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IDENTIFICATION OF A 32—34-KILODALTON POLYPEPTIDE AS A HERBICIDE RECEPTOR PROTEIN IN PHOTOSYSTEM II

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Summary

Photosystem II particles which retained high rates of herbicide-sensitive activity were used to examine the site(s) of action of various herbicides. A polypeptide of 32—34 kdaltons was identified as the triazine-herbicide binding site based upon: (a) parallel loss of atrazine activity and the polypeptide during either trypsin treatment or selective detergent depletion of protein in the Photosystem II complex, and (b) covalent labeling of the polypeptide by a ^{14}C-labeled photoaffinity triazine.

In Photosystem II particles depleted of the 32—34-kdalton polypeptide, electron transport was still active and was slightly sensitive to DCMU and largely sensitive to dinoseb (urea and nitrophenol herbicides, respectively). On the basis of this result it is proposed that the general herbicide binding site common to atrazine, DCMU and dinoseb is formed by a minimum of two polypeptides which determine affinity and/or mediate herbicide-induced inhibition of electron transport on the acceptor side of Photosystem II.

Abbreviations: azidoatrazine, 2-azido-4-ethylamino-6-isopropylamino-*s*-triazine; atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; B, a protein-plastoquinone complex which acts as the secondary electron acceptor in PS II; Chl, chlorophyll; DCIP, 2,6-dichlorophenolindolphenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron); dinoseb, 2-(methyl-*n*-propyl)-4,6-dinitrophenylacetate; DPC, *s*-diphenylcarbazide; LHC-II, chlorophyll *a*/*b* light-harvesting pigment-protein complex serving PS II; PS II, Photosystem II; Q, primary acceptor quinone in PS II; SDS, sodium dodecyl sulfate; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

Photosystem II (PS II) functions in photosynthetic electron transport to mediate light-induced charge separation, generate a strong oxidant, and to stabilize high-energy intermediates produced during primary photochemistry. Stabilization of oxidizing equivalents involves a number of biochemical components which mediate a stepwise charge accumulation with the concomitant release of protons and O_2 [1]. Stabilization of charges on the reducing side of PS II requires a minimum of three intermediates I, Q and B. I is an intermediate carrier which transfers an electron from the reaction center chlorophyll to the first stable electron acceptor Q [2], the latter being a quinone which acts as a one electron acceptor [3]. B is also a protein-bound quinone; it acts as an electron gate mediating a two-electron transfer of electrons from Q to the bulk plastoquinone [4,5].

Previous studies have provided evidence that a large number of photosynthetic herbicides inhibit electron transfer on the reducing side of PS II [6,7]. Velthuys and Amesz [5] reported that addition of the herbicide diuron to thylakoids which were in the state QB^- caused back electron flow from B^- to Q. This phenomenon has also been observed upon addition of atrazine to thylakoids [8]. These observations led to the idea that herbicide binding to PS II lowers the redox potential of B relative to Q, thus inhibiting forward electron flow ($Q \rightarrow B \rightarrow PQ$). The specific mechanism causing the inhibition has not been elucidated although it may involve interaction of a >C-NH- or -CO-NH- group, which is characteristic of PS II herbicides [9,10], with a specific domain within the PS II complex.

In recent years the complexity of PS II reactions has been revealed through EPR, NMR and fast spectroscopic techniques (see review in Ref. 1). Studies utilizing protease digestion of surface-exposed membrane proteins [11,12], analysis of photosynthetically blocked mutants [13], and developmental systems [14] have provided various approaches for analyzing some constituents of PS II. In spite of these efforts, identification of the biochemical components (both polypeptides and associated cofactors) involved in the PS II reactions is at present limited. PS II components which have been tentatively identified include the chlorophyll-protein reaction center (42—48-kdalton polypeptide(s)) [13,15], polypeptides of 25—29 kdaltons associated with the Chl *a*/*b* light-harvesting complex serving Photosystem II (LHC-II) [16], polypeptides of 27—32 kdaltons which may be involved in herbicide inhibition of PS II activity [11], and a polypeptide of 5.6 kdalton identified as the apoprotein of cytochrome *b*-559 [17].

In this manuscript, we report the identification of a 32—34 kdalton polypeptide as the receptor for a specific class of herbicides directed against PS II. The identification is based on studies using an isolated PS II particle. The time course of trypsin treatment or selective extraction of the polypeptide from PS II particles resulted in loss of atrazine-induced herbicide activity. We have also utilized a radiolabeled azido atrazine, which was recently reported to act as a photo-affinity label to covalently tag 32—34 kdalton polypeptides [18].

