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INTERACTIONS OF HERBICIDES AND AZIDOQUINONES AT A PHOTOSYSTEM II BINDING SITE IN THE THYLAKOID MEMBRANE *

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6-Azido-5-decyl-2,3-dimethoxy-*p*-benzoquinone (6-azido-Q_0C_{10}) was found to replace the native plastoquinone at B (the second stable electron acceptor to Photosystem II (PS II)). The 6-azido-$Q_{10}C_{10}$ would accept electrons from the primary electron-accepting quinone, Q, thus allowing electron transport through PS II to the plastoquinone pool in thylakoids. The synthetic azidoquinone also competes with the PS II herbicides ioxynil and atrazine for binding. This observation strongly favors the hypothesis that PS II herbicides block electron transport by replacing the native quinone which acts as the second electron carrier on the reducing side of PS II (termed B). Covalent linkage of 6-azido-Q_0C_{10} to its binding environment by ultraviolet irradiation greatly reduces herbicide-binding affinity but does not lead to a loss in herbicide-binding sites. We take this as evidence that covalent attachment of 6-azido-Q_0C_{10} allows some freedom of quinone head-group movement such that the herbicides can enter the binding site. This indicates that the protein determinants which regulate quinone and herbicide binding are very closely related, but not identical. A compound somewhat related to 6-azido-Q_0C_{10} is 2-azido-3-methoxy-5-geranyl-6-methyl-*p*-benzoquinone (2-azido-Q_2). This compound was found to be an ineffective competitor with respect to herbicide binding. Thus, interactions with protein-binding determinants are highly dependent on the molecular structure of quinones. The 2-azido-Q_2 was an inhibitor of electron flow in the intersystem portion of the chain.

Introduction

PS II herbicides, such as atrazine and ioxynil, are known to inhibit photosynthetic electron transport between the primary PS II electron-accepting quinone (Q) and the two-electron gate, a plastoquinone termed B [1,2]. It has been proposed that herbicides act by replacing the quinone cofactor B, thus inhibiting the transfer of an electron from Q^- to plastoquinone (PQ) [3,4]. Such a hypothesis readily explains [3] both the inhibition of Q^- oxidation by B, and the reverse electron flow from B^- to Q observed after addition of a PS II herbicide (assuming higher binding affinity of B^- than of B) [5]. Furthermore, a replacement of herbicides by synthetic quinones has been shown [6]. However, it was still questionable whether herbicides bind to exactly identical sites as does the quinone, or to a different but closely related site such that

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Abbreviations: Chl, chlorophyll; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; $TMQH_2$, tetramethyl-*p*-benzohydroquinone; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; 6-azido-Q_0C_{10}, 6-azido-5-decyl-2,3-dimethoxy-*p*-benzoquinone; 2-azido-Q_2, 2-azido-3-methoxy-5-geranyl-6-methyl-*p*-benzoquinone; diuron (DCMU), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PQ, plastoquinone.

herbicide and plastoquinone binding strongly influence each other.

One approach to determine whether or not herbicide and plastoquinone binding are mutually exclusive is to attach covalently a quinone or herbicide to its binding environment and to study subsequently the binding characteristics of herbicides or quinones, respectively. The usual interaction of herbicides and plastoquinone with their binding sites is through noncovalent binding. However, covalent linkage of a molecule to its site may occur upon ultraviolet irradiation after introduction of an azido (N_3) group into this molecule: activition of the azido group with light of approx. 300 nm results in the formation of a highly reactive nitrene [7], which spontaneously and nonselectively reacts with molecules in its proximity, forming a covalent bond. A specific covalent attachment of an azido derivative of atrazine to a 32 kDa polypeptide, assumed to be the apoprotein of B, has been shown [8,9]. However, accurate studies of quinone binding to this site are difficult due to the generally extreme hydrophobicity of plastoquinone analogs, which results in a rather high nonspecific 'absorption' of these analogs by the thylakoid membrane. For this reason, attachment of synthetic azidoquinones to their binding site followed by herbicide-binding analysis may give much more reliable results; for many PS II herbicides, the unspecific 'absorption' of the herbicide by the thylakoid membrane is negligible at the low concentrations used [10–12]. The application of azidoquinones has been shown to be an excellent tool for characterization of ubiquinone-binding proteins in mitochondrial systems [13–16].

Materials and Methods

Isolation of chloroplast thylakoids

Seedlings of dwarf pea (*Pisum sativum* L., 'Progress No. 9' Ferry Morse Seed, Mountain View, CA) were grown in a growth chamber at 23°C. Leaves were harvested from 20–25-day-old seedlings, and were homogenized in a blender for 4–5 s at high speed with 50 mM Tricine-NaOH (pH 7.8) buffer containing 0.4 mM sorbitol and 10 mM NaCl. The homogenate was filtered through two and then 16 layers of cheesecloth, and the resulting filtrate was centrifuged at $3000 \times g$ for 5 min. The pellet was resuspended in 10 mM Tricine-NaOH (pH 7.8) buffer containing 10 mM NaCl and 5 mM $MgCl_2$, and recentrifuged for 5 min at $3000 \times g$. The thylakoid pellet was resuspended in 25 mM Tricine-NaOH (pH 7.8) buffer containing 0.1 M sorbitol, 5 mM $MgCl_2$ and 10 mM NaCl (to which we shall refer to as resuspension buffer) to a final chlorophyll concentration of 0.7–2 mg · ml^{-1}. The thylakoid isolation procedure was carried out at 0–4°C. The chlorophyll concentration was calculated from the absorption at 645 and 663 nm in 80% acetone [17].

