Inhibition of a Photosystem II Electron Transfer Reaction by the Natural Product Sorgoleone

Veronica Miranda Gonzalez,[†,‡] Janet Kazimir,[†] Chandrashekhar Nimbal,[‡] Leslie A. Weston,*[,‡] and G. M. Cheniae[†]

The Agronomy Department and The Horticulture and Landscape Architecture Department, University of Kentucky, Lexington, Kentucky 40546-0091

Effects of the alleochemical sorgoleone on photosynthetic electron transport by oxygen-evolving chloroplast thylakoids and Triton X-100-prepared Photosystem II (PSII) membranes were analyzed. The Hill activity of the thylakoids proved to be at least as sensitive to inhibition by sorgoleone as it was to DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], a potent herbicidal inhibitor of PSII. However, a Photosystem I (PSI) partial reaction was not affected by a 10-fold greater concentration of sorgoleone than is required for complete inhibition of Hill activity. Measurements of flash-induced chlorophyll *a* variable fluorescence showed that sorgoleone neither dissipated excitation energy nor diminished the amplitude of chlorophyll *a* variable fluorescence. However, it inhibited the decay of variable fluorescence as effectively as DCMU, which blocks the oxidation of the PSII-reduced primary electron acceptor, Q^-_A, by the PSII secondary electron acceptor, Q_B, by displacing Q_B from the D_1 protein. Additionally, sorgoleone competitively inhibited the binding of [^{14}C]atrazine to the Q_B locus. Increasing durations of trypsin proteolysis of the PSII membranes or thylakoids and of the Q_B-binding niche itself caused parallel losses of inhibition of O_2 evolution from sorgoleone and DCMU, as well as from bromoxynil, a phenol-type herbicide also binding to the Q_B locus.

Keywords: *Allelopathy; sorgoleone; photosystem II; electron transfer; herbicide*

INTRODUCTION

Investigations suggest that the allelopathic traits of crops can be used in agroecosystems to aid weed suppression. Identification of cover crops which suppress weeds effectively may increase the acceptance of soil conserving, no-tillage production techniques. Cover crops are typically utilized in no-tillage production because they provide a surface residue which may decrease soil erosion and increase water retention. Field studies on the allelopathic potential of *Sorghum bicolor* L. Moench showed that prior cropping with grain sorghum suppressed weed populations (Einhellig and Rasmussen, 1989).

Prior studies have shown that the exudates from sorghum roots contain compounds that exhibit allelochemical activity (Abdul-Wahab and Rice, 1967; Brown and Edwards, 1944; Fletcher, 1912; Lehle and Putman, 1983; Pope et al., 1985; Guenzi and McCalla, 1962, 1966; Weston et al., 1989). Biologically active compounds such as chlorogenic, syringic, ferulic, and vanillic acids have been identified from aqueous washings of sorghum root exudates (Abdul-Wahab and Rice, 1967; Guenzi and McCalla, 1962, 1966). At relatively high concentrations, such compounds are known to be nonspecific plant growth inhibitors. Moreover, they occur in the root exudates of many other plants (Putnam, 1985). It thus seems unlikely that such chemicals contribute to the species-specific biological activities found for sorghum (Abdul-Wahab and Rice, 1967; Brown and Edwards, 1944; Fletcher, 1912; Lehle and Putnam, 1983; Pope et al., 1985; Weston et al., 1989).

More recently, studies have been made of the effect(s) of the hydrophobic exudate from root hairs of sorghum. Such studies revealed that the hydrophobic exudate contained compound(s) which strongly inhibited lettuce (*Lactuca sativa* L.) root elongation but not corn (*Zea mays* L.) root elongation, suggesting that this exudate fraction contains species-specific biological inhibitors (Netzly and Butler, 1986; Netzly et al., 1988). Subsequent work showed that most (>90%) of the compounds in the hydrophobic exudate were substituted *p*-benzoquinones with masses of 358−362 Da. The major *p*-benzoquinone species with a mass of 358 Da was identified as 2-hydroxy-5-methoxy-3-[(8*Z*,11*Z*)-8,11,14-pentadecatriene]-*p*-benzoquinone, which was given the common name sorgoleone. The reduced dihydroquinone species of sorgoleone proved to be a potent germination stimulant of witchweed (*Striga asiatica* L. Ktze.) (Chang et al., 1986; Fate et al., 1990); however, the reduced species was rapidly autooxidized and, in its rather stable oxidized form, was inactive in stimulating witchweed germination.