Materials and Methods

Chloroplasts were isolated from pea (*Pisum sativum* L.) leaves as previously described [16]. Photosystem II particles were isolated by a technique described below. Chlorophyll was determined by the method of Arnon [20]. Photosystem II-mediated reduction of 0.02 mM DCIP was measured in the presence of 1 mM DPC using a spectrophotometer modified for direct sample illumination [19]. PS II-mediated reduction of 0.5 mM $K_3Fe(CN)_6$ in the presence of 20 mM KI (I^- used as a donor to PS II) was monitored spectrophotometrically at 420 nm using an extinction coefficient of 1 $mM^{-1} \cdot cm^{-1}$ for ferricyanide [21].

The atrazine used in these studies was provided by Dr. H. LeBaron, Ciba-Geigy, Greensboro, NC. Uniformly ring-labeled azido[^{14}C]atrazine was provided by Dr. G. Gardner, Shell Development Co., Modesto, CA (spec. act. 37.1 μCi/mg). Radioactivity was determined by scintillation techniques as previously described [22]. Chl-containing samples were bleached in 5% H_2O_2 at 80°C prior to liquid scintillation counting. SDS gel electrophoresis techniques used to separate polypeptides have been previously described [23]. Radioactivity in the slab gels was detected after 2,5-diphenyloxazole treatment, drying and X-ray fluorography [24].

For proteolytic modification, PS II particles were treated with trypsin (TR-TPCK, Worthington Biochemical Corp.; 10 μg/ml and 0.2 mg Chl/ml) at 20°C, pH 7.8. The reaction was stopped at various times by the addition of a 10-fold excess of trypsin inhibitor (from soybean, Type I-S, Sigma Chemical Co.).

For selective protein extraction, PS II particles (0.2 mg Chl/ml) were solubilized with 2% sodium cholate/4 M urea, or 1% octyl-β-D-glucopyranoside in 20 mM Tricine/NaOH, pH 7.8, at 4°C for 2 h with stirring. Solubilized polypeptides were then separated from the PS II complexes by centrifugation of 4-ml samples on 0.2—1.0 M sucrose gradients (containing 0.1% sodium cholate, 20 mM Tricine/NaOH, pH 7.8, 4°C) at 100 000 $\times g$ for 10 h in a Beckman SW-28 rotor, using 18 ml total volume sample tubes. Solubilized polypeptides were recovered from the top 2—4 ml of the gradients. After centrifugation, chlorophyll-containing protein-depleted PS II complexes were collected from a band which was approximately midgradient.

Preparation of PS II particles. Chloroplasts were isolated from pea seedlings by grinding leaves in 0.4 M sorbitol, 0.05 M Tricine/NaOH, pH 7.8, 5 mM $MgCl_2$ as previously described [16]. The chloroplast pellet obtained after centrifugation was resuspended in 0.02 M Tricine/NaOH, pH 7.8, 0.01 M $MgCl_2$ (resuspension buffer designated as TNM buffer) and the chloroplast thylakoids were subsequently pelleted by centrifugation at 7000 $\times g$ (4°C). The pelleted thylakoid membranes were resuspended in TNM buffer to 0.75 mg Chl/ml and Triton X-100 was added to a final concentration of 0.6% (w/v). This solution was slowly stirred at 4°C in the dark for 45 min and then centrifuged at 12 000 $\times g$ for 10 min. The chlorophyll-containing pellet obtained after centrifugation was resuspended in 0.02 M Tricine/NaOH, pH 7.8, to 1.0 mg Chl/ml and digitonin (5 mg/mg Chl) and octyl-β-D-glucopyranoside (2 mg/mg Chl) were added. The solution was stirred for 1 h at 4°C in the dark, then centrifuged at 18 000 $\times g$ for 10 min. The supernatant obtained after centrifugation was loaded (4 ml

per tube) on 0.2—1.0 M sucrose linear density gradients (total volume 38 ml) containing 0.02 M Tricine/NaOH, pH 7.8, and 0.1% sodium cholate. The sucrose density gradients were centrifuged in a Beckman SW-28 rotor for 15—20 h at 100 000 × *g* (4°C). After centrifugation the gradients contained three chlorophyll-containing bands; a band near the top of the gradients contained primarily LHC-II, a minor band below LHC-II consisted of Photosystem I particles. PS II particles were collected from the chlorophyll-containing band migrating the longest distance (to approximately midgradient) by removal with a large-bore blunt needle affixed to a syringe.

Results and Discussion

Herbicide inhibition characteristics of PS II particles

Several classes of photosynthetic herbicides have been shown to act by blocking PS II electron transport [6—8]. These include the ureas (example, DCMU), triazines (example, atrazine) and the nitrophenols (example, dinoseb). The urea, triazine and nitrophenol herbicides have been shown to inhibit the same electron transport step in PS II and to compete for a common binding site [25,26].

For analysis of specific PS II constituents involved in herbicide binding, we have developed a rapid isolation procedure for a high yield of PS II particles. The development of the procedure was specifically directed at maintaining herbicide sensitivity in the PS II particles as close as possible to the 'native' in situ responses to herbicides. It should be noted that electron transport in previously reported PS II particle preparations was either totally DCMU resistant or was inhibited partially by high DCMU concentrations [11,27]. Activities of other PS II-directed herbicides against PS II particles have not been characterized.

