Plant Physiol. (1987) 83, 1027–1031
0032-0889/87/83/1027/05/$01.00/0

Interaction of Herbicides and Quinone with the Q_B-Protein of the Diuron-Resistant *Chlamydomonas reinhardtii* Mutant Dr2

Received for publication November 26, 1986

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ABSTRACT

We have used the diuron-resistant Dr2 mutant of *Chlamydomonas reinhardtii* which is altered in the 32 kilodalton Q_B-protein at amino acid 219 (valine to isoleucine), to investigate the interactions of herbicides and plastoquinone with the 32 kilodalton Q_B-protein. The data contained in this report demonstrate that the effects of this mutation are different from those of the more completely characterized mutant which confers extreme resistance to triazines in higher plants. The mutation in *C. reinhardtii* Dr2 confers only slight resistance to a number of inhibitors of photosynthetic electron transport. Extreme triazine resistance results from an increase in the binding constant of the herbicide with the 32 kilodalton Q_B-protein, in contrast the diuron binding constant for chloroplasts isolated from wild-type (sensitive) *Chlamydomonas* and the resistant Dr2 are indistinguishable. We conclude that the altered structure in the 32 kilodalton Q_B-protein of Dr2 does not directly affect the diuron binding site. This mutation appears to alter the steric properties of the binding protein in such a way that diuron and plastoquinone do not directly compete for binding. This steric perturbation confers mild resistance to other herbicidal inhibitors of photosynthesis and alters the kinetics of Q_A to Q_B electron transfer.

Resistance to herbicides which inhibit electron transport has been noted in a number of photosynthetic organisms from bacteria (20) to higher plants (1). In all the documented cases, resistance has been correlated with changes in the primary structure of the 32 kD Q_B-protein, also referred to as the herbicide-binding protein (1, 2). The function of this protein is to reversibly bind a special plastoquinone which serves as the second stable electron acceptor (Q_B) on the reducing side of PSII (17).

The type of herbicide resistance most extensively characterized to date is extreme triazine-resistance in green plants and algae (1, 18). Based on sequence analysis of the chloroplast *psb*A gene encoding the resistance trait, it is known that a point mutation resulting in a single amino acid change (serine 264 to glycine [12] or alanine [6]) in the 32 kD Q_B-protein is responsible for the triazine-resistance. In thylakoids extracted from these triazine-resistant plants the binding constant for atrazine is significantly altered (4×10^{-8} M in a susceptible form *versus* $>10^{-4}$ M in resistant biotypes [18]). This loss of triazine binding is thought to arise directly from a glycine/alanine substitution at serine 264. Although the binding of diuron to the 32 kD Q_B-protein of these triazine-resistant mutants is essentially unaffected, the binding of the plastoquinone Q_B does appear to be altered. This alteration is reflected in decreased kinetics of electron transfer from Q_A to Q_B (11). This mutation has been shown by Ort *et al.* (16) to result in reduced fitness at the whole plant level.

We have further characterized the herbicide binding properties of the diuron-resistant mutant Dr2 of *Chlamydomonas reinhardtii* (8) which has been reported to show 17-fold resistance to diuron and only 2-fold resistance to atrazine (9). The purpose of further characterization of the Dr2 mutant was to clarify the effects of this single amino acid change with respect to the binding of herbicides and plastoquinone and their interactions with the 32 kD Q_B-protein.

MATERIALS AND METHODS

Chlamydomonas reinhardtii Dr2 and 2137c$^+$, the wild-type strain from which Dr2 was isolated (9), were grown phototrophically (20) under constant illumination (100 μE m^{-2} s^{-1}) at 26°C. The Dr2 mutant cells were grown in liquid culture in the presence of 10^{-6} M diuron to maintain resistance, and grew at the same rate as 2137c$^+$ grown in the absence of diuron. The cells were harvested in late log phase, approximately 4 d after inoculation, by centrifugation at 2000*g* for 5 min. Thylakoids suspended in a buffer consisting of 50 mM K-phosphate (pH 7.5), 5 mM $MgCl_2$ and 1% (w/v) BSA at a Chl concentration of 300 μg/ml were isolated by passage through a French pressure cell (1000 p.s.i.). Electron transport studies with *C. reinhardtii* isolated thylakoid membranes were carried out according to Pfister *et al.* (18) measuring 2-6-dichlorophenolindophenol reduction using a Perkin-Elmer model Lambda 3 spectrophotometer. O_2 evolution was measured with an O_2 electrode (model YSI) and whole cell fluorescence transients of *C. reinhardtii* were determined by the procedure described by Arntzen *et al.* (1). Values of F_o, F_i, and F_m were calculated from recordings of these transients.