Electron-transport studies

All electron-transport measurements were performed using an oxygen electrode (Hansatech, U.K.). Saturating light intensities for actinic illumination were achieved with a tungsten lamp after filtering through 1 cm of a saturated $CuSO_4$ solution. The temperature was 22°C. The thylakoids were suspended in the resuspension buffer, to which 5 mM NH_4Cl was added, at a chlorophyll concentration of 30 μg ml^{-1}.

To monitor electron transport through PS II, the O_2-evolution rate with 0.25 mM *p*-benzoquinone (Fluka, Switzerland) as electron acceptor was measured; 1 μM DBMIB was present. For whole-chain electron transport, the methyl viologen-mediated Mehler reaction was monitored using 70 μM methyl viologen. PS I electron transport was measured by the methyl viologen-mediated Mehler reaction (70 μM methyl viologen) in the presence of 1 μM diuron and 2.5 mM ascorbate with 0.5 mM TMPD as electron donor. The Mehler reaction with $TMQH_2$ as donor monitoring electron transport from PQ to the acceptor side of PS I [18,19], was carried out in the presence of 0.5 mM $TMQH_2$, 1 μM diuron, 70 μM methyl viologen and 10 μg · ml^{-1} superoxide dismutase (Sigma, 300 U/mg protein). $TMQH_2$ was prepared by dissolving 50 mM tetramethyl-*p*-benzoquinone (Sigma) in a chilled methanol and ethanol 1 : 1 (v/v) mixture; 3 mg $NaBH_4$ per ml solution was added, dissolved, and allowed to incubate for 5 min (the solution turns colorless); then, 9 μl concentrated HCl per ml solution was added. Addition of 1 part of this $TMQH_2$ solution to 100 parts of resuspension buffer did not change the buffer pH. The $TMQH_2$ stock solution was prepared

immediately before use and was kept in a narrow capped tube on ice.

Azidoquinone attachment

6-azido-Q_0C_{10} and 2-azido-Q_2 were synthesized as described previously [16] and dissolved in ethanol to a final concentration of 140 μM. An aliquot of either quinone solution was added to a thylakoid suspension (100 μg Chl $\cdot$ ml^{-1}) in the resuspension buffer. 8 ml of this mixture were transferred to a petri dish of 9 cm diameter; the petri dish was sealed by two layers of Vitafilm (Goodyear), which transmits long-wavelengths ultraviolet ($\lambda > 300$ nm) but absorbs light of lower wavelengths. The azido group of 2-azido-Q_2 and 6-azido-Q_0C_{10} absorbs maximally at approx. 305 and 315 nm, respectively (data not shown). The petri dish containing the sample was then embedded in ice, and irradiated for 1 h with a germicidal lamp (Sylvania G30T8). The distance between the ultraviolet lamp and the sample was 7 cm. In control experiments, run in parallel, the azidoquinone was added directly after ultraviolet irradiation or not at all. After ultraviolet treatment, the thylakoids were centrifuged ($4000 \times g$ for 5 min) and resuspended into resuspension buffer. The azidoquinone added before or after the ultraviolet treatment was not washed out by this procedure, indicating that almost all quinone had partitioned into the thylakoids.

Herbicide binding

A thylakoid suspension in resuspension buffer (50 μg Chl $\cdot$ ml^{-1}) was divided into 1-ml samples, and different amounts (25–250 nM) of [^{14}C]ioxynil (spec. act. 33.5 μCi $\cdot$ mg^{-1}; May and Baker Ltd., U.K.) or [^{14}C]atrazine (spec. act. 37 μCi $\cdot$ mg^{-1}; CIBA-GEIGY, Greensboro, NC) were added. The ioxynil was allowed to incubate for 30 min with the thylakoid suspension in the dark and the atrazine for 10 min to allow a full equilibration of herbicide binding. Then, the thylakoids were pelleted by centrifugation in a Fisher micro-centrifuge (model 235) for 2.5 min; 0.8 ml of the supernatant was transferred to a scintillation vial containing 10 ml ACS scintillation fluid (Amersham, Arlington Heights, IL). All manipulations were carried out in weak light. The vials were counted in a Beckman scintillation counter (model LS 7500) and the data were converted into values that can be used in a double-reciprocal plot for herbicide binding (1/free herbicide concentration vs. mg Chl per nmol bound herbicide) [10].

