Hitachi Perkin-Elmer 139 spectrophotometer (9).

Rotenone (95–98% purity) was obtained from Sigma. Glyceollin was a mixture of isomers I, II, and III. These were purified from extracts of inoculated soybean plants by TLC followed by HPLC. There was no indication of impurities in the glyceollins used after chromatography in two different solvents on Silica Gel thin-layer chromatograms, HPLC, or in the UV absorption spectra. Glyceollin and rotenone were dissolved in 100% ethanol.

1 Supported by funds from the Illinois Agricultural Experiment Station and United States Department of Agriculture/Science and Education Administration Grant No. 59-2181-1-011-0.
RESULTS

State 3 Inhibition. Mitochondria oxidizing malate in state 3 were inhibited by glyceollin with an apparent $K_i$ of 15 $\mu$M (Fig. 1, A and B). The inhibitory effect of rotenone under similar conditions resulted in an apparent $K_i$ of 5 $\mu$M (Fig. 1, C and D). State 3 malate oxidation, that was inhibited maximally by glyceollin or rotenone, was not released by the addition of the electron transport uncoupler CCCP. This observation was unlike the classical respiratory release by CCCP of oligomycin-inhibited state 3 oxidation (Fig. 2). At lower glyceollin concentrations where state 3 malate oxidation was not completely inhibited to state 4, the addition of ADP stimulated $O_2$ uptake, but to a rate intermediate between the known state 3 and state 4 rates. Measurements of ADP/O ratios in these experiments resulted in values similar to those obtained in the absence of glyceollin (Boydston, unpublished results), and indicate that glyceollin did not uncouple phosphorylation from electron transport.

Virtually no inhibition of succinate or exogenous NADH state 3 oxidation was obtained with glyceollin or rotenone (Table I). Other reports have suggested that the slight inhibitory effects of rotenone may be due to nonspecific inhibitor binding (19, 21). Both compounds appear, in our experiments, to be specific effectors of the NADH-linked malate oxidation at lower concentrations.

When OAA removal is enhanced during malate oxidation, the

---

Abbreviations: CCCP, carbonylcyanide $m$-chlorophenyl hydrazone; FCCP, $p$- trifluormethoxy(carbonylcyanide)-phenylhydrazone; OAA, oxaloacetate.

---

Fig. 1. Determination of apparent $K_i$ values of glyceollin or rotenone on 10 mM malate state 3 oxidation in washed and gradient purified soybean mitochondria. Mitochondria were placed in state 3 by the addition of 1.2 $\mu$mol ADP; glyceollin was added after 1 min. A, Washed; $K_i = 15.1$, $r = 0.986$. B, Purified; $K_i = 14.9$, $r = 0.951$. C, Washed; $K_i = 4.6$, $r = 0.981$. D, Purified; $K_i = 6.6$, $r = 0.828$. 
GLYCEOLLIN INHIBITION OF ELECTRON TRANSPORT

Fig. 2. Comparison of the effects of rotenone, glyceollin, and oligomycin on malate (10 mM) oxidation of isolated corn mitochondria. Additions or final concentrations in the reaction media were: 1.2 μmol ADP, 20 μM rotenone, 15 μM glyceollin, 6 μM oligomycin, or 10 μM CCCP. Standard reaction media were used in all experiments (see Materials and Methods).

Table I. Inhibitory Effects of Glyceollin or Rotenone on State 3 Oxidation of Isolated Soybean Hypocotyl Mitochondria

Reaction media were as presented in "Materials and Methods." State 3 rates resulted from the addition of 1.2 μmol ADP following substrate addition. Inhibitors were added after mitochondria were oxidizing substrate for 1 min in state 3.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>State 3</th>
<th>+Inhibitor</th>
<th>Inhibition</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Malate</td>
<td>97</td>
<td>21</td>
<td>78</td>
<td>40 μM glyceollin</td>
</tr>
<tr>
<td>0.5 mM NADH</td>
<td>251</td>
<td>235</td>
<td>6</td>
<td>40 μM glyceollin</td>
</tr>
<tr>
<td>0.5 mM Succinate</td>
<td>182</td>
<td>174</td>
<td>4</td>
<td>40 μM glyceollin</td>
</tr>
<tr>
<td>10 mM Malate</td>
<td>93</td>
<td>16</td>
<td>83</td>
<td>10 μM rotenone</td>
</tr>
<tr>
<td>0.5 mM NADH</td>
<td>246</td>
<td>246</td>
<td>0</td>
<td>10 μM rotenone</td>
</tr>
<tr>
<td>0.5 mM Succinate</td>
<td>185</td>
<td>176</td>
<td>5</td>
<td>10 μM rotenone</td>
</tr>
</tbody>
</table>

