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The influence of diphenylcarbazide and hydrazobenzene on the binding of the herbicide chlorotoluron to isolated thylakoids

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To investigate the overlap of the effects of Q_B site inhibitors and donors of Photosystem II we analyzed the binding of [^{14}C]chlorotoluron, a representative of Q_B site inhibitors, to isolated thylakoids in the presence of two electron donors, *sym*-diphenylcarbazide or hydrazobenzene. Chlorotoluron binds to pea thylakoids with a K_d of 30 nM and a maximum number of binding sites of 2 nmol/mg Chlorophyll, which corresponds to one ligand molecule per Photosystem II reaction center. Both *sym*-diphenylcarbazide and hydrazobenzene decreased the affinity of chlorotoluron for the Q_B site. The decrease in binding could not be explained by a simple competition for the Q_B niche. Comparison of the dissociation of chlorotoluron to electron transport in normal and Tris-treated thylakoids indicated that this effect of donors on the Q_B niche is independent of their known capacity to donate electrons to PSII.

Introduction

Two classes of chemicals have been important for investigating electron transport in Photosystem II of photosynthesis [1]. In the first class are various herbicides that block on the acceptor side of PS II nearest the chloroplast stroma (for a review see [4]). For example,ureas (DCMU), triazines (atrazine), phenols, are all known to inhibit electron transport by displacing plastoquinone from its binding site Q_B [2,3]. The donor side of PS II, which is oriented towards the lumen of the thylakoid [5], has been studied using a second class of chemicals which donate electrons to PS II [1], for example, *sym*-diphenylcarbazide [6,7] and hydrazobenzene [8,9]. These chemicals are efficient donors when the capacity for oxygen evolution of PS II is destroyed [10,11].

[illegible] of Q_B site inhibitors on the donor side of PS II were also reported [12–16] and their inhibiting actions were overcome in the presence of DPC [17–19] or hydrazobenzene [8,9]. This led to the proposal of the existence of a second binding site for Q_B-site inhibitors located at the donor side of PS II [16,19]. Recent measurements of chlorophyll fluorescence and luminescence provided support for such a proposal [20–22]. In the context of a hypothetical model for the functioning of PSII, it was proposed that these apparent interactions of donors and inhibitors resulted from their competition for the Q_B site at the acceptor side of PS II [23].

In this report, we investigated the physical interaction of PS II donors and inhibitors by analyzing the binding of radioactive chlorotoluron, a DCMU-type herbicide (Q_B-site inhibitor) [24] to thylakoids in the presence of DPC and HB. We show that chlorotoluron binds to only one site and that its dissociation constant is increased by the presence of DPC or HB.

Materials and Methods

Peas (*Pisum sativum* L., var. Petit Provencal, Truffaud, Vierneuil, France) were grown for 10 days on wet vermiculite at 18°C with photosynthetic photon flux density of 400 μE/m^{2}s for 16 h per day. They were kept in darkness for 18 h before use and pre-il-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, *sym*-diphenylcarbazide; HB, hydrazobenzene; Chl, chlorophyll; *r*, rate; DCIP, 2,6-dichlorophenolindophenol; CT, chlorotoluron (3-(3-chloro-*p*-tolyl)-1,1-dimethylurea); K_d, dissociation constant; K_m, Michaelis constant; ED_{50}, dose of unlabeled ligand yielding 50% displacement of the labeled ligand; B_{max}, maximal ligand binding.

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luminated for 25 min at 800 μE/m^{2}s. Intact chloroplasts were prepared as in [25]. The preparations were 85–90% intact as judged by ferricyanide penetration [26]. Chlorophyll concentration was determined according to Bruinsma [27].

A medium containing 330 mM sorbitol, 10 mM KCl, 1 mM EDTA, and 50 mM Hepes buffer, adjusted to pH 7.9 with KOH, prepared as double strength, was the basis of all other media used. The isolation medium was the basic medium diluted 25-fold with 330 mM sorbitol. The resuspending medium was the basic medium with the addition of 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 10 mg/ml of bovine serum albumin.

For Tris-treatment [10] 0.5–1 ml of chloroplasts (2 mg Chl) were mixed with 10 ml of water, followed by 10 ml of 1.6 M Tris (pH 8). After 20–30 min incubation on ice, the suspension was centrifuged at 1500 × *g* for 10 min. The pellet was re-suspended in 20 ml of chloroplast isolation medium and sedimented as before. The final pellet (Tris-treated thylakoids) was resuspended in basic medium.