Fluorescence induction

A cuvette with 1 ml of dark-adapted thylakoids in resuspension buffer (10 μg Chl $\cdot$ ml^{-1}) was illuminated by opening an electronically controlled shutter (Uniblitz 26 L2 AOX 5; opening time approx. 1.0 ms) between a tungsten lamp and the sample. The actinic light was filtered through a blue Corning 4-96 filter. The fluorescence was detected by a photodiode (UDT 500D) shielded from scattered actinic light by a red 3 mm Corning 2-64 filter. The signal was recorded and stored by a Nicolet Explorer III oscilloscope.

Oxygen flash yield

A drop of thylakoids in resuspension buffer (0.5 mg Chl $\cdot$ ml^{-1}) was applied to the Pt electrode surface of an unmodulated Joliot-type O_2 electrode [20] and was covered by a single dialysis membrane. The compartment between membrane and the Ag/AgCl electrode was filled with resuspension buffer. The thylakoids were allowed to settle and to dark adapt for 10 min; then they were illuminated with saturing 1 Hz xenon flashes (3 μs half-width) from a Stroboslave (type 1539 A, General Radio, Concord, MA). The signals were recorded and stored by a Nicolet Explorer III oscilloscope. The best-fitting miss parameter, α, double-hit parameter, β, and the percentage of centers in state S_0 and S_1 after dark adaptation were calculated from the relative O_2 evolution in the first 10 flashes using the four-step model of Kok et al. [22].

Results

A first approach to investigate the site(s) of action of the azidoquinones was to test their effects on different parts of the electron-transport chain. As is shown in Fig. 1, PS II electron transport to PQ as measured by the *p*-benzoquinone Hill reaction is virtually insensitive to the quinones. This electron transport was also measured by oxygen evolution with diaminodurene/ferricyanide as electron acceptor in the presence of 1

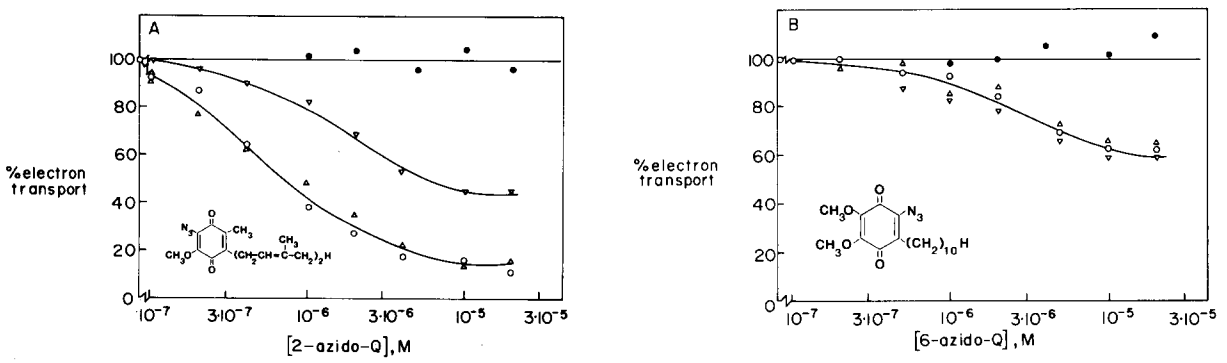

Fig. 1. Effects of 2-azido-Q_2 (A) and 6-azido-Q_0C_{10} (B) on electron transport. The chemical structures of each compound are indicated as part of the figure. (●) $H_2O \rightarrow$ *p*-benzoquinone in the presence of 1 μM DBMIB; (○) $H_2O \rightarrow$ methyl viologen; (△) $TMQH_2 \rightarrow$ methyl viologen; (▽) reduced TMPD → methyl viologen. The control rates were 48 μmol O_2/mg Chl per h for $H_2O \rightarrow$ *p*-benzoquinone + 1 μM DBMIB, 160 μmol O_2/mg Chl per h for $H_2O \rightarrow$ methyl viologen, 361 μmol O_2/mg Chl per h for $TMQH_2 \rightarrow$ methyl viologen, and 730 μmol O_2/mg Chl per h for $TMPDH_2 \rightarrow$ methyl viologen.

μM DBMIB, and was also found to be insensitive to azidoquinone addition (data not shown). However, when 2-azido-Q_2 is added to an assay for PS I electron transport with $TMQH_2$ as electron donor, the reaction was more sensitive to azidoquinone addition than when $TMPDH_2$ served as electron donor; this suggests that 2-azido-Q_2 may interfere with the PQH_2 oxidation by the Rieske iron-sulfur center; with 6-azido-Q_0C_{10} there was a partial inhibition of both whole-chain and PS I electron transport (Fig. 1B). No part of the electron-transport chain can be fully inhibited by azidoquinone concentrations of up to 20 μM (this concentration is equivalent to 1 azidoquinone per 1.5 Chl molecules; in the following sections we will refer to azidoquinone/Chl ratios instead of azidoquinone concentrations because the azidoquinone partitions into the thylakoid membrane, resulting in a very low free quinone concentration).