Analysis of PS II-mediated electron transport over a range of herbicide concentrations has previously been used to characterize inhibition constants for various chemicals. K_i values calculated from binding data are related to a measured I_{50} value (the herbicide concentration required for 50% inhibition of the

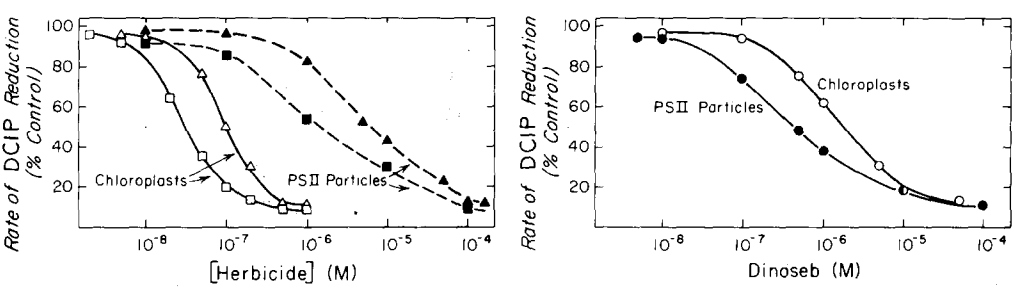

Fig. 1. Inhibition of PS II-mediated DCIP photoreduction in chloroplasts (H_2O as the donor) and PS II particles (DPC as the donor) by various concentrations of azidoatrazine or DCMU. Samples containing 5 μg Chl/ml were illuminated (broad band blue) for 1 min at 20°C. Control rates were 350 μmol DCIP reduced · mg^{-1} Chl · h^{-1} for PS II particles and 290 μmol DCIP reduced · mg^{-1} Chl · h^{-1} for chloroplasts. (△, ▲ = azido-atrazine; □, ■ = DCMU).

Fig. 2. Inhibition of PS II-mediated DCIP photoreduction in chloroplasts and PS II particles in the presence of various concentrations of dinoseb. Assay conditions are described in Fig. 1.

stated photoreaction). I_{50} values are proportionally related to the true herbicide binding constant [25]. DCMU, azidoatrazine, and dinoseb inhibit chloroplast photoreactions over a narrow concentration range (Figs. 1 and 2). (Atrazine data, not presented, were nearly identical to those for azidoatrazine.) In contrast, the herbicides expressed inhibition over a much wider concentration range in PS II particle preparations (note the flattening of the inhibition response curve in Figs. 1 and 2). The increase in the concentration range needed to saturate inhibition in the PS II particles may be related to increased heterogeneity of binding sites, and may reflect perturbation in the herbicide-binding sites with respect to affinity for the various herbicides. The total number of herbicide inhibition sites in the PS II particles, measured by total inhibition, remained constant.

I_{50} values for each of the herbicides can be determined from Figs. 1 and 2; these provide a measure of affinity between a herbicide and its binding site [25]. The results show that PS II particles have an apparent increased affinity for dinoseb and decreased affinity for atrazine and DCMU as compared to chloroplasts. Preliminary evidence suggests that the decreased affinity for the latter herbicides is due to a slight depletion of certain PS II polypeptides even during the mild isolation procedures utilized.

Trypsin modification of herbicide inhibition of PS II particle photoreactions

Table I shows the effect of herbicides on PS II activity in isolated particles. The partial reaction of electron transfer from I^- to ferricyanide was found to be nearly insensitive to herbicides. In contrast, electron transport from DPC to DCIP was strongly inhibited. It has been previously suggested that ferricyanide is able to very slowly accept electrons directly from the primary acceptor Q in intact thylakoids [28], but more rapidly in silicomolybdate-treated membranes or from thylakoids after mild trypsin treatment [12,29]. Electron transport to DCIP is thought to require the participation of the secondary quinone B [30]. These suggestions are consistent with the results shown in Table I. Herbicides which block electron transport in PS II particles are able to inhibit reduction of DCIP but not ferricyanide. We conclude that ferricyanide accepts electrons directly from Q in isolated particles and the herbicides do not influence electron transfer reactions before or at the level of Q.

The two partial reactions mediated by PS II (DCIP or ferricyanide reduction) have been utilized to probe the site(s) of action of trypsin, a water-soluble

TABLE I

HERBICIDE INHIBITION OF PS II ACTIVITY IN ISOLATED PS II PARTICLES

Rate is μmol DCIP or ferricyanide reduced · mg^{-1} Chl · h^{-1}. Data in parentheses indicate percent inhibition.