Oxygen evolution was measured at 20°C by a thermostated water-jacketed Clark-type electrode (Beckman, Palo Alto, CA, USA). The reaction mixture (2 ml) consisted of basic medium with 10 or 20 μg Chl, 90 μM DCIP, 5 mM NH_4Cl, 2000 units catalase, and DPC, chlorotoluron and ethanol as indicated on figures. DCIP photo-reduction was monitored at 550 nm with a single beam spectrophotometer (DU-40, Beckman, Gagny, France) equipped with a thermostated sample holder, a magnetic stirrer, a Kinetic Soft-Pak Module and home-made fiber-optic illumination accessory. The photo-detector was protected with a blue glass filter (4–96, Corning, New York, USA) from the actinic light of 2000 μE/m^{2}s defined with a cold heat filter (Achervex TA3 MTO, Palaiseau, France) and red glass filter (RG 645, Shott Mainz, FRG). The reaction mixture (1.5 ml final volume) contained in addition to basic medium 7.5 μg Chl, 90 μM DCIP, 5 mM NH_4Cl, 1500 units catalase, and was maintained at 20°C.

[^{14}C]Chlorotoluron binding was conducted under dim light in 1 ml of basic medium. Intact chloroplasts (30 μg) were broken in water and isotony was restored by addition of the double strength basic medium. Different amounts of [^{14}C]chlorotoluron (1.95 MBq/mg) was then added followed, when appropriate, by DPC, HB, or unlabelled chlorotoluron to perform saturation or dissociation studies ("hot", "cold", "dissociation" [28]). Care was taken to have always a constant ethanol concentration in a series of samples with varied DPC concentration. Samples were incubated for 15 or 20 min in a darkened water bath at 20°C and then centrifuged at 15000 × *g* for 3 min. Aliquots of the supernatants (0.5 or 0.8 ml) were analyzed in a liquid scintillation counter (LS-6000IC, Beckman, Gagny, France) equipped with the facility for automatic DPM calculations (Auto DPM). Each sample was compared to a blank without thylakoids present, and bound and free chlorotoluron calculated according to Tisher and Strotmann [29].

Computer programs LIGAND (Mc Pherson, Interchim, Montluçon, France) and SigmaPlot (Jandel Scientific, Corte Madera, California, USA) were used to fit and test different binding models to experimental data, and to calculate binding parameters. Catalase (EC 1.11.1.6) was purchased from Sigma (St. Louis, MI, U.S.A.). Radioactive (Ciba-Geigy, Basel, Switzerland) and unlabelled chlorotoluron (Riedel-de Hoen) were kindly provided by Dr. J.-M. Ducruet (CEA-INRA, Saclay, France). The other reagents were of proanalysis grade.

Results

Chlorotoluron binds to pea thylakoids with a K_d of 30 nM and a maximum number of binding site of 2 nmol/mg Chl, when corrected for non-specific binding (Fig. 1). This amounts to 550 Chl per binding sites which corresponds well to the ratio of chlorophyll to Q_B sites, characterized by other herbicides [29] or other techniques in spinach [30] and peas [31]. From the Scatchard plot of non-corrected data (Fig. 1) an apparent positive cooperativity for binding can be discerned at concentrations lower than 20 nM free chlorotoluron, similar to what has been shown for DCMU binding (Groom, Q., personal communication). At concentrations higher than 600 nM free chlorotoluron there is an increase of non-specific binding. Using the program LIGAND [28] only a one-site binding model

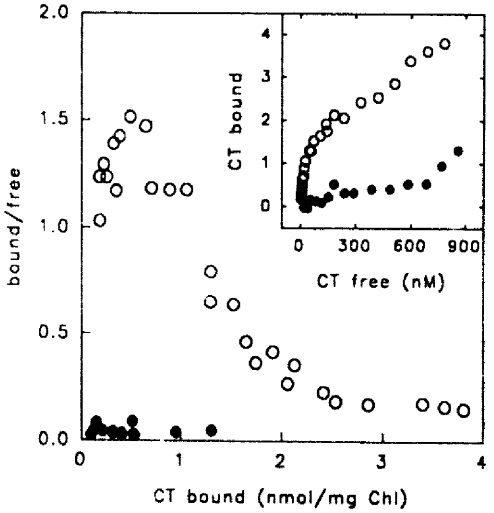

Fig. 1. Binding of chlorotoluron to thylakoids. Saturation binding experiments were performed as indicated in Material and Methods. To assess the part of non-specific binding, in the experiments marked by filled symbols a 1000-fold amount of unlabelled chlorotoluron was added to each sample.