The procedure for calculating herbicide binding constants using [^{14}C-1-ethyl]atrazine (Amersham) was adopted from the protocol of Pfister *et al.* (18). Determination of Chl content and Chl *a*/*b* ratios were according to the procedure of Mackinney (13).

RESULTS

A detailed characterization of mutant Dr2 requires complete analysis of the biochemical changes in the structure and function of thylakoids resulting from this mutation. The first step of this investigation was to analyze cells of *Chlamydomonas reinhardtii*, grown under phototrophic conditions, for Chl content. Wild-type cells contained 33 ± 3 μg Chl/10^7 cells with a Chl *a*/*b* ratio of 3.1 ± 0.2. The Dr2 mutant had 29 ± 3 μg Chl/10^7 cells and a Chl *a*/*b* ratio of 2.9 ± 0.2. The whole-cell O_2 evolution rate as a function of actinic light intensity for wild-type and Dr2 cells is shown in Figure 1. The light-saturated O_2 evolution rate for wild-type cells was 0.236 mmol O_2/mg Chl·h, while the rate for Dr2 under the same conditions was 0.188 mmol O_2/mgChl·h, some 20% less. Similar decreases in photosynthetic function were reported by Ort *et al.* (16) for triazine-resistant biotypes of

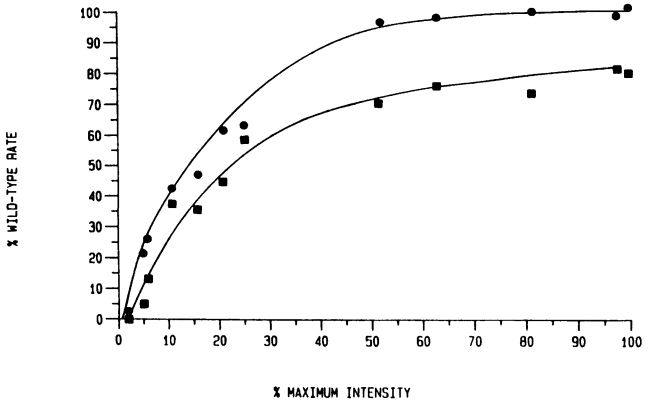

FIG. 1. Intensity dependence of O_2 evolution from cells of *C. reinhardtii* wild-type (2137c$^+$) (●) and Dr2 (■). Chl concentration 50 μg/ml, volume 3 ml, temperature 30°C, maximum actinic intensity 1.3×10^3 μE m^{-2} s^{-1}, resuspension buffer; consisting of 50 mM K-phosphate (pH 7.5), 5 mM $MgCl_2$, and 1% BSA. Maximum rates for 2137c$^+$ and Dr2 of 0.23 and 0.18 mmol O_2/mg Chl, respectively.

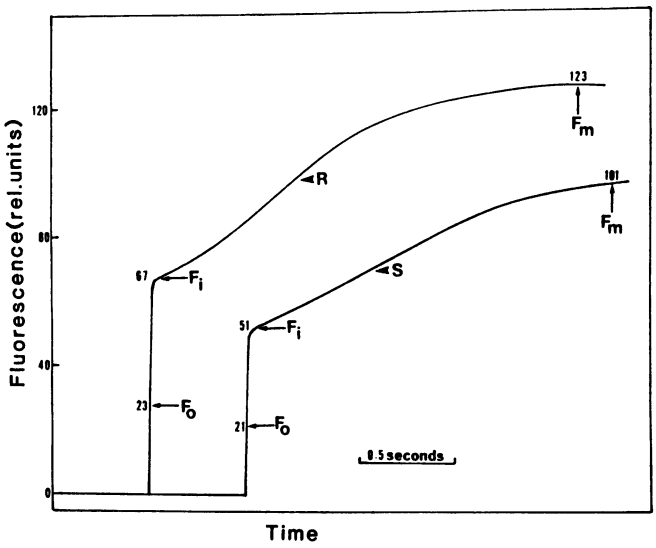

FIG. 2. Fluorescence transients from cells of *C. reinhardtii* 2137c$^+$ and Dr2. Transients were recorded using the procedure of Arntzen *et al.* (1), values of F_o, F_i, and F_m are presented in arbitrary units; see text for detail.