Condition	Rate	
	$I^- \rightarrow$ ferricyanide	DCP → DCIP
Control	336 (—)	310 (—)
+ 10^{-4} M atrazine	329 (4)	93 (70)
+ 10^{-4} M DCMU	311 (7)	40 (87)
+ 10^{-4} M dinoseb	306 (9)	22 (93)

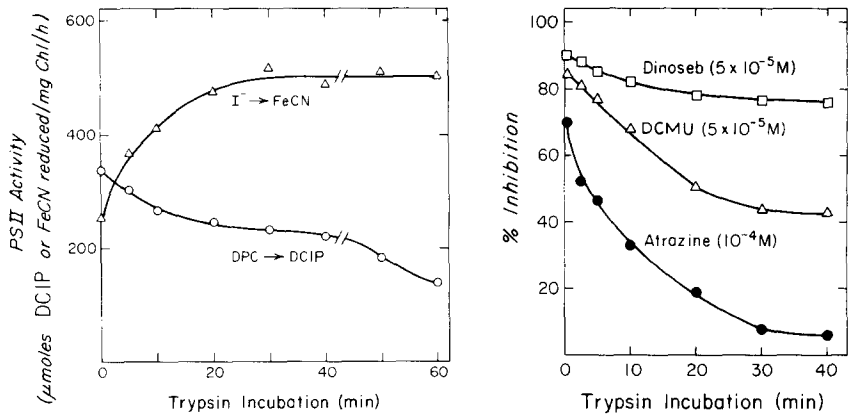

Fig. 3. Time course of trypsin treatment of PS II particles. PS II activity was monitored by following the photoreduction of ferricyanide (FeCN) or DCIP. Trypsin concentration was 10 μg/ml for the first 45 min and 20 μg/ml from the 45 min time point to 60 min. The change in trypsin concentration is indicated by a break in the activity curves.

Fig. 4. Time course of trypsin treatment of PS II particles. Conditions were the same as described in Fig. 3. Inhibition of DCIP photoreduction induced by 10^{-4} M atrazine, $5 \cdot 10^{-5}$ M DCMU or $5 \cdot 10^{-5}$ M dinoseb were monitored after various times of trypsin digestion.

protease, on PS II particles. Fig. 3 shows a time course of PS II activity in the presence of trypsin. Electron transport from I^- to ferricyanide approximately doubled in the first 10 min and then remained relatively constant. A trypsin-induced increase in ferricyanide reduction has been previously observed in chloroplasts and has been attributed to increased exposure of Q to the ferricyanide [12]. Trypsin treatment of PS II particles gradually inhibited DCIP reduction over a 60-min time course. The difference in time course and in rate of the action of trypsin on ferricyanide and DCIP reduction suggests two different sites of trypsin digestion, or two sequential effects of trypsin on one polypeptide.

The effect of trypsin treatment on herbicide inhibition of DCIP reduction in PS II particles is shown in Fig. 4. The data reveal two central features. First, atrazine is an effective inhibitor in the untreated PS II particles, but trypsin digestion results in atrazine-resistant electron flow after 10—20 min of trypsin treatment; this time course is similar to the trypsin-induced increase in electron transport to ferricyanide observed in Fig. 3. Second, dinoseb, and to a lesser extent DCMU, are able to inhibit PS II activity when inhibition mediated by atrazine is lost. This suggests that the electron transport step which is inhibited by these herbicides is functional in trypsin-treated PS II particles even when the site for atrazine binding is severely altered or lost.

Polypeptide alterations in PS II particles were analyzed during the time course of trypsin digestion in an attempt to identify the polypeptides modified concomitant with altered electron transport and herbicide inhibition. Fig. 5 reveals that polypeptides of 32—34 and 25—29 kdaltons are modified with a time course corresponding to the loss of atrazine inhibition. Trypsin modification of a 27- and 32-kdalton polypeptide have been previously reported concomitant with the loss of DCMU inhibition of PS II activity, and a two-step alter-