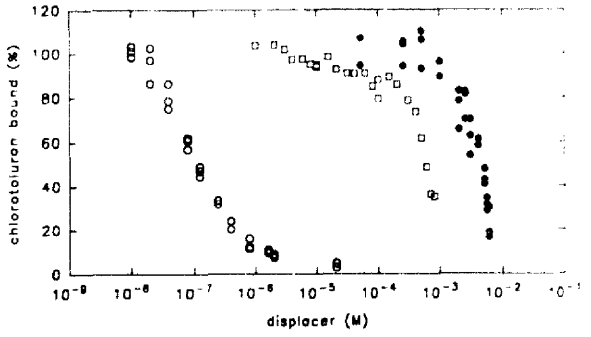

Fig. 2. Displacement or dissociation of [^{14}C]chlorotoluron by unlabelled chlorotoluron (○), hydrazobenzene (□) and diphenylcarbazide (●) from thylakoids. Each sample contained 50 nM [^{14}C]chlorotoluron. The maximal amount of [^{14}C]chlorotoluron bound was 27 pmol for experiments with chlorotoluron and HB as displacers, and 8 pmol for the experiments with DPC due to the presence of 6% ethanol throughout in the last case.

could be fitted to data corrected for non-specific binding. If non-corrected data are analyzed, an equally good fit is obtained for a one-site binding model with 7% non-specific binding, or a two binding model with a second site with a K_d of 100 μM and no non-specific binding.

Fig. 2 shows the results of displacement or dissociation of radioactive chlorotoluron added at a concentration close to its K_d, by unlabelled chlorotoluron, DPC and HB. The data for displacement by unlabelled chlorotoluron confirm the presence of only one binding site with the same K_d as in Fig. 1. The dissociation of chlorotoluron in the presence of DPC is prominent and occurs in the milimolar range of DPC (ED_{50} = 4.2 mM). The K_d for DPC could not be calculated and the ED_{50} is only an approximation, because the shape of the dissociation curve is atipic (convex on a linear scale) with a high apparent cooperativity coefficient (equal to 3). A competitive type of displacement could not be fitted to the data. This is why it is more appropriate to describe the results as a dissociation which is due to a decrease in affinity of chlorotoluron for its binding site in the presence of increasing concentration of DPC. Chlorotoluron was also dissociated in the presence of HB (Fig. 2). The ED_{50} for the effect of HB was smaller (0.6 mM) than for DPC but the shape of the dissociation curve was similar to the situation with DPC and therefore no model could be assigned. At these high concentrations of HB a change in the sedimentation pattern and even of the color of thylakoids could be seen. At concentrations below 50 μM HB a true displacement curve could be fitted with an apparent ED_{50} of 6 μM. However, it accounted for only 10% of chlorotoluron bound.

We repeated the saturation experiments for chlorotoluron binding in the presence and absence of donors close to their apparent ED_{50} concentrations in order to

TABLE I

The effect of diphenylcarbazide and hydrazobenzene on the binding constants of chlorotoluron

Saturation binding experiments of chlorotoluron were performed for the 20 to 200 nM range of concentrations as indicated in Material and Methods. Where indicated 3 mM DPC or 0.5 mM HB were included in the chloroplast incubation mixture and control. Data represents the result ± standard deviations of a one-site binding model fitted to curves comprising 10 to 12 points.

Addition	K_d (nM)	B_{max} (nmol / mg Chl)
Non-treated thylakoids		
none	55 ± 9	1.40 ± 0.10
3 mM DPC	91 ± 14	1.53 ± 0.12
none	42 ± 11	2.07 ± 0.32
3 mM DPC	114 ± 23	2.54 ± 0.36
none	34 ± 4	1.88 ± 0.08
0.5 mM HB	44 ± 8	1.71 ± 0.14
Tris-treated thylakoids		
none	143 ± 29	1.78 ± 0.24
3 mM DPC	119 ± 55	0.67 ± 0.16

determine the type of interaction based on the effect of the donors on the K_d and B_{max} for chlorotoluron. As seen from Table I (and Scatchard plots, not shown) the presence of DPC has a stronger impact on the K_d than B_{max} in non-treated thylakoids. The presence of HB decreases both the K_d and B_{max}, although to a smaller degree. In Tris-treated thylakoids, where the K_d for chlorotoluron is already increased to a level similar to

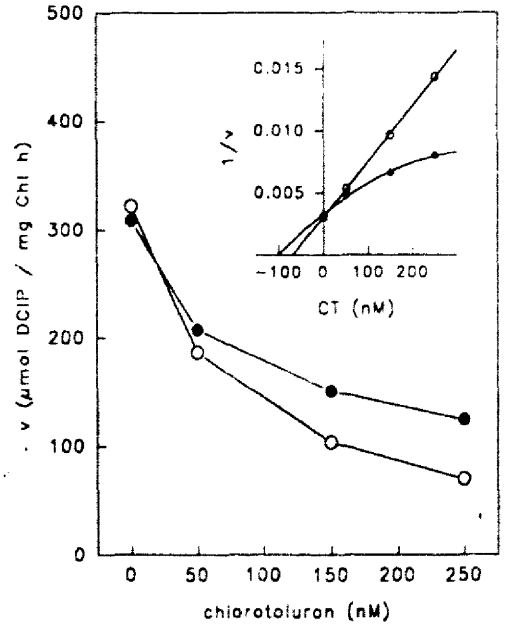

Fig. 3. Inhibition of electron transport by chlorotoluron as affected by the presence of diphenylcarbazide. When present (●) DPC was 2.5 mM. Initial rates of DCIP reduction was measured 5 min after the addition of chlorotoluron (and DPC) to thylakoids.

non-treated thylakoids in the presence of 3 mM DPC, the presence of DPC affects primarily the B_{max}. These results, in addition to those of Figs. 2 and 6, indicate that the effect of donors on the Q_B site is not specific, and that their interpretation alone can not be conclusive.