The inhibitory effects of rotenone are less dramatic (16, 25). When glutamate was added in our experiments, the inhibitory effects of glyceollin or rotenone were reduced. The addition of glutamate after that of glyceollin or rotenone increased the inhibited state 3 oxidation rate of malate (Fig. 3). This indicates that the removal of OAA, probably by transamination, reduces the effective concentrations of the two inhibitors. Wiskich and Day (25) concluded that rotenone inhibition of malate oxidation was related to factors affecting the equilibrium poise of malate dehydrogenase. Our results indicate that this also appears to be the case with glyceollin.

State 4 Inhibition. The CCCP-uncoupled malate state 4 oxidation was inhibited by both glyceollin and rotenone (Table II), providing further evidence that these inhibitors act directly on electron transport and not as phosphorylation inhibitors. Uncoupled succinate state 4 oxidation also was inhibited 25% by glyceollin or rotenone at concentrations of 20 and 10 μM, respectively (Table II). Little or no inhibition of uncoupled exogenous NADH state 4 oxidation was observed (Table II). Glyceollin did not inhibit the rate of malate, exogenous NADH, or succinate state 4 oxidation. Likewise, rotenone had little effect on the rate of exogenous NADH or succinate state 4 oxidation, but did inhibit the rate of malate state 4 oxidation.

Inhibition of Ferricyanide Reduction. In experiments to further establish the site of glyceollin inhibition, ferricyanide was used as the terminal electron acceptor, as described by Klingenberg (9). He explains that ferricyanide accepts electrons from Cyt c if cyanide is added to eliminate terminal electron transfer to oxygen. When malate or succinate oxidation is linked to ferricyanide reduction, complete inhibition by antimycin A is achieved, as it specifically inhibits electron transfer between Cyt b and Cyt c. Glyceollin-inhibited malate oxidation linked to ferricyanide (apparent $K_i = 5.4 μM; r = 0.882$), but had no effect on the succinate-ferricyanide reduction rate (Table III). Succinate donates electrons through flavin adenine dinucleotide to coenzyme Q, therefore bypassing the rotenone inhibitory site at complex I of the electron transport chain (16). Inasmuch as malate, but not succinate, oxidation was inhibited by glyceollin, the site of glyceollin inhib-
similar or identical to rotenone. Results were consistent from day to day between numerous mitochondrial preparations, and among the various tissues from which mitochondria were isolated. Glyceollin specifically inhibited malate oxidation, but not that of other substrates which affect electron flow into the electron transport chain by-passing the site I coupling site. The oxidation rates of exogenous NADH or succinate were generally not affected by the glyceollin concentrations used in our research. Kaplan et al. (6) have suggested, however, that glyceollin acts at some point on the O₂ side of succinate dehydrogenase in electron transport. Those authors did not demonstrate any substrate differentiation as we have in the present study.

In vivo local concentrations of glyceollin near invading hyphae have been reported to be 100-fold higher than the apparent glyceollin Kᵣ of 15 μM determined by our studies (26). These observations support a hypothesis that the effect of glyceollin on substrate oxidation of mitochondria, both in the pathogen and in the host, may be an important factor in the expression of soybean resistance to pathogen invasion. The inhibition of nematode respiration by glyceollin has been demonstrated (6), and we suspect that some of the physiological and biochemical mechanistic responses to glyceollin may be similar in both host and parasite. Through an inhibition of the mitochondrial electron transport system of invading pathogen and host cells, in the localized area of infection, glyceollin could inhibit further invasion of the microbe into surrounding host tissue. Whether the inhibition of mitochondrial electron transport is the major mode of glyceollin action in situ has not been shown in our experiments, but based on the low apparent Kᵣ of 15 μM for malate state 3 oxidation, it is likely that the effect of glyceollin on mitochondrial electron transport could play a role in preventing the spread of a pathogen in host tissue.

