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A second site for herbicide action in Photosystem II

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The results in this report confirm the existence of a second site of herbicide inhibition on the oxidizing side of Photosystem II (PS II). The inhibitors which were examined in this study were initially found to inhibit the photoreduction of silicomolybdate with chloroplast thylakoids utilizing water as the electron donor. This indicated a site other than the well-characterized inhibition site on the reducing side of PS II at the 32 kDa polypeptide, Q_b apoprotein. Further studies were conducted utilizing Triton X-100 extracted PS II oxygen-evolving particles from spinach and atrazine-resistant and susceptible *Amaranthus hybridis*. Fluorescence data and the ability to overcome the inhibition of DCIP reduction by diphenylcarbazide both indicated that the oxidizing side of PS II was affected. ^{14}C-atrazine binding studies are also presented.

Introduction

Two sites of inhibition in Photosystem II (PS II) have been reported for a number of herbicides [1–4]. The primary site of action which has been studied in some detail has been localized to Q_b, the secondary stable quinone acceptor molecule of PS II [5,6]. The apoprotein of this quinone-binding protein has been visualized in SDS polyacrylamide gel electrophoresis and found to display an anomalous migration behavior (30–34 kDa) dependent on the presence of urea in the polyacrylamide gel system [7] making it difficult for simple visual analysis. Another difficulty stems from the rather poor staining ability exhibited by this protein to Coomassie blue [8]. Most studies localizing a secondary site of action of these PS II herbicides have utilized higher concentrations of the inhibitor [2]. This secondary site has been speculated to be on the oxidizing side of PS II from luminescence, EPR and electron transport studies [1–4].

The present study was conducted with the use of chloroplast thylakoid membranes and PS II oxygen-evolving preparations derived from Triton X-100 extraction of stroma-freed pea, spinach and *Amaranthus hybridis* chloroplast thylakoids. These PS II preparations were used in this report for studies on the site of action of inhibitors in Photosystem II in which the lumenal surface of the granal lamellae was exposed to the medium. Re-

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Abbreviations: PS II, Photosystem II; EPR, electron paramagnetic resonance; DCMU, 3-(3,4′-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-di-chlorophenolindophenol; DPC, diphenylcarbazide.

cent reports from our laboratory have shown that a site of inhibition of the oxidizing side of PS II was observed for calmodulin-type inhibitors and other Ca^{2+} and Cl^- antagonists with similar PS II-O_2-evolving preparations [9,10]. The aim of this communication is, therefore, to determine whether or not the more classical PS II inhibitors act at a definitive secondary site on the oxidizing side of PS II with this type of preparation.

Materials and Methods

Chloroplast thylakoids (stroma free) were isolated from 2–3 week old pea seedlings, market spinach and *Amaranthus hybridis* as previously described [11]. Triton X-100 extraction of thylakoids to obtain PS II submembrane fractions from spinach and *A. hybridis* (atrazine-sensitive and resistant biotypes) was performed as described [10]. The incubation time in the presence of Triton X-100 was 30 min for spinach and 10 min for *A. hybridis*. Oxygen evolution was monitored at 20°C by a thermostated water-jacketed Clark-type electrode. To monitor silicomolybdate photoreduction, the thylakoid preparations were assayed in a 20 mM Tricine-NaOH buffer (pH 7.8) containing 25 μg Chl · ml^{-1}/100 mM sorbitol/5 mM NaCl/10 μM DCMU/0.5 mM potassium ferricyanide and 200 μM silicomolybdate. The thylakoids were preincubated with the herbicide for 15 min and silicomolybdate was added immediately prior to the assay measurements. DCIP photoreduction by PS II submembrane fractions was monitored at 580 nm in 20 mM Mes-NaOH buffer (pH 6.5) containing 10 μg Chl · ml^{-1} and 40 μM DCIP as described [11]. [^{14}C]atrazine binding was conducted under dim light in 1 ml of 20 mM Mes-NaOH buffer (pH 6.5) containing 5 mM $MgCl_2$/10 mM NaCl/50 μg Chl per ml. The samples containing $5 \cdot 10^{-8}$ to $5 \cdot 10^{-7}$ M [^{14}C]atrazine were incubated for 5 min in the dark and then centrifuged. Half of the supernatant was analyzed by scintillation spectrometry.