In order to test the azidoquinone affinity for the binding environment near Q, to which plastoquinone and herbicides are able to bind, we measured the [^{14}C]atrazine affinity [10] in the presence and absence of azidoquinones (Fig. 2). None of these samples were ultraviolet treated and, thus, the azidoquinones are noncovalently bound to binding sites. A strong interaction between 6-azido-Q_0C_{10} and atrazine binding was observed. At a 6-azido-Q_0C_{10}/Chl ratio of 1 : 140, the [^{14}C]atrazine affinity (proportional to the distance between origin and the abscissa intercept) decreased by 25% compared to the control; after an increase in the 6-azido-Q_0C_{10}/chl ratio to 1 : 40, which means that there are approx. 10 6-azido-Q_0C_{10} molecules per PS II reaction center, the atrazine affinity is decreased to approx. 1/3 of the original value. The observation that all binding curves intersect at the *y*-axis (Fig. 2) indicates that all 6-azido-Q_0C_{10} can be removed from the quinone/herbicide-binding site at high concentra-

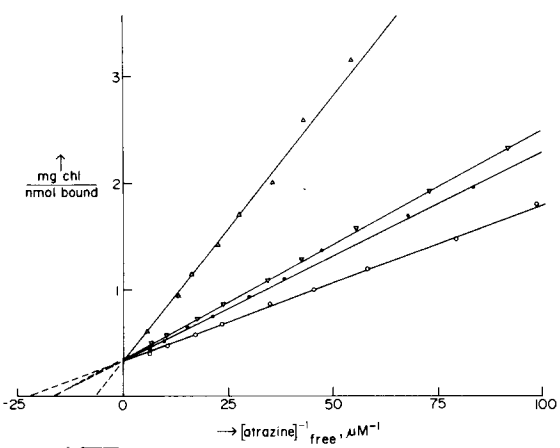

Fig. 2. Double-reciprocal plot of free and thylakoid bound [^{14}C]atrazine in the presence and absence of azidoquinones. (○) No azidoquinone addition; (△) + 6-azido-Q_0C_{10} (6-azido-Q_0C_{10}/Chl = 1 : 40); (▽) + 6-azido-Q_0C_{10} (6-azido-Q_0C_{10}/Chl = 1 : 140); (●) + 2-azido-Q_2 (2-azido-Q_2/Chl = 1 : 3).

tions of atrazine. This demonstrates that the interaction between 6-azido-Q_0C_{10} and [^{14}C]atrazine is competitive. In this type of double-reciprocal herbicide-binding plot, the intercept of a line with the ordinate multiplied by 10^6 divided by the molecular weight of the chlorophyll (approx. 1000) is equal to the number of Chl molecules per herbicide binding site (see also Table I).

Addition of 2-azido-Q_2 was found to cause much less replacement of [^{14}C]atrazine than 6-azido-Q_0C_{10} (Fig. 2); a 2-azido-Q_2/Chl ratio of 1 : 3 is needed to observe a measurable [^{14}C]atrazine displacement. Decreasing the ratio to 1 : 10 or less resulted in the disappearance of any significant atrazine displacement (data not shown). Another azidoquinone, 2,3-dimethoxy-5-methyl-6-{10-[4-(azido-2-nitroanilinopropionoxy)]decyl}-*p*-benzoquinone (Q_0C_{10}-NAPA, synthesized as described in Ref. 21), was also found to be an ineffective atrazine displacer (data not shown).

Qualitatively, the interaction between [^{14}C]-ioxynil, a nitrilic herbicide, and 6-azido-Q is identical to the one between [^{14}C]atrazine and 6-azido-Q; 6-azido-Q decreases the ioxynil affinity for its binding site, but does not change the number of ioxynil-binding sites (the lines intersect at the ordinate) (Fig. 3). Therefore, 6-azido-Q_0C_{10} and ioxynil interact for binding to the quinone/herbicide-binding environment competitively (see also Table II). Quantitatively, ioxynil affinity is slightly less affected by 6-azido-Q_0C_{10} addition than is the atrazine affinity.

The observation that 6-azido-Q_0C_{10} binds to the quinone/herbicide-binding environment (Figs. 2 and 3), but does not inhibit PS II electron transport (Fig. 1), implies that 6-azido-Q_0C_{10} may be able to oxidize Q^- rather efficiently. To test this, we measured oxygen evolution as a function of flash number using an unmodulated Joliot-type electrode. If 6-azido-Q_0C_{10} is able to oxidize Q^- completely in the dark time between the flashes, then neither a loss in O_2 flash yield nor an increased damping in the period-of-four oscillation of O_2 evolution with flash number [22] would be expected. The results of two experiments are shown in Fig. 4. The O_2-evolution pattern in the absence of 6-azido-Q_0C_{10} can be fitted by assuming the following parameters: miss parameter $\alpha = 0.16$; double-hit parameter $\beta = 0.05$; fraction of thylakoids in S_0 state after dark adapation, 26%; and fraction in S_1 state, 74%. In the presence of 6-azido-Q_0C_{10} (6-azido-Q_0C_{10}/Chl ratio = 1 : 10) the best-fitting parameters are $\alpha = 0.29$, $\beta = 0.04$, $S_0 = 25\%$ and $S_1 = 75\%$. We observed consistently that only the miss parameter α is significantly changed upon 6-azido-Q_0C_{10} addition (Fig. 5). The oxygen flash yield does not decrease more by