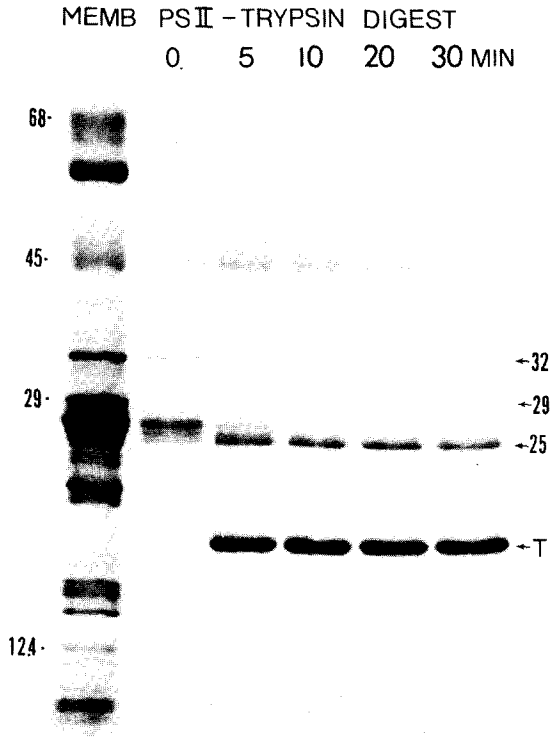

Fig. 5. Time course of trypsin treatment of PS II particles showing polypeptide changes over a 30 min time course. Time zero shows the polypeptide composition of PS II before addition of trypsin. The trypsin digestion was stopped at the times shown by addition of a 10-fold excess of trypsin inhibitor (T). Molecular weight standards are shown to the left of separated chloroplast polypeptides (MEMB). Molecular weight standards used include: bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (29 000) and cytochrome c (12 400).

ation in polypeptides of 32—34 kdaltons were related to stepwise changes in atrazine binding in trypsin-treated thylakoids [31].

Selective extraction of PS II polypeptides

The results shown in Fig. 3 and 4 revealed correspondence between the loss of atrazine-induced inhibition of PS II activity and exposure of Q to ferricyanide under conditions where DCMU- or dinoseb-sensitive electron transport to DCIP was partially or largely unaffected, respectively. These results could be explained by the selective cleavage of a single polypeptide which contains multiple herbicide-binding sites as well as the quinone cofactor, B. For this possibility, one would have to assume that trypsin cleaves the atrazine receptor portion of the polypeptide leaving the quinone cofactor and the DCMU and dinoseb receptors either partially or largely unmodified. An alternative explanation for the results in Figs. 3—5 would involve the participation of separate polypeptides in these effects; one polypeptide would be hypothesized to contain the atrazine receptor and one or more other polypeptides which would bind the quinone cofactor of B and/or DCMU and dinoseb receptors. In order to distinguish between these possibilities, polypeptides present in the PS II particles

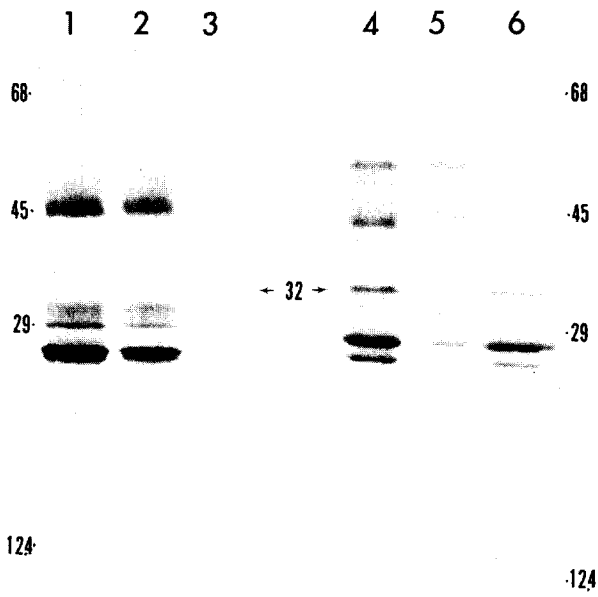

Fig. 6. SDS-polyacrylamide slab gel of PS II (lanes 1 and 4), PS II after cholate/urea extraction (lane 2), polypeptide extracted from PS II by cholate/urea (lane 3), PS II after octyl-β-D-glucopyranoside extraction (lane 5), and polypeptides extracted from PS II by octyl-β-D-glucopyranoside (lane 6). The polyacrylamide gel (lanes 4—6) shown on the right contained 4 M urea. The molecular weight standard shown on the far-left and far-right sides are listed in Fig. 5.

were selectively removed from the particles and remaining activity and herbicide sensitivity were monitored.

Two procedures were utilized to selectively remove polypeptides from isolated PS II preparations. The first involved solubilization of PS II particles with

TABLE II

HERBICIDE INHIBITION OF PHOTOSYSTEM II ACTIVITY (DPC → DCIP) AFTER SELECTIVE EXTRACTION OF PS II PARTICLES

Rate is μmol DCIP reduced · mg^{-1} Chl · h^{-1}. Data in parentheses are percent inhibition.

Sample	Control rate	Rate		
		+ 10^{-4} M atrazine	+ 10^{-4} M DCMU	+ 10^{-4} M dinoseb
Control (untreated PS II particles)	310	93 (70)	40 (87)	22 (93)
Cholate/urea-extracted PS II	274	260 (5)	181 (33)	105 (61)
Octyl-β-D-glucopyranoside-extracted PS II	300	296 (2)	272 (10)	149 (51)

cholate/urea and the second with octyl-β-D-glucopyranoside (see Materials and Methods). The resultant PS II particles were then separated from the extracted polypeptides on sucrose gradients. The extracted polypeptides and the particles were individually recovered and analyzed.