Results

A wide range of known herbicides were assayed for inhibition of electron transport from water to silicomolybdate in chloroplast thylakoid membranes isolated from pea as shown in Table I. The percent inhibition of oxygen evolution is given for equal concentrations of the inhibitor (300 μM). At this concentration several herbicides inhibited almost 100% of the oxygen-evolving activity, while others acted only minimally. Pyrazon and bentazon did not inhibit this reaction at 300 μM. We assumed that the inhibition of silicomolybdate photoreduction implicated an inhibition site either on the oxidizing side of PS II or, minimally, before Q_a, the primary quinone acceptor of PS II [12].

Partial reactions using DPC or NH_2OH as artificial electron donors to PS II could not be used to test this assertion with silicomolybdate, since these compounds too rapidly reduced this acceptor directly in the dark. In order to alleviate this problem, H_2O_2 and DPC electron donation were monitored in PS II submembrane fractions with DCIP as the terminal electron acceptor (Table II). In Table II, the percent inhibition of DCIP photoreduction is listed for each herbicide used at a concentration where the inhibitory effect approached the I_{50} concentration (concentration corresponding to 50% inhibition of DCIP photoreduction). The order of effectiveness was different for PS II preparations compared to intact thylakoid membranes (cf. Tables I and II). None of these inhibitors allowed electron transport to proceed via H_2O_2 to P-680. However, the photoreduction of DCIP was restored by DPC except in the case of buthidazole (see Table II). Nitrofluorfen did not inhibit electron transport in PS II preparations whereas, in intact thylakoids, silicomolybdate photoreduction was fully inhibited (Tables I and II). For all of the herbicides used (Table II), the addition of salts (NaCl, $CaCl_2$, $NaNO_3$, $Ca(NO_3)_2$) to the PS II sample before or after the introduction of the herbicide did not prevent the inhibition [10].

The restoration of DCIP photoreduction activity upon addition of 0.5 mM DPC to inhibited PS II preparations is shown in Fig. 1. No direct electron donation from DPC to DCIP was recorded at this DPC concentration (see Fig. 1). However, at higher concentrations (1–2 mM DPC) a slow but continuous dark reduction of DCIP was observed (not shown). The data presented in Fig. 1 indicates a linear relationship between the restoration of DCIP photoreduction by 0.5 mM DPC and

TABLE I

INHIBITION OF SILICOMOLYBDATE PHOTOREDUCTION IN THYLAKOID MEMBRANES AT AN HERBICIDE CONCENTRATION OF 300 μM.

Photoreduction was monitored as described in Materials and Methods. Initial rate, 605 μequiv. per mg Chl per h (control).

Herbicide	Inhibition (%)
Nitrofluorfen [2-chloro-1-(4-nitrophenoxy-4-trifluoromethyl)benzene]	100
SWEP methyl-3,4-dichlorocarbanilate	100
Karsil 2-methyl-valeric-3,4-dichloroanilide	100
JNP-867 [a] 2-propenoic acid-2-cyano-3-(decylamino)-2-ethoxyethyl ester	100
Ioxynil 4-hydroxy-3,5-diiodobenzonitrile	94
Buthidazole [3-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-4-hydroxy-1-methyl)-2-imidazolimide	83
DCMU	61
Metribuzin 4-amino-6-tert-butyl-3- (methylthio)-as-triazin-5(4H)-one	49
Tebuthiuron *N*-[5[(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N*,*N'*-dimethyl urea	35
Propanil 3',4'-dichloropropionilide	29
Bromoxynil 3,5-dibromo-4-hydroxybenzonitrile	28
Bromacil 5-bromo-3-*sec*-butyl-6-methyluracil	27
Desmediphan ethyl-*m*-hydroxycarbanilate	25
Dinoseb 2-*sec*-butyl-4,6-dinitrophenol	18
Atrazine	15
Pyrazon 5-amino-4-chloro-2-phenyl-3-(2H)-pyridazinone	0
Bentazon 3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one-2,2-dioxide	0

[a] See J. Phillips and J. Huppatz (1984) Z. Naturforsch. 39C, 335–337.

the percent inhibition. This relationship was identical for all of the herbicides tested (see Fig. 1) and clearly indicates an active site on the oxidizing side of PS II. Higher concentrations of DPC brought about a larger restoration of the activity after inhibition (not shown). However, addition of

TABLE II

INHIBITION OF DCIP PHOTOREDUCTION IN PS II PREPARATIONS BY DIFFERENT HERBICIDES.

Salts used: 10 mM NaCl, 5 mM $CaCl_2$, 10 mM $NaNO_3$ and 5 mM $Ca(NO_3)_2$. H_2O_2 and DPC concentrations are, respectively, 0.03% and 0.5 mM. (−) denotes no restoration or prevention of the activity; (+) denotes restoration of the activity. See text for more details. Initial rate, 545 μequiv. per mg Chl per h (control).