Amaranthus hybridus. This difference in overall photosynthetic rate is observed at all but limiting light intensities, where the two rates are indistinguishable.

Fluorescence transients obtained from the wild-type and the resistant Dr2 cells are presented in Figure 2. Using these transients it is possible to calculate the ratio $(F_m - F_i)/(F_m - F_o)$, where F_o is dead fluorescence, F_i is the level of the initial rapid fluorescence rise, and F_m is the maximum steady state fluorescence (1). The calculated ratio normalizes the F_i level as a proportion of total fluorescence. It has been observed that plant biotypes resistant to atrazine have a higher ratio (lower F_i) than sensitive biotypes (1), a phenomenon that has been directly correlated to a slower Q_A to Q_B electron transfer step (11). The ratios calculated from Figure 2 for wild type and Dr2 are 0.63 and 0.56, respectively, suggesting that the reduced O_2 evolution rate of Dr2 is also due to a slower Q_A to Q_B electron transfer step.

We have determined the degree of resistance to diuron at the whole cell level by measuring O_2 evolution as shown in Figure 3A. Mutant Dr2 is approximately 8.5-fold resistant to diuron.

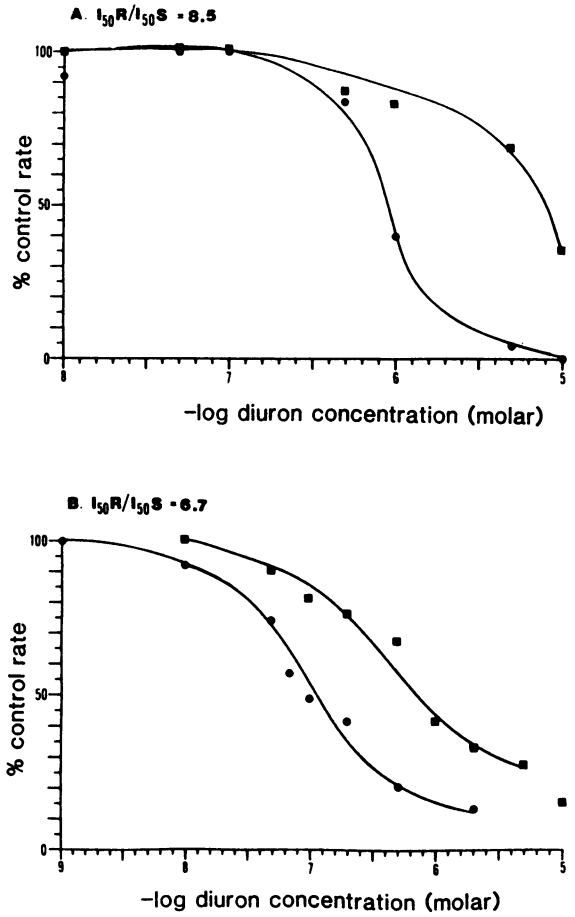

FIG. 3. Diuron dose response for inhibition of photosynthetic oxygen evolution (A) and electron transport:water to DCPIP (B) in wild-type (●) and Dr2 (■) *C. reinhardtii*. Each data point is the mean of two separate experiments. I_{50} value, the molar inhibitor concentration giving 50% inhibition, was calculated directly from these curves. Methods listed in Table II.

At the chloroplast level we have measured electron transport (H_2O to DCPIP) and detect a 6.7-fold resistance, (Fig. 3B). Although the level of diuron resistance observed in this study is less than the 17-fold resistance reported by Galloway and Mets (9), these data consistently indicate only a moderate level of diuron resistance. Because most inhibitors of photosynthesis act by binding to the 32 kD Q_B-protein, a mutant which has an altered sensitivity to one class of inhibitors will often display resistance or hypertensitivity to others (4, 23). Accordingly, we have tested the sensitivity of *C. reinhardtii* Dr2 to a number of photosynthesis inhibitors from diverse chemical classes and compared them directly to the wild-type strain 2137c$^+$. These data are summarized in Table I. The *C. reinhardtii* mutant Dr2 displays moderate resistance to almost all classes of photosynthesis inhibitors except the s-triazines (atrazine and terbutryn). Compounds tested on both the whole cells and isolated chloroplasts display the same level of resistance for both systems, an observation which indicates that the basis of resistance does lie at the thylakoid membrane level for all of these photosynthesis inhibitors.