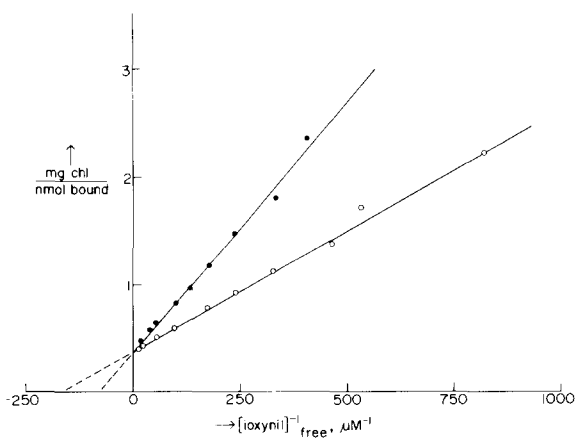

Fig. 3. Double-reciprocal plot of free and thylakoid-bound [^{14}C]ioxynil in the presence (●) and absence (○) of 6-azido-Q_0C_{10} (6-azido-Q_0C_{10}/Chl = 1 : 10).

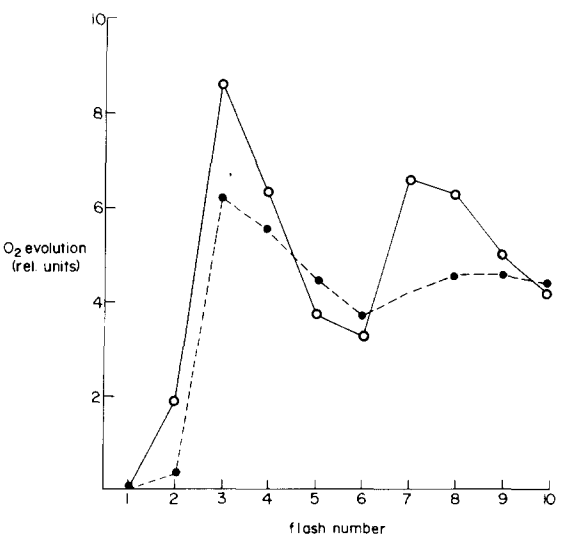

Fig. 4. Oxygen evolution as a function of flash number in the presence (●) and absence (○) of 6-azido-Q_0C_{10} (6-azido-Q_0C_{10}/Chl = 1 : 10).

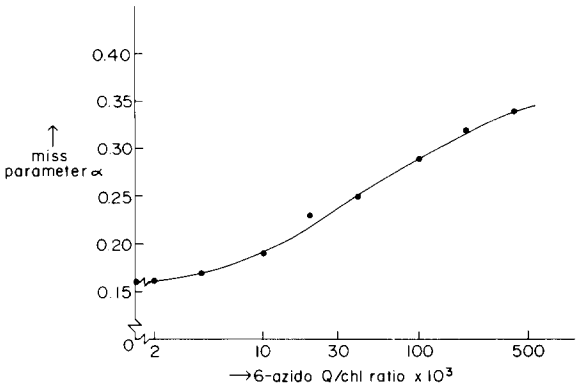

Fig. 5. Miss parameter (α) dependence on addition of 6-azido-Q_0C_{10}. α was calculated from the O_2-evolution pattern as a function of flash number.

6-azido-Q addition than would be expected from the increased α value. This implies that 6-azido-Q_0C_{10} can be reduced by Q^-, but that the Q^- concentration present 1 s after a flash is higher than when PQ oxidizes Q^- (due to a slower electron transfer from Q^- to the synthetic quinone and/or a different equilibration state between the two electron carriers. We consistently observed that the O_2 yield in the second flash decreased much more upon 6-azido-Q_0C_{10} addition than would be expected from the increased α value. Perhaps 6-azido-Q_0C_{10} may also have effects on the donor side of PS II, for example on processes involving the molecule responsible for EPR Signal II (which may be a quinone [23]).

In the experiments described above, 6-azido-Q_0C_{10} was noncovalently attached to its binding site, and could be replaced by herbicides. The next question posed was whether herbicide binding is still possible when the azidoquinone is covalently attached to its binding environment. For these experiments, [^{14}C]atrazine or [^{14}C]ioxynil binding was measured with thylakoids to which 6-azido-Q_0C_{10} (6-azido-Q_0C_{10}/Chl ratio = 1 : 40) was added before or after ultraviolet treatment (Fig. 6). K_b values and the number of herbicide-binding sites for these experiments are listed in Tables I and II. Although neither the ioxynil affinity nor the number of binding sites is changed by covalent linkage of 6-azido-Q_0C_{10} to its binding environment, atrazine affinity is decreased by a factor of two. The number of atrazine-binding sites, however, is not affected. It should be noted that the ultraviolet treatment itself seems to change some of the binding properties of the herbicides to the binding sites that are left. We observe a decrease in herbicide affinity after ultraviolet treatment. The treatment also seems to influence the quinone binding; although in controls the [^{14}C]atrazine binding constant increases by a factor of more than three upon addition of 6-azido-Q_0C_{10} (6-