On the other hand, experiments done with the unpurified hydrophobic exudate showed the following: (1) ∼50 and ∼100% inhibition of *Eragrostis tef* radical elongation by 125 and 250 μM sorgoleone, respectively; (2) a stunting of the growth of *Lemna minor* by 50 μM sorgoleone and a decrease (∼11%) of its chlorophyll (Chl) abundance by a 100 μM concentration; and (3) a suppression of growth over a 10-day period of several different broadleaf and grass weed seedlings at a concentration of only 10 μM (Einhellig and Souza, 1992; Nimbal et al., 1996a). Such inhibitory effects by sorgoleone at these low concentrations suggest that sorgoleone may be a major contributor to sorghum allelopathy.

Few biochemical insights are available as to how sorgoleone may contribute to sorghum allelopathy. According to Rasumussen et al. (1992), sorgoleone blocks mitochondrial electron transport by inhibiting the reduction of cytochrome c_1 by cytochrome *b*, a site of

† The Agronomy Department.
‡ The Horticulture and Landscape Architecture Department.
* To whom correspondence should be addressed.

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inhibition of several hydroxyquinone analogues (von Jagow and Link, 1986). Corn and soybean (*Glycine max* L. Kerr.) mitochondria showed essentially equivalent sensitivity to inhibition by a range of sorgoleone concentrations. Experiments addressing the possible effects of sorgoleone on photosynthesis revealed that 10 μM sorgoleone inhibited photosynthesis by soybean leaf disks by ~50%. Only ~0.2 μM sorgoleone was sufficient for 50% inhibition of photosynthesis by intact pea chloroplasts (Einhellig et al., 1993). Nimbal et al. (1996a) have reported that sorgoleone inhibited photosynthetic oxygen evolution in potato (*Solanum tuberosum* L.) and common groundsel (*Senecio vulgaris* L.). Most recently, Nimbal et al. (1996b), reported the results of studies with triazine-susceptible potato and redroot pigweed (*Amaranth retroflexus* L.) thylakoids which suggested that sorgoleone was a competitive inhibitor of atrazine binding. Here we report the further results of experiments directed toward determining the site(s) of action of sorgoleone causing inhibition of photosynthesis. (The data published in Nimbal et al., 1996b, suggesting that sorgoleone was a competitive inhibitor of atrazine binding, were generated long after the collection of data described in this manuscript. The data presented in this manuscript, with the exception of Figures 1 and 4, were collected by Veronica Miranda Gonzalez and Janet Kazimir under the supervision of Dr. George Cheniae. Data collected in Figures 1 and 4 were generated by C. I. Nimbal and L. A. Weston.)

MATERIALS AND METHODS

Isolation and Purification of Sorgoleone. Sorgoleone was isolated and purified by procedures outlined by Netzly et al. (1988) with the following exceptions: (1) *Sorghum bicolor* (cv. Pioneer 8333) seed (25 per sterile petri dish lined with moistened filter paper) were germinated at 29 °C; (2) after 5 days, roots were excised, then briefly (~20 s) dipped into CH_2Cl_2 containing 1% acetic acid; and (3) the resulting crude extract was evaporated to dryness at room temperature under a stream of N_2. Subsequently, the crude dried extract was solubilized in acetonitrile and subjected to high-pressure liquid chromatography (HPLC) (Novapak C-18 column, 3.9 × 150 mm^2) using a solvent system mixture of acetonitrile:acidified water (75:25 v/v). The acidified water was milli-Q-water: glacial acetic acid (97.5:2.5 v/v). The eluate containing sorgoleone (MW 358) was collected, evaporated to dryness under a stream of N_2, dissolved in 95% ethanol, and stored at −15 °C.

Preparation of Oxygen-Evolving Thylakoids and PSII Membranes. Thylakoids from leaves of 9−11 day old wheat seedlings or from leaves of locally grown spinach (*Spinacea oleracea* L.) were isolated essentially as described by Callahan and Cheniae (1985). The thylakoids were resuspended (>3 mg of Chl/mL) in SHM buffer (0.35 M sucrose/25 mM Hepes-NaOH/2 mM $MgCl_2$, pH 7.5), then used immediately or stored at −80 °C for ≤1 week before use. PSII membranes (Berthold et al., 1981), with rates of O_2 evolution in excess of 700 μmol of O_2 (mg of Chl·h)$^{-1}$ were prepared essentially as described by Radmer et al. (1986) and stored at −80 °C before use.