The binding of [^{14}C]atrazine to thylakoid membranes of higher plants is a well characterized phenomenon (14, 18) and has been a valuable tool in quantitation of resistance to photosynthesis inhibitors. The theory behind this double-reciprocal analysis of herbicide binding is adapted directly from the Lineweaver-Burk principal of enzyme kinetics. In this application atrazine is

Table I. *Comparison of Herbicide Activity in C. reinhardtii 2137c⁺ (S) and Dr2 (R); Determined by Inhibition of Photosynthetic and Electron Transport*

The resuspension medium included: 50 mM K-phosphate (pH 7.5), 5 mM $MgCl_2$, and 1% BSA.

Herbicide	Class	I_{50}					
		Whole cell electron transport[a]			Chloroplast electron transport[b]		
		S	R	R/S	S	R	R/S
		O_2 Evolution		*ratio*	*Water to DCPIP*		*ratio*
Diuron	Urea	9.4×10^{-7} M	7.9×10^{-6} M	8.5	1.0×10^{-7} M	6.7×10^{-7} M	6.7
Monuron	Urea				1.0×10^{-6} M	1.5×10^{-5} M	15.0
Terbutryny	Triazine				2.5×10^{-8} M	4.0×10^{-8} M	1.5
Atrazine	Triazine	2.7×10^{-6} M	5×10^{-6} M	2.0	2.2×10^{-7} M	7.9×10^{-7} M	4.5
Metribuzin	Triazinone	7.5×10^{-7} M	7.9×10^{-6} M	10.5			
Propanil	Amide	2.0×10^{-6} M	1.8×10^{-5} M	10.0	5.0×10^{-7} M	5.7×10^{-6} M	11.4
Ioxynil	Nitrile				3.5×10^{-7} M	2.5×10^{-6} M	7.1

[a] Measured in a Yellow Springs YSI model O_2 electrode: (Chl) = 50 μg/ml, 30°C, light intensity = 1.3×10^3 μEm^{-2}, s^{-1}. [b] Measured with Hitachi single beam spectrophotometer, actinic illumination 430 nm, (Chl) = 30 μg/ml.

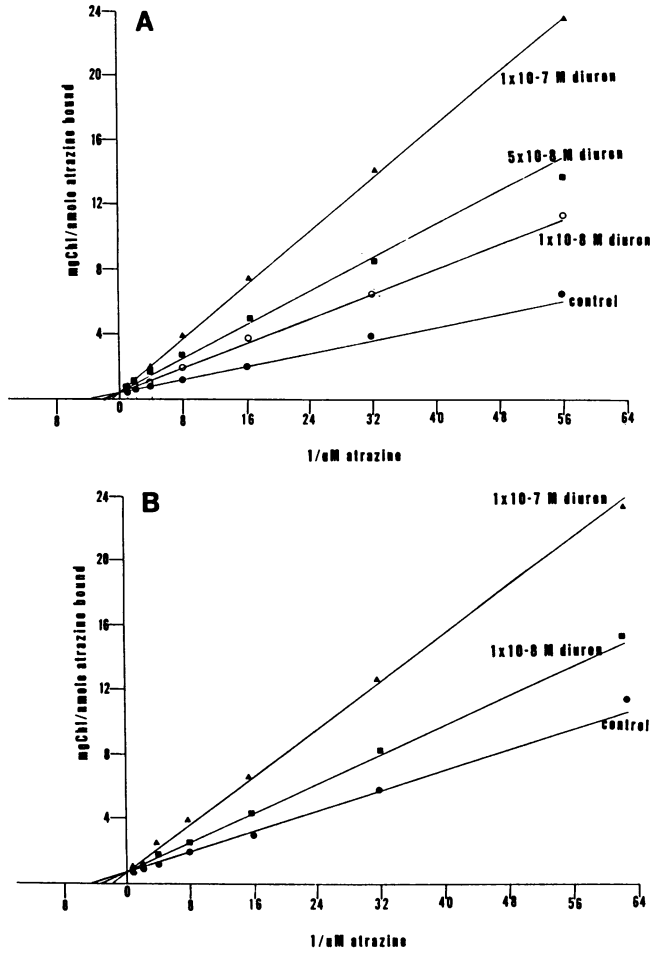

FIG. 4. Lineweaver-Burk analysis of atrazine binding data for chloroplast membranes isolated from 2137c⁺ (wild-type) (A) and Dr2 (B). Diuron binding constants were determined by competitive binding in the presence of diuron as described in "Materials and Methods" follow the protocol of Pfister *et al.* (18). Values of K_b and Chl/binding site calculated from these data are summarized in Table II. Each plot is the mean of three separate experiments.