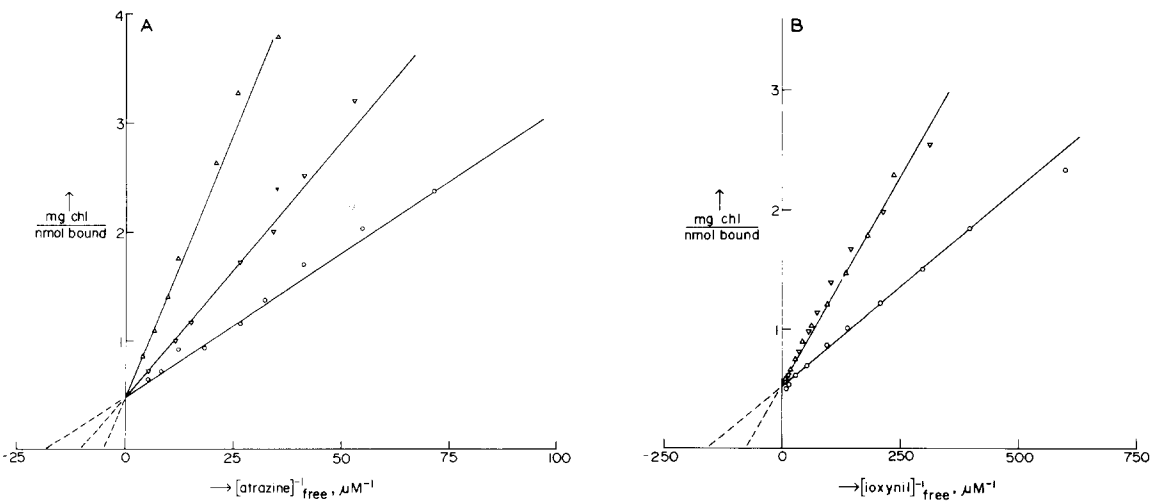

Fig. 6. Double-reciprocal plot of free and bound [^{14}C]atrazine (a) and ioxynil (b) in ultraviolet-treated thylakoids. Azidoquinone addition, if any, occurred before (△) or after (▽) ultraviolet treatment. Binding of the herbicide was then measured in the absence (○) or presence (△, ▽) of 6-azido-Q_0C_{10} (6-azido-Q_0C_{10}/Chl = 1 : 40).

TABLE I

[^{14}C]ATRAZINE-BINDING CONSTANT (K_b) AND NUMBER OF CHLOROPHYLL MOLECULES PER ATRAZINE-BINDING SITE AS A FUNCTION OF AZIDOQUINONE CONTENT AND ULTRAVIOLET IRRADIATION

Data are taken from Figs. 2 and 6A. K_b is defined as the concentration of free herbicide needed to cause occupation of 50% of the binding sites by that herbicide; K_b is equal to the negative reciprocal of the intersection of the double-reciprocal binding curve with the abscissa. +Q+UV means that the azidoquinone was added before ultraviolet treatment of the thylakoids; +UV+Q indicates that 6-azido-Q was added after ultraviolet illumination of the thylakoids.

Addition	Azidoquinone/Chl ratio	Ultraviolet	K_b (nM)	Chl per binding site
None	–	–	45	340
2-Azido-Q_2	1:3	–	63	340
6-Azido-Q_0C_{10}	1:140	–	61	340
6-Azido-Q_0C_{10}	1:40	–	150	340
None	–	+	55	490
6-Azido-Q_0C_{10}	1:40	+Q+UV	208	490
6-Azido-Q_0C_{10}	1:40	+UV+Q	96	490

azido-Q_0C_{10}/Chl = 1 : 40), in ultraviolet-treated samples the increase is less than a factor of two upon 6-azido-Q_0C_{10} addition. For ioxynil binding, this difference appears to be less.

One might argue that the lack of loss of herbicide-binding sites upon covalent azidoquinone attachment, expected to occur in the case of identical binding sites for quinones and herbicides, is due to a lack of an efficient quinone attachment to the binding environment. In order to test such a suggestion, we monitored fluorescence induction kinetics of ultraviolet-treated thylakoids with and without 6-azido-Q_0C_{10} addition (6-azido-Q_0C_{10}/Chl = 1 : 20). The thylakoids were incubated with 5mM NH_2OH for 10 min prior to the fluorescence induction measurement to exclude possible effects related to the donor site of PS II. The results are shown in Fig. 7. In the absence of 6-azido-Q_0C_{10} a rather normal fluorescence induction curve was observed. Addition of 6-azido-Q_0C_{10} after ultraviolet treatment resulted in a markedly lower initial fluorescence yield F_0, caused by the fluorescence quenching properties of the oxidized 6-azido-Q_0C_{10}. The I level in this induction curve is rather low, indicating that no significant Q^- accumulation due to a blockage of electron flow between Q and the PQ pool occurs. This strengthens our proposal that 6-azido-Q_0C_{10} accepts electrons from Q^- effectively. The area over the fluorescence induction curve (curve 2) is much larger than the area obtained using samples without 6-azido-Q_0C_{10}, which indicates that 6-azido-Q_0C_{10}

TABLE II

[^{14}C]IOXYNIL BINDING CONSTANT (K_b) AND NUMBER OF CHLOROPHYLL MOLECULES PER IOXYNIL-BINDING SITE AS A FUNCTION OF AZIDOQUINONE CONTENT AND ULTRAVIOLET IRRADIATION

Data are taken from Figs. 3 and 6B. See the legend to Table I for explanation of K_b, +Q+UV and +UV+Q.