Activity Assays. O_2 evolution and PSI donor photooxidation were measured polarographically in saturating light at 23 °C. Light from a Dolan-Jenner high-intensity illuminator (Series 180) was filtered by two Schott KG-1 filters, one Corion FR-400-S filter, and one Corning 2-63 filter and focused on the polarograph vessel. Oxygen evolution reaction mixtures for thylakoids and PSII membranes contained 0.4 M sucrose/40 mM Tricine-NaOH/1 mM FeCN/30 mM methylamine, pH 7.5, and 0.8 M sucrose/50 mM Mes-NaOH/15 mM $CaCl_2$/1 mM FeCN/300 μM PBQ, pH 6.2, respectively. PSI donor photooxidation activity of thylakoids was measured as oxygen consumption using a reaction mixture containing 0.4 M sucrose/50 mM tricine-NaOH/15 mM NaCl/100 μM methylviologen/400 μM sodium azide/10 μM DCMU, pH 7.5, with 50 μM $DCIPH_2$ (present as 50 μM DCIP and 10 mM sodium ascorbate) as a PSI electron donor. The Chl concentration was 10 μg/mL in all assay mixtures.

Chlorophyll *a* Fluorescence. Flash-induced Chl *a* fluorescence and its decay was measured using a pulse-modulated fluorometer (Walz Co., Effeltrich, Germany) described in detail by Schrieber et al. (1986). The weak, noninductive measuring light (λ = 660 nm) was modulated at a frequency of 100 kHz for 30 ms, beginning 2 ms before the actinic flash, provided by a Walz XST-103 xenon flash lamp (8 μs flash width). Fluorescence detection began 120 μs after the flash, and the signal captured on a digital oscilloscope then transferred to an electronic plotter. PSII membranes, 200 μg of Chl/mL in 0.4 M sucrose/50 mM Mes-NaOH/15 mM NaCl, pH 6.5, were dark adapted for >30 min at 4 °C, then 0.75 mL of the suspension was added in near darkness to the cuvette (Model KS 101). The adaptor and the FL 101 fiber illuminator were then added to the cuvette. After a 5 min incubation in total darkness at room temperature, any addition to the suspension was made through the cuvette's needle port with the use of a Hamilton syringe. The suspension was briefly stirred then incubated for 5 min more in total darkness before measuring flash-induced Chl *a* fluorescence and its decay.

Trypsin Treatment of Thylakoids and PSII Membranes. Trypsin treatment of wheat thylakoids and spinach PSII membranes was done at pH 6.5 to minimize proteolysis of peptides essential for O_2 evolution and to maximize proteolysis of the D_1-peptide domain on the acceptor side of PSII that contains the binding site for Q_B and for DCMU-type herbicides (Renger et al., 1976, 1981; Völker et al., 1985). Freshly prepared wheat thylakoids and spinach PSII membranes that had been frozen (−80 °C) were washed (~250 μg of Chl/mL) twice and once, respectively, with buffer A (0.4 M sucrose/50 mM Mes-NaOH/15 mM NaCl, pH 6.5) and resuspended in this buffer. Trypsin treatment of the thylakoids or the PSII membranes was carried out by incubation (100 μg of Chl/mL in buffer A) for times indicated with TPCK-treated bovine pancreas trypsin (Sigma Chemical Co.) at 50 μg/mL (500 BAEE units/mL). Trypsinization was stopped by rapid addition and mixing of 2.5 volumes of buffer A containing 80 μg/mL of type 1-S trypsin inhibitor from soybean (Sigma Chemical Co.) per volume of trypsin digestion mixture. Subsequently, the thylakoids or PSII membranes were pelleted (27000*g*/10 min), washed twice (~100 μg of Chl/mL), and resuspended with buffer A containing 5 μg/mL of the trypsin inhibitor. In the presence of the trypsin inhibitor, the initial rate of O_2 evolution by the trypsinized thylakoids or the PSII membranes remained constant for at least 2−3 h.

Other Procedures. Concentration of chlorophyll was determined as described by MacKinney (1941). Atrazine binding to thylakoids was analyzed essentially as outlined by Tischer and Strotmann (1977) and Pfister et al. (1979). The ^{14}C-ring labeled atrazine (Sigma Chemical Co.) had a specific activity of 17.7 mCi/mmol. Measurements of radioactivity were made using LSC (Tri-Carb Model 2200 CA, Packard Instrument Co.).

RESULTS AND DISCUSSION

Purity of Sorgoleone and Its Effect on the Hill Reaction. Figure 1 shows a HPLC chromatogram of sorgoleone (MW = 358) that was purified by following the isolation procedure described by Netzly et al. (1988). Only one major symmetrical peak is observed. After subsequent examination using GC−MS and ^{1}H NMR analysis (data not presented), this purified sorgoleone-358 fraction proved free of sorgoleone species having molecular weights of >358 and also of an unidentified chemically unstable component occurring in the crude CH_2Cl_2-soluble fraction of the hydrophobic exudate of sorghum seedling roots (Netzly et al., 1988). The inset of Figure 1 shows the UV-absorbance spectrum of