regarded as the substrate and the amount of atrazine binding per unit Chl is equivalent to the enzyme rate. With this analogy, a double-reciprocal plot of atrazine binding (Fig. 4) yields a y axis intercept ($1/V_{max}$) equivalent to the number of Chl/herbicide

Table II. *Summary of Diuron Binding Data for C. reinhardtii: 2137c⁺ and Dr-2*

These values were calculated from Figure 4.

	Diuron	K_b Diuron
Wild-type (2137c⁺)	1×10^{-7}	4.3×10^{-8}
	5×10^{-8}	4.5×10^{-8}
	1×10^{-8}	1.5×10^{-8}
	Mean	3.4×10^{-8}
Dr-2	1×10^{-7}	6.6×10^{-8}
	1×10^{-8}	2.3×10^{-8}
	Mean	4.5×10^{-8}

binding site and the x axis intercept ($-1/K_b$) is a measure of the atrazine binding constant. Applying the same principals of Lineweaver-Burk analysis to competitive binding in the presence of diuron, the y axis intercept still gives a measure of Chl/binding site while the x axis intercept is displaced by the factor ($1/1 + [I]/K_I$), where $[I]$ is the concentration of the competing diuron and K_I is the diuron binding constant. This competitive binding approach overcomes the unavailability of radiolabeled herbicides with high specific activity. In theory, this technique can be used for any molecule which displaces atrazine binding, either competitively or noncompetitively. We have confirmed the validity of this approach by calculating the diuron binding constant in atrazine sensitive *A. hybridus* (1), data not shown. The atrazine/diuron binding studies on thylakoid membranes of *C. reinhardtii* 2137c⁺ and Dr2 are presented in Figure 4. This figure clearly demonstrates that binding of diuron and atrazine are competitive in both wild-type and resistant strains since all plots intersect the y axis at the same place. The calculated values of Chl/binding site and binding constants are summarized in Table II. These data confirm the results of Table I and the data of Galloway and Mets (9) in that an atrazine binding constant of 2.5×10^{-7} M is found for both 2137c⁺ and Dr2. While this value is slightly higher than that observed in *Senecio vulgaris* (1.4×10^{-8} M) (20) and *Amaranthus* sp. (7×10^{-8} M) (1), it is consistent with the atrazine binding constant for *C. reinhardtii*, of 2.3×10^{-7} M previously reported by Tellenbach *et al.* (22). With wild-type *C. reinhardtii* the calculated binding constant for diuron is 3.4×10^{-8} M, in agreement with the high-affinity binding reported for *Chlamydomonas* by Arntzen *et al.* (2) and Boschetti *et al.* (5). In contrast to expectation, a diuron binding constant of 4.5×10^{-8} M was determined for mutant Dr2 suggesting that loss of diuron binding does not cause the observed resistance. The only parameter which is significantly altered in the mutant Dr2 is the apparent number of Chl/binding site; 445 in 2137c⁺ and 670 in mutant Dr2. This

increase in the number of Chl/binding site has also been reported for other mutants which have altered herbicide binding properties (1, 18). This change in the number of binding sites between 2137c$^+$ and Dr2 cannot be correlated to the small difference in Chl *a*/*b* ratios.

DISCUSSION

Much has been written on the function of the 32 kD Q_B-protein in the binding of photosynthesis inhibitors and its role in resistance to these herbicidal compounds (1, 2, 4, 11, 15, 21–23, 26). The system characterized in most detail is that of the naturally occurring triazine-resistant weed biotypes (20), although triazine resistance in purple photosynthetic bacteria (19), cyanobacteria (10), and green algae (9) also has been reported. In every case, triazine resistance results from an altered structure of the 32 kD Q_B-protein. More specifically, amino acid 264, serine in sensitive biotypes (12), is altered to either glycine (12) or alanine (6) in resistant biotypes. This difference is the result of a single base pair change in the chloroplastic *psb*A gene which encodes the 32 kD Q_B-protein (7). The mutation results in an almost complete loss of atrazine binding, suggesting that serine 264 is directly involved in the binding process, presumably through some ionic interaction not provided by the glycine or alanine substitution (12). As reviewed by Gressel (11) this triazine resistance confers a penalty in that the kinetics of Q_A to Q_B electron transfer are slowed. This slower kinetic results in a 23% decrease in the quantum yield of CO_2 reduction (16). It is assumed that the altered Q_A to Q_B electron transfer step reflects the role of serine 264 in binding or stabilizing one or more of the redox states of Q_B (12). Support for the concept that atrazine and quinone binding are structurally and functionally linked was reported by Vermaas *et al.* (25) who demonstrate that competition in the binding of atrazine and a plastoquinone analog occurs on the thylakoid membrane. The binding properties of the 32 kD Q_B-protein are further complicated by the fact that a large number of different chemical inhibitor classes also interact with this protein (2) and resistance to one class of compounds does not necessarily confer resistance to another (23).