Addition	Azidoquinone/Chl ratio	Ultraviolet	K_b (nM)	Chl per binding site
None	–	–	6.0	380
6-Azido-Q_0C_{10}	1:40	–	12.8	380
None	–	+	6.7	530
6-Azido-Q_0C_{10}	1:40	+Q+UV	13.3	530
6-Azido-Q_0C_{10}	1:40	+UV+Q	13.3	530

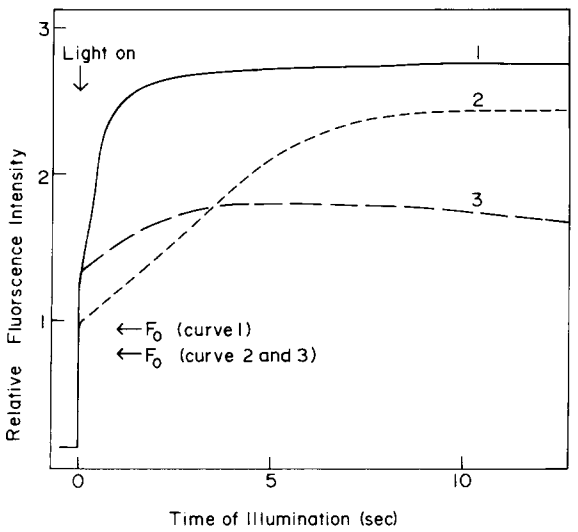

Fig. 7. Fluorescence induction curves of ultraviolet-treated thylakoids. Curve 1 (———), no 6-azido-Q_0C_{10} addition; curve 2 (- - - - -), 6-azido-Q_0C_{10} added after ultraviolet treatment; curve 3 (— —), 6-azido-Q_0C_{10} added before ultraviolet treatment. The 6-azido-Q/Chl ratio is 1 : 20 for curves 2 and 3. The initial fluorescence yield F_0 is indicated.

is photoreduced by PS II. The maximal fluorescence yield in the presence of 6-azido-Q_0C_{10} approaches the yield obtained without addition of the azidoquinone, suggesting that most of the quinone is reduced after a few seconds of illumination. However, when 6-azido-Q_0C_{10} is added before ultraviolet treatment, a fast rise in the fluorescence yield occurs, which, on a decreased time scale, appears to be just as fast as the rise in the presence of diuron (data not shown). The fast initial rise indicates a blockage of Q^- oxidation in a large portion of the PS II electron-transport chains. The slower following rise is due to electron transport in PS II electron chains which are still active. Only a small part of the 6-azido-Q_0C_{10} population appears to be photoreducible after covalent attachment of the azidoquinone. These data indicate that ultraviolet irradiation in the presence of 6-azido-Q_0C_{10} leads to a disturbance of Q^- oxidation, which does not occur if 6-azido-Q_0C_{10} is added after ultraviolet irradiation. This strongly suggests that, under the conditions used, covalent attachment of 6-azido-Q_0C_{10} is at the domain of the PS II complex which is normally the binding site for the quinone which accepts electrons from Q^-.

Discussion

The data presented here indicate that 6-azido-Q_0C_{10} binds with a high affinity to the site where herbicides (and plastoquinone) also bind (see Figs. 2 and 3, and Tables I and II) and, moreover, is able to oxidize Q^- (Figs. 4 and 7). To our knowledge, reports of oxidation of Q^- by a quinone other than the native plastoquinone are scarce. Recently, Lavergne [24] has suggested that the *p*-benzosemiquinone anion may be able to bind to the quinone/herbicide-binding environment and to oxidize Q^-. The residence time of that semiquinone at the site was estimated to be more than 2 min. However, *p*-benzoquinone in the oxidized form cannot oxidize Q^- to any significant extent [24]. Velthuys [3] proposed a Q^- oxidation by both the oxidized and semiquinone form of DBMIB at rather high DBMIB concentrations (approx. 1 μM). However, at such DBMIB concentrations PS II electron transport is partly inhibited [25]. In contrast, 6-azido-Q_0C_{10} does not lead to any significant inhibition of the steady-state rate of PS II electron flow. Thus, 6-azido-Q_0C_{10} appears to be a better electron acceptor from Q^- than other synthetic quinone analogs studied thus far.