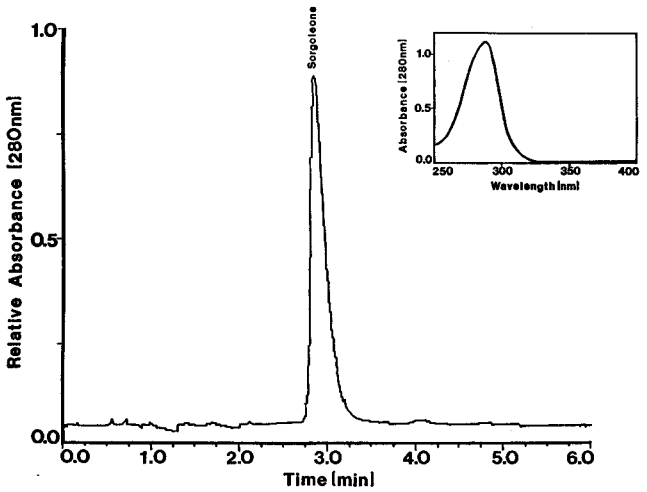

Figure 1. HPLC chromatogram of sorgoleone previously purified by HPLC chromatography (Materials and Methods). Inset: UV-absorbance spectrum of purified sorgoleone.

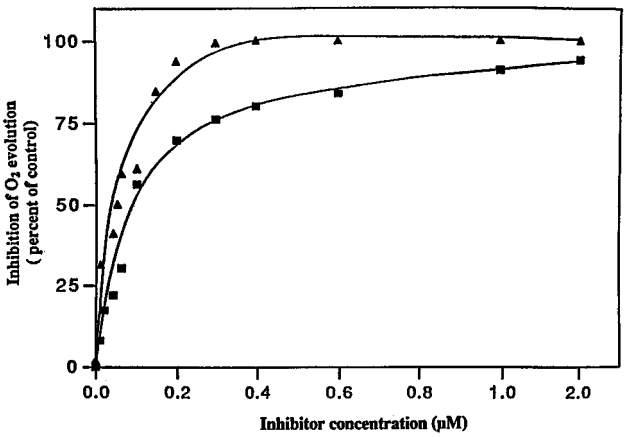

Figure 2. DCMU and sorgoleone concentration dependence for inhibition of FeCN supported O_2 evolution by wheat chloroplast thylakoids. The closed triangles and closed square data were obtained with purified sorgoleone and DCMU, respectively.

purified sorgoleone-358. This spectrum is essentially equivalent to the spectrum of oxidized purified sorgoleone-358 previously reported (Netzly et al., 1988). These two considerations lead us to believe that the sorgoleone used in the following experiments was essentially pure oxidized sorgoleone-358. Using an unpurified, CH_2Cl_2-soluble fraction of sorghum root exudate, Einhellig et al. (1993) determined that only ~0.2 and 4.5 μM unpurified sorgoleone was sufficient to give 50 and 100% inhibition, respectively, of photosynthesis measured with intact pea chloroplasts. These studies, however, offered no clues regarding the specific photosynthetic reaction(s) inhibited by the crude sorgoleone extract.

Figure 2 shows typical results from experiments measuring the effect(s) of purified sorgoleone on the rates of O_2 evolution by broken wheat chloroplasts with FeCN as the electron acceptor. In these experiments, 30 mM methylamine was included in the reaction mixtures to dissipate any light-induced electromotive force and thus permit measurements of maximum rates of water oxidation coupled to FeCN reduction. Increasing concentrations of sorgoleone resulted in increasing inhibition of O_2 evolution (closed triangles). Only 0.093 and 0.6 μM sorgoleone were required to give 50% and nearly 100% inhibition of the Hill reaction, respectively. These values are significantly less than those reported

Table 1. Effect of Sorgoleone in Photosystem I-Mediated Oxidation of Ascorbate/DCIP

sorgoleone concentration (μM)	rate of ascorbate/ DCIP photooxidation (μequiv mg of Chl^{-1} h^{-1})	% inhibition
0.0	1749.0	0.0
0.3	1749.0	0.0
0.6	1711.0	2.2
1.8	1903.0	−8.8
6.0	1617.0	7.6

by Einhellig et al. (1993) for comparable inhibitions of photosynthesis by intact pea chloroplasts. The inhibition obtained with increasing concentrations of DCMU (diuron), a potent herbicidal inhibitor of PSII, is also shown in Figure 2 (closed squares). Inhibitions of 50 and ~100% were observed at DCMU concentrations of 0.12 and ~2 μM, respectively. This comparison shows that sorgoleone is at least as effective as DCMU for blocking the FeCN-supported Hill reaction. An entirely similar conclusion was reached when the autooxidizable low-potential electron acceptor, methylviologen, was used as a Hill-reaction acceptor (data not shown). Such results indicate that the previously reported inhibition of photosynthesis by unpurified sorgoleone (Einhellig et al., 1993) can be attributed to inhibition(s) of electron-transfer reaction(s) at undetermined sites within the electron transfer "chain" in chloroplasts.