Phytoalexins are produced in many plant tissues in response to pathogen infection. Several phytoalexins including phasellin, rishitin, and pisatin apparently disrupt various cellular membranes (2–4, 12–14, 18, 23). Phasellin and rishitin have been reported to inhibit respiration of whole tissue (12, 14, 20, 23). Ipomeamarone, a sequiterpene phytoalexin, produced by sweet potato roots, inhibits substrate oxidation and concomitant phosphorylation in rat liver mitochondria (22). Kaempferol, a flavonoid found in higher plants, inhibits mitochondrial electron flow (17) and phosphorylation (10). Other isoflavonoid phytoalexins (e.g. coumestrol and daidzen) associated with the hypersensitive response in soybeans (8) are currently being studied for their effects on mitochondrial substrate oxidation.

We therefore believe that our experiments indicate a glyceollin-specific site in the mitochondrial electron transport chain associated with the inner membrane. We cannot rule out the possibility that glyceollin also binds to and influences other membranes and/or enzymes. In vivo experiments are currently underway to further assess the nature of the glyceollin-mitochondrial membrane interaction. If this interaction is unique and specific, it represents the first observation with plant material of such a specific phytoalexin effect.

**Acknowledgments**—The authors gratefully acknowledge the donation of soybean seed from the Illinois Foundation Seed Company, Tolono, IL and corn seed from Crow Seed Company, Milford, IL. Extensive discussions of the research with Prof. Robert Klein were of great value in developing the thrust of the research. Roberta Steward is acknowledged for her laboratory maintenance and assistance.

**LITERATURE CITED**


**DISCUSSION**

Data presented here strongly suggest that glyceollin acts as a site I inhibitor of mitochondrial electron transport in a manner

---

**Table III. Effect of Glyceollin on Substrate-Ferricyanide Reduction by Isolated Soybean Hypocotyl Mitochondria after Purification on Sucrose Density Gradients**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Initial Rate</th>
<th>Glyceollin-</th>
<th>Inhibition</th>
<th>Glyceollin Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>109</td>
<td>109</td>
<td>0</td>
<td>0.04 ml ethanol control</td>
</tr>
<tr>
<td>Succinate</td>
<td>130</td>
<td>104</td>
<td>20</td>
<td>5 μM</td>
</tr>
<tr>
<td>Succinate</td>
<td>133</td>
<td>78</td>
<td>41</td>
<td>10 μM</td>
</tr>
<tr>
<td>Malate</td>
<td>127</td>
<td>45</td>
<td>65</td>
<td>20 μM</td>
</tr>
<tr>
<td>Malate</td>
<td>136</td>
<td>9</td>
<td>93</td>
<td>40 μM</td>
</tr>
<tr>
<td>Malate</td>
<td>304</td>
<td>363</td>
<td>0</td>
<td>40 μM</td>
</tr>
<tr>
<td>Malate</td>
<td>1793</td>
<td>1818</td>
<td>0</td>
<td>20 μM</td>
</tr>
</tbody>
</table>

**Table IV. Effects of Glyceollin on State 3 Oxidation by Mitochondria Isolated from Different Tissues**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glyceollin Conc.</th>
<th>Inhibition of State 3 Oxidation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>0.5 mm NADH</td>
<td>40  13  7  6</td>
</tr>
<tr>
<td>Succinate</td>
<td>10 mm Succinate</td>
<td>40  0  0  4</td>
</tr>
<tr>
<td>Malate</td>
<td>10 mm Malate</td>
<td>40  79  74  78</td>
</tr>
<tr>
<td>Malate</td>
<td>10 mm Malate</td>
<td>15  68  53  61</td>
</tr>
</tbody>
</table>

---

All reactions were as described in "Materials and Methods." Initial rates were determined 1 min after addition of the substrate. Measurable ferricyanide reduction rates were obtained with 0.3 to 0.4 mg mitochondrial protein with malate or succinate as substrates, and ~0.1 mg mitochondrial protein was used with exogenous NADH as the substrate.
GLYCEOLLIN INHIBITION OF ELECTRON TRANSPORT

and surfactants on zoospores of Oomycetes. Physiol Plant Pathol 11: 163-169
7. KEEN NT 1971 Hydroxyphaseollin production by soybeans resistant and susceptible to Phytophthora megasperma var. sojae. Physiol Plant Pathol 1: 265-275
14. LYON GD 1980 Evidence that the toxic effect of rishitin may be due to membrane damage. J Exp Bot 31: 957-966