At first glance, it may seem contradictory to propose that 6-azido-Q_0C_{10} is an efficient electron acceptor (Figs. 1 and 2) and to show also an increase in α (the miss parameter) which is presumably due to an increase in the 'dark' Q^- concentration measured 1 s after a flash in the presence of 6-azido-Q_0C_{10} (Fig. 5). We suggest that the high α value is due to a greater probability of finding an electron on Q in the complex $(Q \cdot A)^-$, in which A represents 6-azido-Q_0C_{10} reversibly bound to the quinone/herbicide-binding environment, than of finding a reduced Q in the complex $(Q \cdot B)^-$, in which B is the native PQ bound to the site. In other words, we propose that the midpoint potential of the A/A^- couple is lower than that of the B/B^- couple. Furthermore, as soon as B^{2-} is formed, it is assumed to be protonated and to have lost a high affinity for the binding site [3]; however, we consider it probable that 6-azido-Q_0C_{10} has a high affinity for its binding site in the

oxidized form (6-azido-Q_0C_{10} competes very effectively with atrazine and ioxynil binding; Figs. 2 and 3; Tables I and II), and may also have a reasonable affinity in the fully reduced form. In this respect we note that the replacement of herbicides by most chlorinated *p*-benzoquinones appears to be virtually independent of the redox state of these synthetic quinones [6]. Therefore, the change in midpoint potential of A/A^- compared to B/B^- may not be the only reason for a higher equilibrium Q^- concentration. The total number of relatively free electrons in the Q-bound quinone complex is 0 or 1 if the bound quinone is PQ (assuming that B^{2-} is protonated and released immediately), whereas this number may be up to 3 when 6-azido-Q_0C_{10} is bound (assuming a relatively high affinity for the fully reduced quinone, and a relatively low pK_a). The residence time of 6-azido-Q_0C_{10} at its site in the quinone/herbicide-binding domain appears to be less than the time of the rate-limiting step in Hill reactions because no Hill reaction inhibition by 6-azido-Q_0C_{10} in PS II is observed (Fig. 1).

An understanding of 6-azido-Q_0C_{10} binding to the PS II complex allows an interpretation of the interaction between 6-azido-Q_0C_{10} and herbicides. Surprisingly, after covalent azidoquinone attachment to thylakoids, the number of binding sites remains unchanged for both types of herbicides tested (Fig. 6). In addition, covalent linkage of 2-azido-Q_0C_{10} (azidoquinone/Chl ratio = 1 : 3) does not result in a loss of atrazine-binding sites (data not shown). To explain these observations, one might argue that both atrazine and ioxynil do not replace the quinone head (the ring), but only the flexible hydrophobic tail of quinones. In this case, herbicide binding would still be observed, though with changed affinity, after covalent attachment of the azidoquinone to the quinone/herbicide-binding environment. However, chlorinated *p*-benzoquinones that lack the hydrophobic tail are still able to compete with ioxynil and atrazine [6]. Thus, this hypothesis is highly unfavorable. We think it to be much more plausible that the covalent linkage of the azidoquinone to the protein creating the binding site still allows a large degree of freedom of movement of the quinone. This would allow the herbicide to enter the binding site, although with a low probability. The result is that the herbicides can bind with weak affinity without a loss of binding sites. There may also be some conformational change in the herbicide-binding protein concomitant with covalent quinone attachment that forces the azidoquinone slightly out of its binding site. The fluorescence data presented support this idea; covalently linked 6-azido-Q_0C_{10} is no longer able to oxidize Q^- (the fast rise in fluorescence induction has kinetics identical to those of the fluorescence induction in the presence of diuron), possibly due to a disturbance of the spatial arangement of 6-azido-Q_0C_{10} relative to Q. Alternatively, the covalently linked quinone may be in the same site as the noncovalently linked quinone, but with altered redox properties due to the chemical bonding to the site.

The ideas presented above imply that the binding determinants in the protein creating the binding site for the herbicides ioxynil and atrazine are not absolutely identical to those of the quinones (if this were true we would have expected an absolute loss of herbicide-binding sites upon covalent attachment of the quinone). However, the observation that 6-azido-Q_0C_{10} and atrazine or ioxynil compete for binding to the quinone/herbicide-binding environment implies that the binding sites are spatially overlapping but not identical.

To conclude, we believe that 6-azido-Q_0C_{10} is at this time a unique compound that can: (a) replace PS II herbicides, but does not bind identically to atrazine and ioxynil; (b) oxidize Q^- efficiently; and (c) be covalently attached to its binding environment. The related compound 2-azido-Q_2 does not show these features at low concentrations, indicating that binding to the quinone/herbicide-binding environment requires stringent, specific interactions between the small molecules and the protein determinants creating the binding site. Interestingly, 2-azido-Q_2 turns out to be a much better tool for research on the mitochondrial succinate-cytochrome *c* reductase than 6-azido-Q_0C_{10} [16]. The data of Fig. 1a can be interpreted to indicate that 2-azido-Q_2 also blocks electron transfer in thylakoids at the micromolar range; this inhibition might be at the level of the Rieske Fe-S and cytochrome b_6-*f* region of thylakoids. The 2-azido-Q_2 may therefore be a useful tool for analysis of those oxidation-reduction processes.

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