Effect of Sorgoleone on a PSI Partial Reaction. Thermodynamically, the electron acceptor, FeCN (ϵ_O = +0.42 V), used in the Hill-reaction experiments has the capacity to drain off electrons at the Q_A/Q_B locus on the reducing side of PSII. However, the Q_A/Q_B locus is essentially inaccessible to this highly polar oxidant, except in some subchloroplast membranes (see Izawa (1980) for a review) and in PSII particles isolated from PSII membranes using specific detergents (Ikeuchi and Inoue, 1986). Thus, FeCN acts more as a PSI electron acceptor than as a PSII electron acceptor in the chloroplast thylakoids used in the Hill reaction experiments of Figure 2. In efforts to define the site(s) of inhibition by sorgoleone, we measured the effect(s) of sorgoleone on an electron transfer reaction catalyzed only by PSI.

Rates of photooxidation of $DCIPH_2$, an electron donor to cytochrome *f*/plastocyanin (Izawa, 1980), were measured using methylviologen as an electron acceptor and DCMU to block electron transfers from PSII (Table 1). As shown, high rates of $DCIPH_2$ photooxidation were observed in the absence of sorgoleone and the additions of sorgoleone had no inhibitory effect, with the possible exception of 6 μM sorgoleone causing only 7.6% inhibition. This concentration is 10-fold greater than the concentrations of sorgoleone required for nearly complete inhibition of the Hill reaction (Figure 2). We conclude that sorgoleone causes no significant inhibition of electron transfers from cytochrome *f*/plastocyanin to P700 and from P700 to the low-potential Fe−S electron acceptor(s) of PSI.

Effects on Flash-Induced Chl *a* Variable Fluorescence. Light absorption by the PSII reaction center results in charge separation ($P680^+/Q_A^-$) which normally is rapidly stabilized by the reduction of $P680^+$ by Tyr_Z and the oxidation of Q_A^- by Q_B. Any impairment of the reduction of $P680^+$ results in a diminished amplitude of fluorescence, due to quenching by $P680^+$ and rapid $P680^+/Q_A^-$ charge separation; thus, the yield of flash-induced variable Chl *a* fluorescence is maximum in the $P680/Q_A^-$ state. Without impairment of the reduction of $P680^+$, the relative amplitude of the vari-

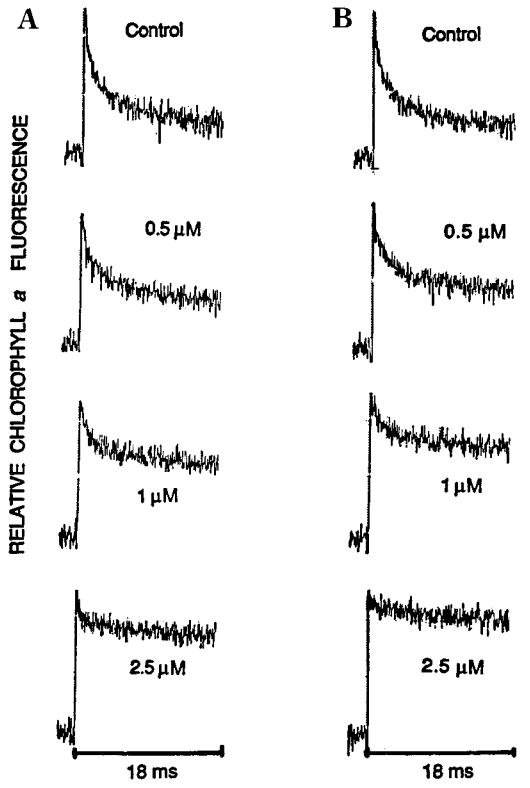

Figure 3. Effects of sorgoleone and DCMU on the amplitude and decay of flash-induced Chl *a* variable fluorescence. Measurements were made using spinach PSII membranes. Panels A and B were recorded using sorgoleone and DCMU, respectively, at their indicated concentrations.

able fluorescence is an indicator of $[Q_A^-]$ formed and its decay reveals the kinetics of Q_A^- reoxidation. Generally, Q_A^- is reoxidized by Q_B with a half-time of 200−300 μs in thylakoids and green algae; however, in BBY-type PSII membranes, this half-time is slower (for a review, see van Gorkom (1986)). Accordingly, measurements of flash-induced Chl *a* variable fluorescence and subsequent fluorescence decay can give clues as to the site(s) of inhibition in PSII by compounds affecting PSII electron transfers.