Lanes 1 and 4 of Fig. 6 demonstrate the polypeptide complement of isolated PS II particles. For comparative purposes, the polyacrylamide gels used contained 0 and 4 M urea for lanes 1 and 4, respectively. The inclusion of urea in gels is known to sometimes alter polypeptide migration [15]; comparison of the PS II particles shows that changes in apparent molecular weight occurred in the 42—48- and 25—29-kdalton region. In addition, a diffuse band at approx. 30 kdaltons was more distinct in urea-free gels.

Fig. 6 shows that a polypeptide (32—34 kdaltons) was extracted from PS II particles (lane 2) by cholate/urea; the single non-pigmented polypeptide was recovered at the top of the gradient (lane 3). Solubilization of PS II particles by octyl-β-D-glucopyranoside removed the 32—34-kdalton polypeptide plus polypeptides of 25—29-kdaltons from the remaining PS II complex (lane 5). The 32—34- and 25—29-kdalton polypeptides (lane 6) were recovered in the upper portion of the gradient; this mixture contained both Chls *a* and *b*. Previous studies have identified the 25—29-kdalton polypeptides as constituents of the LHC-II [16].

The polypeptide changes described above for PS II particles were correlated with only small changes in electron transport rates (DPC → DCIP, see Table II). These results indicate that the 32—34-kdalton polypeptide (and light-harvesting pigment-proteins of 25—29-kdaltons) are not required for DPC → DCIP photoreduction in PS II particles. The major change caused by polypeptide extraction involved alteration of herbicide inhibition of DPC → DCIP electron transport. The results in Table II show that selective removal of the 32—34-kdalton polypeptide is correlated with a virtually complete loss of PS II inhibition mediated by atrazine. Extraction of PS II particles with octyl-β-D-glucopyranoside also caused a decrease in DCMU-induced inhibition of PS II activity. This treatment removed several polypeptides including the 32—34 kdalton polypeptide. Inhibition mediated by DCMU or dinoseb are modified but to a lesser extent when the 32—34-kdalton protein is selectively extracted via cholate/urea. These results are consistent with the hypothesis that the 32—34-kdalton polypeptide is necessary for atrazine-induced inhibition of PS II activity, but that this polypeptide does not, by itself, fully determine the site of DCMU or dinoseb binding.

Photoaffinity labeling of the PS II triazine-binding site

The herbicide, azidoatrazine, has been reported to act as a photo-affinity label [18]. It inhibits PS II electron transport with an I_{50} value similar to atrazine (Fig. 1). These data demonstrate that substitution of an N_3 group in place of chloride on the triazine ring did not significantly alter the inhibition properties of this herbicide.

Exposure of the azidoatrazine to ultraviolet illumination at 254 nm activates the azido group to form a highly reactive nitrene which can undergo nucleophilic attack of susceptible groups (for a general review see Ref. 32). ^{14}C-Labeled azidoatrazine, if activated while occupying the herbicide-binding site

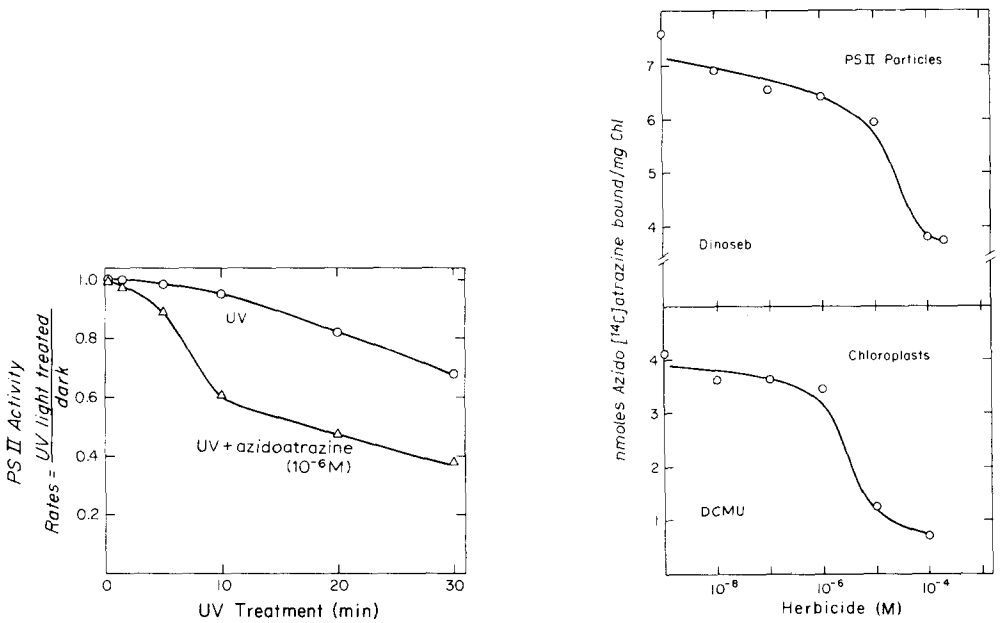

Fig. 7. Time course of modification of PS II activity (DPC → DCIP) in chloroplasts by 254 nm illumination in the presence or absence of azidoatrazine. Samples containing 25 μg Chl/ml, 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$ were illuminated at a distance of 4 cm or kept in the dark at 20° C in the presence or absence of $1 \cdot 10^{-6}$ M azidoatrazine. After treatment the chloroplasts were washed twice with 20 mM Tricine/NaOH, pH 7.8, 5 mM $MgCl_2$ prior to assay. Rates are expressed as a ratio of ultraviolet light-treated activity over dark sample activity.