The recent characterization of a number of *Chlamydomonas reinhardtii* mutants (8, 9), each with a single amino acid change in the 32 kD Q_B-protein, (7) provides a new opportunity to study the interaction of herbicides and quinones with a protein binding site. Galloway and Mets (8) have selected a mutant of *C. reinhardtii* 2137c$^+$ which has a 17-fold resistance to diuron and 2-fold resistance to atrazine. This mutant, cataloged as Dr2, was originally reported to exhibit wild-type electron transport rates (9) and thus appeared to be of potential agronomic interest.

In this study we observed that the rate of O_2 evolution from Dr2 was approximately 20% less than the wild-type cells under saturating light intensities. Calculations based on the whole cell fluorescence transients shown in Figure 2, indicate that the lower than normal O_2 evolution rate occurs when the rate of electron transfer from Q_A to Q_B is reduced the same electron transfer step which is altered in higher plant biotypes resistant to triazines.

Because of the analogy between diuron-resistant Dr2 and the triazine-resistant weed biotypes, we assumed that the change of valine 219 to isoleucine might result in a loss of diuron binding at the thylakoid membrane of Dr2. The data in Figure 4 and Table II demonstrate that neither the binding of atrazine nor diuron is affected by this mutation. Initially this observation did not appear consistent with the data in Table I which indicates resistance to diuron. The apparent contradiction can be explained in a number of ways. One possibility is that the level of resistance observed in Dr2 is too small to expect a measurable difference in the diuron binding constant. However, changes in diuron binding constant have been reported for mutants with similar levels of resistance *e.g. C. reinhardtii* dr-412 (5). An alternative explanation for this observation was proposed by Theil and Böger (23) who speculated that binding of a herbicide to the 32 kD Q_B-protein might not always result in inhibition of photosynthetic electron transport. It is reasonable to assume that in diuron sensitive biotypes, the binding of the herbicide correlates directly to inhibition of the Q_A to Q_B electron transfer step. Indeed, all of the data on higher plants and green algae support this idea. According to the 'lock and key' model of Pfister *et al.* (18), the diuron binding site partially overlaps with the atrazine binding site. The bound diuron then inhibits qiunone binding either through a steric hindrance or an induced protein conformational change (3). Although some of the amino acid residues of these sites are directly involved with binding (*e.g.* serine 264), the remainder determine the optimal structure conformation for the binding site(s). Alteration of one of these amino acids is unlikely to affect binding directly. However, it could subtly alter the conformation, and hence properties, of the 32 kD Q_B-protein. The evidence in this paper leads us to suppose that the Dr2 mutant of *C. reinhardtii* is an example of this type of conformational change. The valine to isoleucine change represents only a change in steric bulk and neither amino acid can take part in ionic binding. Models of the 32 kD Q_B-protein proposed by Hirschberg *et al.* (12) and Trebst (24), based on the primary amino acid sequence, each show valine 219 to be adjacent to, or in, the proposed binding region. This proposal is significant since we expect the effect of a valine/isoleucine to be localized in the 32 kD Q_B-protein. Finally, a small steric alteration could be expected to effect the activity of herbicide acting at this site in a moderate and nonspecific fashion. Table II demonstrates that Dr2 displays moderate levels of resistance to a group of chemically diverse inhibitors. It is interesting to note that although the valine 219 to isoleucine change gives only minor herbicide resistance, its effect on the rate of Q_A to Q_B electron transfer is equivalent to that of the serine 264 to glycine change reported in extreme triazine-resistant weed biotypes. This observation suggests that the interaction of quinone with this binding site is more precise than previously supposed.

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