Herbicide	Concn. (μM)	% Inhibition	Restoration/prevention		
			H_2O_2	DPC	salts
DCMU	0.1	60	−	+	−
Bromoxynil	0.1	78	−	+	−
Ioxynil	0.1	67	−	+	−
JNP-867	0.1	47	−	+	−
Karsil	1.0	74	−	+	−
Atrazine	1.0	68	−	+	−
Dinoseb	1.0	65	−	+	−
Propanil	1.0	57	−	+	−
SWEP	1.0	55	−	+	−
Tebuthiuron	1.0	49	−	+	−
Desmediphan	10.0	66	−	+	−
Pyrazon	10.0	64	−	+	−
Buthidazole	10.0	45	−	−	−
Bentazon	100.0	62	−	+	−
Nitrofluorfen	1000.0	0	−	+	−

herbicide at concentrations needed for total inhibition of DCIP photoreduction led to complete loss of DPC donation even if DPC were present at 2.5 mM.

The above-mentioned results imply that at relatively low concentrations, the herbicides inhibit PS II submembrane preparations principally on the oxidizing side of PS II whereas upon increasing the herbicide concentration, an inhibition at the reducing side becomes apparent. The transition from inhibition on the oxidizing side to the inhibition on the reducing side is well illustrated by the fluorescence induction kinetics presented in Fig. 2. The modification of the fluorescence induction curve by inhibition at the oxidizing side (10^{-8} M DCMU) and the reducing side (10^{-5} M DCMU) of PS II is shown in Fig. 2A; $1 \cdot 10^{-7}$ M DCMU induced an intermediate state (see Ref. 10). Two other typical examples of inhibition on both sides of PS II by different concentrations of ioxynil and bromoxynil are shown in Fig. 2B.

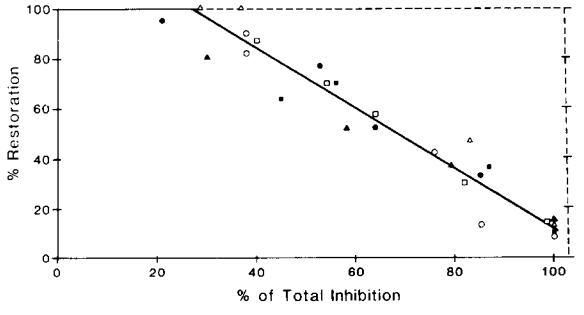

Fig. 1. Restoration of DCIP photoreduction upon addition of 0.5 mM DPC to inhibited PS II preparations (spinach). The concentration range studied is indicated in the brackets: ▲, DCMU (10^{-4}–10^{-8} M); △, atrazine (10^{-3}–10^{-7} M); ●, dinoseb (10^{-4}–10^{-8} M); ○, ioxynil (10^{-4}–10^{-9} M); □, bromacil (10^{-4}–10^{-9} M), ■, propanil (10^{-4}–10^{-7}); data points represent increasing concentrations from left to right (see Table II).

Two herbicide-binding sites were further characterized with PS II submembrane fractions isolated from wild type and atrazine resistant biotypes of *A. hybridis*. The percent inhibition of DCIP photoreduction and restoration of the activity by addition of 0.5 mM DPC are listed in Table III for atrazine, DCMU and dinoseb. The inhibitory effect of atrazine was very pronounced with the wild type, and 0.5 mM DPC was inefficient in restoring the activity. However, only high con-

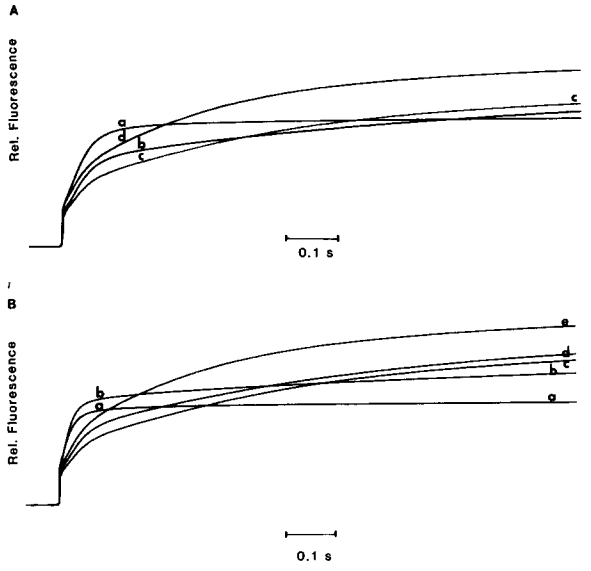

Fig. 2. (A) Effect of DCMU on fluorescence induction in spinach PS II preparations: (a) 10^{-5} M; (b) 10^{-6} M; (c) 10^{-8} M; (d) control. (B) Effect of ioxynil and bromoxynil on fluorescence induction in PS II preparations (spinach): (a) 10^{-4} M ioxynil; (b) 10^{-6} M bromoxynil; (c) 10^{-8} M ioxynil; (d) 10^{-8} M bromoxynil; (e) control.