Panel A of Figure 3 shows the traces of flash-induced Chl *a* variable fluorescence obtained with PSII membranes in the presence of the indicated concentrations of purified sorgoleone. The traces shown in panel B were obtained in a similar manner in the presence of increasing concentrations of DCMU, a herbicide specifically inhibiting the oxidation of Q_A^- by Q_B by displacing PQ from the Q_B site (Velthuys, 1981). An inspection of the traces of panel A reveals that the amplitude of variable fluorescence was not affected by increasing concentrations of purified sorgoleone. This result indicates that sorgoleone caused no impairment of the reduction of $P680^+$ by $TyrY_Z$. Moreover, these concentrations of sorgoleone ($\leq$2.5 μM) did not dissipate excitation energy as is sometimes caused by quinones (Amesz and Fork, 1967); thus, despite the lipophilic property of this substituted *p*-benzoquinone (sorgoleone), it seems that bulk Chl and sorgoleone molecules were not closely associated.

Second, a comparison of the traces obtained with increasing concentrations of sorgoleone (panel A) with those obtained with DCMU (panel B) shows that the kinetics of Q_A^- reoxidation were slowed by sorgoleone in a concentration-dependent manner similar to that of DCMU. Under the conditions employed (200 μg of Chl/mL), essentially complete inhibition of Q_A^- reoxidation was obtained with either 2.5 μM sorgoleone or DCMU. Clearly, these results identify the Q_A/Q_B locus as at least one site of inhibition of electron transfer by sorgoleone. This result/conclusion was somewhat surprising for the following reasons: (1) both sorgoleone and PQ are substituted 1,4-benzoquinones, each with a side chain serving to promote lipophilicity; (2) 1,4-benzoquinone and many substituted 1,4-benzoquinones ($\leq$ 300 μM) act as effective exogenous electron acceptors at the Q_B locus (Izawa, 1980); but (3) sorgoleone at $\leq$300 μM apparently acts strictly as an inhibitor of the oxidation of Q_A^-.

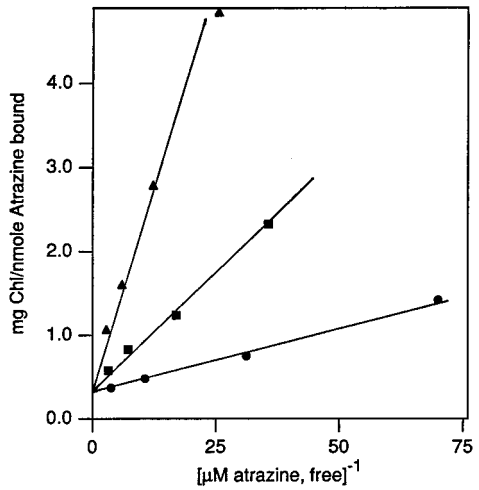

Figure 4. Analysis of the effects of sorgoleone on the binding of [^{14}C]atrazine by wheat chloroplast thylakoids. The closed circle data were obtained without additions of sorgoleone, and the closed square and closed triangle data were obtained with addition of 0.25 and 0.5 μM purified sorgoleone, respectively.

Analysis of Sorgoleone Binding. Two different classes of herbicides have been recognized to inhibit PSII by interfering with Q_B function. The first class consists of a number of chemically different compounds (e.g., DCMU and atrazine), all having a common structural feature, N − C = X, where X indicates N or O. The second class is comprised entirely of phenols which have a nitro and/or halogen and/or nitrile substituent in addition to the phenolic hydroxy group. Whereas compounds within a given class exhibit competitive binding with each other, compounds from the two different classes show noncompetitive binding with each other (Oettmeier et al., 1982; Draber et al., 1991).

The experiments summarized in Figure 4 were made to verify the tentative conclusion reached in the preceding section and to establish if sorgoleone behaved in competition binding studies as a DCMU/atrazine-type inhibitor or as a phenol-type inhibitor. Accordingly, [^{14}C]atrazine binding by thylakoids was measured in the absence and the presence of 0.25 and 0.5 μM sorgoleone. The double-reciprocal plot shown permits the distinction between sorgoleone/atrazine competitive binding versus noncompetitive binding of molecules at the Q_B locus (Tisher and Strotmann, 1977). As shown, the ordinate intercepts of the three regression lines obtained using zero, 0.25 , and 0.50 μM purified sorgoleone proved equivalent, clearly indicating competitive binding of sorgoleone with atrazine at the Q^-_B binding pocket of the D_1 protein.