Fig. 8. Incorporation of azido[^{14}C]atrazine into PS II particles (top) and chloroplast membranes (bottom) in the presence of various concentrations of dinoseb (top) or DCMU (bottom). PS II particles or chloroplasts were suspended at 25 μg Chl/ml in 50 mM Tris-HCl, pH 7.8, exposed to 254 nm illumination for 15 min. The PS II particle samples contained $5 \cdot 10^{-5}$ M azido[^{14}C]atrazine and dinoseb. The chloroplast samples contained $1 \cdot 10^{-6}$ M azido[^{14}C]atrazine and the DCMU concentration indicated. Illuminated samples were pelleted, washed once in 20 mM Tricine/NaOH, 5 mM $MgCl_2$, bleached and counted for radioactivity.

within the PS II complex, can thus become covalently cross-linked to a protein creating the binding site. In these experiments, the organic buffer Tris was included to act as a scavenger for non-selectively activated azidoatrazine. Fig. 7 shows a time course of ultraviolet-induced inhibition of chloroplasts in the presence or absence of azidoatrazine. For this experiment chloroplasts were suspended in 0 or $1 \cdot 10^{-6}$ M azidoatrazine, illuminated for stated times with 254 nm light, washed twice by centrifugation and then assayed for PS II activity (DPC → DCIP). The results presented in Fig. 7 show that ultraviolet illumination for 20 min in the presence of azidoatrazine causes more than 50% inhibition of electron transport which was not reversed by washing. This indicates that covalent binding of the azidoatrazine permanently inhibited PS II activity.

It has previously been shown that triazine, urea and nitrophenol herbicide act competitively in radiolabel-binding assays [22,25,26]. Fig. 8 shows competition of diuron (in chloroplasts) and dinoseb (in PS II particles) for the specific triazine-binding site via measurement of reduced azidoatrazine covalent labeling

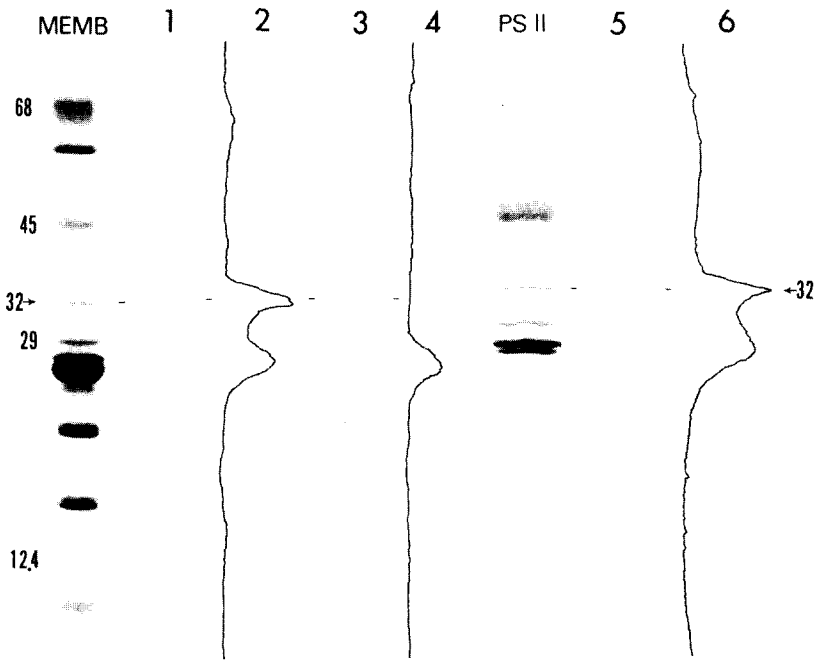

Fig. 9. SDS-polyacrylamide slab gel showing Coomassie-staining polypeptides of chloroplast membranes (MEMB) and PS II particles (PS II). Lanes 1 and 3, autoradiograms of chloroplast polypeptides which were labeled by azido[^{14}C]atrazine in the absence or presence of 10^{-4} M DCMU, respectively. Lanes 2 and 4, densitometric scans of the autoradiograms in lanes 1 and 3, respectively. Lane 5, autoradiogram of azido[^{14}C]atrazine-labeled PS II polypeptides. Lane 6, densitometric scan of the autoradiogram in lane 5. Conditions of labeling are described in Fig. 8. The molecular weight standards shown at the left of the figure are listed in Fig. 5.

of chloroplast and PS II particles in the presence of the competing herbicide. The higher background binding in PS II particles (i.e., triazine binding not competitively displaced by dinoseb) as compared to chloroplasts may be partially due to increased concentrations of azidoatrazine which were necessary to attain maximal inhibition of electron transport in PS II particles.