centrations of atrazine inhibited electron transport from water to DCIP in the case of the resistant biotype. In addition, 100% of the inhibitory effect was overcome by DPC electron donation to P-680 (Table III), showing that this remaining inhibitory effect was located on the oxidizing side of PS II. Conversely, the atrazine resistant biotype was not resistant to DCMU (Table III) and, moreover, susceptible to dinoseb [13].

Binding studies with [^{14}C]atrazine were conducted with thylakoids and PS II submembrane fractions isolated from spinach and *A. hybridis*. The double reciprocal plots of [^{14}C]atrazine binding to the PS II preparations are presented in Fig. 3. An atrazine binding contant (K_b) of 54 nM was obtained for spinach thylakoid membranes (data not shown) and the number of chlorophyll molecules per binding site was calculated to be 477 (similar to literature values, see Refs. 13 and 17). A binding constant of 1300 nM was found for PS II preparations obtained from spinach with a chlorophyll to binding site ratio of 150 (Fig. 3A). The binding data obtained from PS II preparations isolated from the wild type of *A. hybridis* (Fig. 3B) were in the same order of magnitude as that obtained with PS II preparations extracted from spinach (K_b, 1200 nM; chlorophyll ratio, 165). As expected from the results presented in Table III, no [^{14}C]atrazine binding was found with PS II preparations isolated from the resistant biotype at the low concentrations used for this assay [13]. Furthermore, addition of 0.5 mM DPC to the assay mixture prior to the radiolabelled atrazine

TABLE III

INHIBITION OF DCIP PHOTOREDUCTION IN PS II PREPARATIONS ISOLATED FROM *A. HYBRIDIS*

DPC concentration is 0.5 mM. See text for more details.

Herbicide	Concn. (M)	Wild type [a]		Atrazine-resistant [b]	
		% inhibition	% restoration	% inhibition	% restoration
Atrazine					
	10^{-3}	100	0	57	100
	10^{-4}	100	0	11	100
	10^{-5}	92	25	0	–
DCMU					
	10^{-5}	100	17	85	32
	10^{-7}	47	74	23	100
Dinoseb					
	10^{-4}	100	10	100	0
	10^{-5}	92	24	100	0
	10^{-6}	59	76	91	37

[a] Initial rate, 450 μequiv. per mg Chl per h.
[b] Initial rate, 430 μequiv. per mg Chl per h.

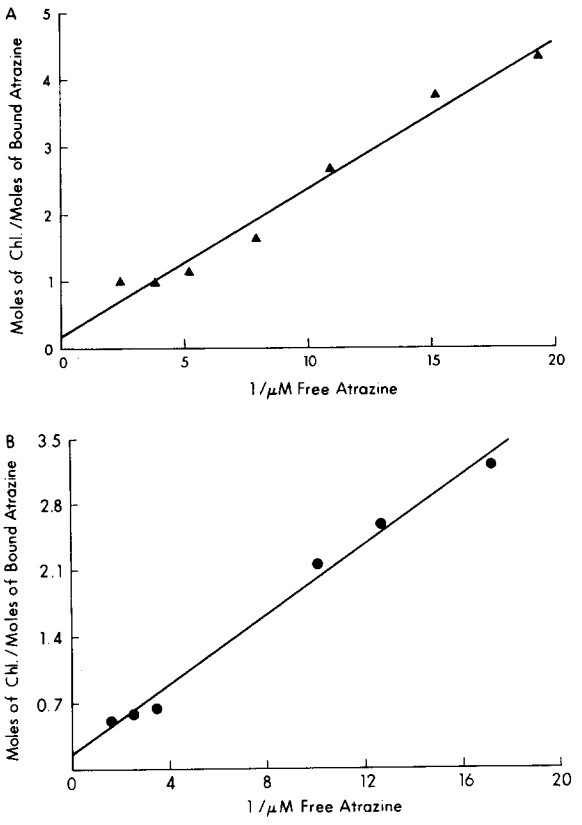

Fig. 3. Double reciprocal plots representing binding of [^{14}C]atrazine; (A) spinach PS II; (B) PS II of wild type *A. hybridis*. Conditions are given in Materials and Methods.

did not alter the results of the binding experiment, thus indicating that DPC did not displace the herbicide from its active site.