Effects of Trypsinization on Inhibition by Sorgoleone. Photoaffinity labeling procedures and mutagenesis approaches have led to the identification of D_1 amino acid residues that contribute to the binding of Q_B and the herbicides that bind at the Q_B site. These

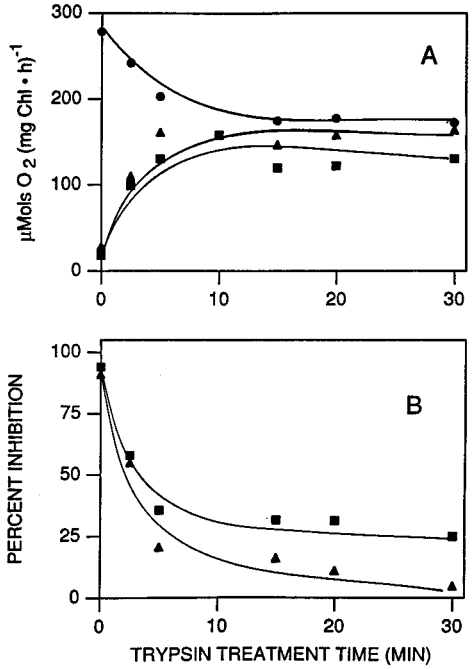

Figure 5. (A) Effects of trypsin treatment of wheat chloroplast thylakoids on rates of oxygen evolution and the susceptibility to inhibition by DCMU and sorgoleone. The rates shown by the closed circles, triangles, and squares were obtained with no addition, additions of 1 μM DCMU, and 1 μM purified sorgoleone, respectively. (B) Percent inhibition of oxygen evolution by trypsin-treated wheat chloroplast thylakoids from 1 μM DCMU (closed triangles) and 1 μM sorgoleone (closed squares). The data shown are a replot of the results of Figure 5A to show the percent inhibitions relative to trypsinized thylakoids assayed in the absence of any inhibitors.

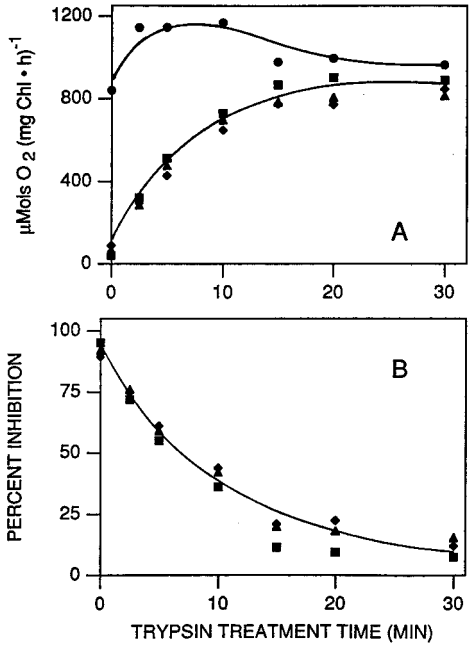

Figure 6. (A) Effects of trypsin treatment of spinach PSII membranes on rates of oxygen evolution and the susceptibility to inhibition by DCMU, bromoxynil, and purified sorgoleone. The closed circle data show rates observed in the absence of addition of inhibitors. The closed triangles, squares, and diamonds are the rates observed in the presence of 1 μM DCMU, 1 μM sorgoleone, and 4 μM bromoxynil, respectively. (B) Percent inhibition of O_2 evolution by trypsin-treated PSII membranes from 1 μM DCMU (closed triangles), 1 μM sorgoleone (closed squares), and 4 μM bromoxynil (closed diamonds). The data shown represent a replot of the results of Figure 6A to show the percent inhibitions relative to trypsinized PSII membranes assayed in the absence of any inhibitors.

amino acid residues reside within the stromal-side epitope linking helix D (IV) and E (V) of the D_1 protein (see Draber et al. (1991) for a review). In the near vicinity of these residues is Arg 238, a trypsin cleavage point. Treatment of thylakoids or PSII membranes with the proteolytic enzyme trypsin diminishes and abolishes the high-affinity binding of the DCMU/atrazine-type herbicides (Renger et al., 1976; Trebst, 1979; Tisher and Strotmann, 1979; Völker et al., 1985). On the other hand, the binding constants for the phenol-type inhibitors (e.g., ioxynil) are not affected by trypsin treatment of thylakoids, although some decrease in the number of ioxynil binding sites of thylakoids occurs (Oettmeier et al., 1982). Accordingly, measurements were made of the effects of increasing duration of trypsin treatment of thylakoids and PSII membranes on the sensitivity of O_2 evolution to inhibition by DCMU and sorgoleone in efforts to verify and extend the conclusion reached in Figure 4, indicating that sorgoleone and atrazine compete for a common site within the Q_B binding domain of the D_1 protein.

