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

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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.

Glyceollin, a phytoalexin produced during the hypersensitive response in soybeans, is hypothesized to be responsible for the expression of cultivar-specific resistance of soybeans to Phytophthora megasperma var sojae (7, 26), and its accumulation has been correlated with the incompatible response of soybeans to Meloidogyne incognita (5), and Pseudomonos glycinea (8). Glyceollin inhibits fungal and nematode growth in vitro at concentrations similar to localized concentrations found in vivo (6, 26).

Inhibition of nematode respiration and electron transport from several substrates to O2 in isolated soybean mitochondria by glyceollin has been reported by Kaplan, Keen, and Thomason (6), but the specific site of inhibition by glyceollin was not determined. We thus began our study with the a priori assumption that glyceollin could play a primary role in plant-affected pathogen toxicity, and autophtotoxicity through effects on mitochondrial electron transport. The experiments reported here deal only with the autophtotoxicity of glyceollin on electron transport of mitochondria isolated from etiolated soybean hypocotyls and cotyledons and corn shoots. All studies were designed to determine the unique site of glyceollin effect on electron transport and utilized various substrates, electron acceptors, and known inhibitors.

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 CaCl2 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 CaCl2 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 (Chiopece Mills, Inc., Milltown, NJ) before the first centrifugation.

Purification of washed mitochondria consisted of resuspending washed mitochondria in 1 ml of standard resusension medium consisting of 0.3 mM mannitol, 20 mM Hepes, 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 60,000 g 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 KH2PO4, 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 14,600 g 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.

O2 uptake experiments were measured polarographically in a 4.0-ml cell (28°C), fitted with a Clark O2 electrode (Yellow Springs Instrument Co., Yellow Springs, OH).

The mitochondrial reaction media consisted of 0.3 mM mannitol, 10 mM Tris-HCl (pH 7.2), 5 mM MgCl2, 10 mM KCl, 4 mM KH2PO4, 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 media (excluding BSA) containing 20 mM KCl. Mitochondria, followed by additions to give 1 mM KCN and 1 mM K3Fe(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.
RESULTS

State 3 Inhibition. Mitochondria oxidizing malate in state 3 were inhibited by glyceollin with an apparent $K_i$ of 15 μM (Fig. 1, A and B). The inhibitory effect of rotenone under similar conditions resulted in an apparent $K_i$ of 5 μM (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

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2 Abbreviations: CCCP, carbonylcyanide $m$-chlorophenyl hydrzone; FCCP, $p$-trifluormethoxy(carbonylcyanide)-phenylhydrazone; OAA, oxaloacetate.
inhibitory effects of rotenone are less dramatic (16, 25). When glutamate was added in our experiments, the inhibitory effects of glycine or rotenone were reduced. The addition of glutamate after that of glycine 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 poised of malate dehydrogenase. Our results indicate that this also appears to be the case with glycine.

State 4 Inhibition. The CCCP-uncoupled malate state 4 oxidation was inhibited by both glycine 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 glycine 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). Glycine 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 glycine 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. Glycine-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 glycine, the site of glycine inhibi-
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 O$_2$ 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 $K_i$ of 15 $\mu M$ 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 $K_i$ of 15 $\mu M$ 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 phaseollin, rishitin, and pisatin apparently disrupt various cellular membranes (2–4, 12–14, 18, 23). Phaseollin and rishitin have been reported to inhibit respiration of whole tissue (12, 14, 20, 23). Ipomeamorein, 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 initial observation with plant material of such a specific phytoalexin effect.

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