Discussion

Silicomolybdate is assumed to accept electrons from Q_a, the primary quinone acceptor of PS II [12]. As shown in Table I, silicomolybdate photoreduction by thylakoid membranes was inhibited by a wide range of herbicides. This inhibition indicated the presence of a site for herbicides other than the 32 kDa protein, the apoprotein of the secondary quinone acceptor of PS II [5,6]. The presence of a second site in the thylakoid membranes was previously reported for ioxynil and dinoseb [1], buthidazole [2,3], atrazine and DCMU [4]. It could also have been suspected from the inhibitory effect of high concentrations of herbicides in resistant biotypes (for example, see Ref. 14). For instance, these mutants are not affected by herbicide concentrations (which presumably affect the reducing side of PS II in intact thylakoids) that are lethal to the wild types. The requirement for higher concentrations of inhibitor may represent incomplete resistance of the biotypes to herbicides or an inhibition at another site as indicated by the results presented in this report.

The few dispersed results available to date which indicate an inhibition site on the oxidizing side of PS II [1–4,18,19] are due to the rather minimal effect of most herbicides at this site in intact thylakoid membranes. However, with PS II submembrane preparations in which the lumenal surface is exposed, the herbicide inhibition is detected primarily on the oxidizing side of PS II. In fact, at low herbicide concentrations, all of the inhibition by the compounds examined in this report with the exception of buthidazole (see Tables I and II) could be overcome by the addition of DPC. In contrast, buthidazole exerted an inhibition of electron transport between the DPC donation site and Q_a, since DPC was unable to overcome the effect of this latter compound (Fuerst, E.P., Nakatani, H.Y., Penner, D. and Arntzen, C.J., unpublished data). The sensitivity of the oxidizing side of PS II to herbicides may be the result of protein(s) modification (conformation) which increases the herbicide affinity at this site or conversely, damage to the 32 kDa protein could lead to a decrease of the herbicide affinity at this site. In support of this latter hypothesis, the 32 kDa polypeptide was found to be less effective in binding atrazine or DCMU in other PS II preparations [15]. The existence of another site is also confirmed by the results presented in Table III in an atrazine resistant biotype of *A. hybridis*. Compared to the wild type, the resistant biotype was more sensitive to dinoseb and somewhat less sensitive to DCMU. However, the resistant biotype was sensitive only to high concentrations of atrazine and, as stated earlier, this inhibitory effect (fully overcome by 0.5 M DPC) is likely due to the inhibition on the oxidizing side of PS II. The incomplete resistance to triazines [14] conferred by a single amino acid change in the 32 kDa polypeptide [16], may thus, in part, be explained by the

presence of a secondary active site of herbicides on the oxidizing side of PS II. A candidate for another quinone-binding protein is D_2 which has been found to have amino acid sequence similarities to the Q_b protein [20]. However, no physiological role has yet been ascribed to this latter polypeptide. It is interesting to note that the EPR signal from Z has been postulated to arise from a quinone cation radical species [21].

The results of the [^{14}C]atrazine binding studies (Fig. 3) with spinach thylakoids were similar to that found in the literature [13,17]. However, the atrazine binding constant obtained with PS II preparations isolated from spinach and atrazine-susceptible *A. hybridis* ($K_b = 1.2–1.3$ μM) was approx. 10–20-fold higher than for spinach thylakoids ($K_b = 50–100$ nM). Two facts thus suggest that this binding constant in PS II preparations represents the interaction of atrazine with a site on the oxidizing side of PS II: (i) at the low concentration used for this assay ($5 \cdot 10^{-8}–5 \cdot 10^{-7}$ M), all of the inhibition is overcome by DPC (Table II); (ii) the ratio of chlorophyll molecules per binding site in PS II preparations (150–200) represents the binding of one atrazine molecule (one binding site) for one electron-transport chain.

Several polypeptides have previously been radiolabelled by herbicides [4,5,15], and it is known that the triazine and urea-types of herbicides interact with a 32 kDa polypeptide (Q_b apoprotein) whereas the effect of the phenolic-type herbicides was purported to involve one or more polypeptides in the 40–53 kDa range [15]. In the present report, we demonstrate another site of herbicide action prior to or at the level of Z (see scheme presented in Fig. 3, Ref. 10).

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