As shown by the closed circles of Figure 5A, increasing duration of trypsin treatment of thylakoids led to decreasing rates of O_2 evolution measured in the absence of any inhibitors during the first 10 min but then leveled off after ~38% loss of the original activity. The closed triangles and closed squares record the rates of O_2 evolution measured in the presence of 1 μM DCMU and sorgoleone, respectively, by the trypsin-treated thylakoids. Initially, the rates of O_2 evolution were nearly zero in the presence of either DCMU or sorgoleone; however, with increasing time of trypsin treatment of thylakoids, the inhibitory effectiveness of both of these compounds declined before leveling off after ~5−10 min of trypsin treatment.

Figure 5B is a plot of the percent inhibition by 1 μM DCMU and 1 μM sorgoleone of the rate of O_2 evolution by thylakoids subjected to the trypsin treatment. This replot of the data of Figure 5A shows that the time courses of trypsin-induced destruction of the inhibitor activities of sorgoleone and DCMU occur almost in parallel. Apparently, trypsin hydrolysis of D_1 at Arg 238 diminishes the high-affinity binding of DCMU and sorgoleone similarly, such that their effectiveness for blocking the oxidation of Q_A^- by Q_B is almost abolished.

This conclusion is clearly shown in the results presented in Figure 6A. In these experiments, spinach PSII membranes were trypsin treated for various time intervals and subsequent rates of O_2 evolution were determined in the absence of any additions (closed circles) or in the presence of either 1 μM DCMU (closed triangles), 1 μM sorgoleone (closed squares), or 4 μM bromoxynil (closed diamonds), a phenol-type inhibitor. In the absence of addition of any of the inhibitors, we noted that the rates of O_2 evolution actually increased before decreasing to a level that still was slightly greater than the initial, zero time rate. However, with increasing time of trypsinization, the rate of O_2 evolution measured in the presence of DCMU, sorgoleone, or bromoxynil increased from near zero to a value essentially equivalent to that observed with control membranes after 20−30 min trypsinization. We attribute the ~40% increase in rate of O_2 evolution observed with control membranes after 5−10 min treatment to a trypsin-induced increase of the accessibility of the electron acceptors, PBQ/FeCN, to reduced Q_A/Q_B thereby

overcoming a normally occurring rate constraint of electron transfers from reduced Q_A/Q_B to the exogenous electron acceptors.

Figure 6B is a replot of the data of Figure 6A in which the percent inhibition of O_2 evolution by DCMU (closed triangles), sorgoleone (closed squares), or bromoxynil (closed diamonds) relative to control membranes is plotted versus time of trypsin treatment. An inspection shows that the inhibitory effectiveness of all three compounds decreases in parallel from nearly 100% to nearly 0% inhibition after 15−30 min trypsin treatment.

The combined results of Figures 4-6 show that atrazine, DCMU, bromoxynil, and sorgoleone all bind to a common domain within the Q_B-binding niche of the D_1 protein. Mutants of the green alga *Chlamydomonas reinhardtii* exhibit differential tolerance to the classical herbicides (e.g., atrazine, DCMU) in contrast to the phenol-type inhibitors (Draber et al., 1991). Studies of the effects of sorgoleone on these mutants may permit identification of the specific amino acid residues in the Q_B-binding niche that contribute to the binding of sorgoleone.

CONCLUDING REMARKS

We conclude that sorgoleone is a very effective inhibitor of electron transfer between Q_A^- to Q_B at the reducing side of PSII; thus it is potentially a very effective herbicide. However, the significance of this finding to sorghum allelopathy remains unclear. If sorgoleone is a significant factor in the allelopathic activity of *Sorghum* spp., we speculate that sorgoleone is reasonably persistent in the soil, can be absorbed by root cells, and then can be transported to shoots. Finally, in this scenario, we speculate that sorghum itself, and other plants species resistant to sorghum interference, exclude uptake/transport and/or inactivate sorgoleone by unknown mechanisms.

ABBREVIATIONS USED

Chl, chlorophyll; D_1, a 33 kDa polypeptide which contains the Q_B binding locus and helps form the PSII core; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, $DCIPH_2$, the oxidized and reduced forms, respectively, of 2,6-dichlorophenolindophenol; FeCN, ferricyanide; GC−MS, gas chromatography coupled to mass spectroscopy; HPLC, high-pressure liquid chromatography; ^1H NMR, proton nuclear magnetic resonance spectroscopy; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PBQ, phenyl-*p*-benzoquinone; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; P680, $P680^+$, the reduced and oxidized forms, respectively, of the PSII primary electron donor chlorophyll; Q_A^-, Q_A, the reduced and oxidized forms, respectively, of the primary plastoquinone electron acceptor of PSII; Q^-_B, Q_B, the reduced and oxidized forms, respectively, of the secondary plastoquinone acceptor of PSII; $TyrY_Z$, the redox-active tyrosine 161 of the D_1 polypeptide of the PSII core.

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