PS II particles and chloroplast samples which were covalently labeled with azido[^{14}C]atrazine were subjected to analysis on SDS-polyacrylamide gels followed by autoradiography in order to determine which polypeptides were labeled by the azidoatrazine. Fig. 9 (lane MEMB) shows the polypeptide content (Coomassie blue staining) of chloroplasts; the adjacent lane 1 shows the corresponding fluorograph. A densitometer scan of the fluorogram (lane 2) revealed maximal specific activity of label at a polypeptide of 32—34 kdaltons. In addition, the 25—29-kdalton polypeptides of the LHC-II [16] incorporated activity. When chloroplasts were inhibited in $5 \cdot 10^{-6}$ M azidoatrazine plus 10^{-4} M DCMU during ultraviolet activation, the resultant autoradiogram of the labeled sample (lane 3) showed a specific decrease in the [^{14}C]triazine labeling of the 32—34-kdalton polypeptide (see lane 4). This indicates competitive herbicide interaction at the 32—34-kdalton constituent but not for the binding to LHC-II polypeptides.

Isolated PS II particles were illuminated (254 nm) in the presence of azido-[^{14}C]atrazine. A comparison of the stained polypeptide profile (slot PS II) and the corresponding autoradiogram (slots 5 and 6) reveals maximum specific activity of labeling in a 32—34-kdalton polypeptide. The LHC-II polypeptides also show a reduced amount of ^{14}C-labeling.

Conclusions

Besides their obvious economic importance in agriculture, photosynthetic herbicides which inhibit electron transport mediated by PS II are important as probes for elucidating the functional organization of the PS II complex. In the present investigation we have utilized a herbicide-sensitive PS II particle to identify polypeptides involved in herbicide binding.

In this study a 32—34-kdalton polypeptide of the PS II complex has been identified as the atrazine receptor protein. This identification is based upon: (a) the concomitant loss of the 32—34-kdalton polypeptide and atrazine-induced inhibition of PS II during trypsin treatment of PS II particles (Figs. 3—5), or (b) selective extraction of the 32—34-kdalton polypeptide from PS II particles (Fig. 6 and Table II). The identification was further supported by the labeling of the 32—34-kdalton polypeptide by an azido-atrazine photoaffinity label; the labeling could be prevented by competition with other herbicides (Figs. 7—9).

The 32—34-kdalton polypeptide must be localized on the external surface of PS II particles based upon the ability of trypsin, a hydrophilic protease, to cleave the polypeptide (Fig. 5 and Ref. 11). In intact thylakoid membranes, it has been observed that this polypeptide is also susceptible to mild trypsin treatment indicating exposure on the external surface [31]. Proximity to Q is suggested by the time course of trypsin treatment which revealed concomitant cleavage of the 32—34-kdalton polypeptide, loss of atrazine-induced inhibition of PS II activity, and exposure of Q as measured by an increase in the accessibility of ferricyanide as an electron acceptor (Fig. 3). Trypsin treatment of chloroplast membranes has previously been reported to cause exposure of Q [12], and to modify herbicide inhibition of PS II activity [33,34].

The results in Fig. 6 and Table II show that selective extraction of the 32—34-kdalton polypeptide caused the loss of atrazine-induced inhibition of PS II activity without a large decrease in PS II-mediated DCIP reduction. The fact that other herbicides, for example dinoseb and DCMU retain at least partial inhibition properties in PS II particles depleted of the 32—34-kdalton polypeptide (Table II), indicates that the binding of these herbicides does not have an absolute requirement for the 32—34-kdalton polypeptide.

It has been recently reported that dinoseb exhibits high-affinity and low-affinity binding [35] which may correspond to inhibition sites on the acceptor side and donor side of PS II, respectively. The results in Table I indicate that 10^{-4} M dinoseb does not significantly inhibit electron flow from I^- to ferricyanide yet inhibits DCIP photoreduction by more than 90%. These data are consistent with the action of dinoseb on the acceptor side of PS II particles. A photoaffinity dinoseb inhibitor has been found to specifically label polypeptides of approx. 35—45-kdalton [35]. This result may indicate that a polypeptide other than the 32—34-kdalton atrazine-binding polypeptide is involved in

binding dinoseb. We thus suggest that the general herbicide-binding site common to atrazine, DCMU and dinoseb consists of a minimum of two polypeptides which determine affinity and/or mediate herbicide-induced inhibition of electron transport from Q to B.

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