Chlorotoluron inhibits DCIP reduction (Fig. 3) and oxygen evolution (data not shown). In the presence of 2.5 mM DPC the inhibition was much less pronounced, as seen in PS II particles [19]. The inhibition data presented in a Dixon plot (Fig. 3) are reminiscent of a situation when non-competitive inhibition becomes partial non-competitive (or pure competitive becomes partial competitive) [32]. For a ligand it would mean a change in its affinity for the binding site, not a displacement. Fig. 3 is therefore best explained as an effect of DPC on the Q_B site which changes the affinity of chlorotoluron, and not a direct displacement of chlorotoluron by DPC. A direct displacement by 2.5 mM DPC should be larger in the presence of 50 nM than 250 nM chlorotoluron, which is not the case here (Fig. 3).

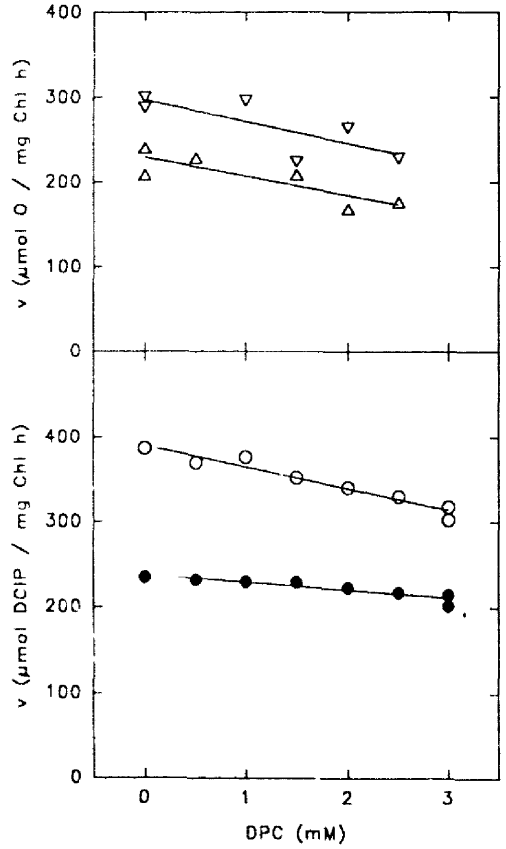

Fig. 4. Effects of diphenylcarbazide on oxygen evolution and DCIP reduction in non-treated thylakoids. Two series (△, ▽) of measurements of oxygen evolution in the presence of increasing concentrations of DPC is presented (top part). The rate is expressed in atoms of oxygen reduced to permit direct comparison to the rate of DCIP reduction which is presented at the bottom part of the figure. The presence (●) and absence (○) of 50 nM of chlorotoluron in the reaction mixture is compared. Measurements were made immediately upon DPC (and chlorotoluron) addition.

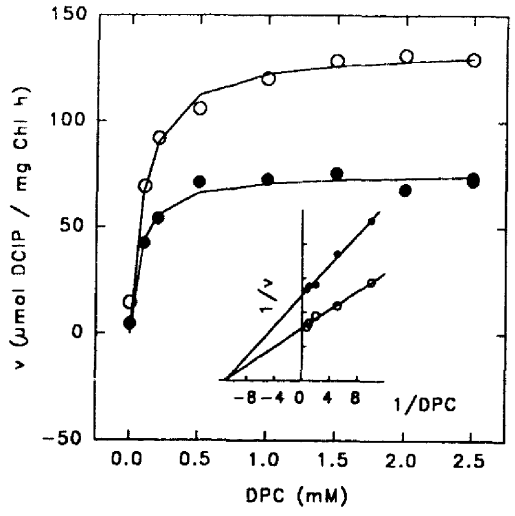

Fig. 5. Effect of diphenylcarbazide on the reduction of DCIP in Tris-treated thylakoids. After the treatment by Tris, thylakoids retained less than 5% of their initial capacity for oxygen evolution. The rate of DCIP reduction was measured in the absence (○) and presence (●) of 100 nM chlorotoluron at different DPC concentrations.

We investigate the effect of increasing concentrations of DPC on electron transport by non-treated pea thylakoids, under the same conditions used for the binding experiments. Similar experiments reported in the literature have given contradictory results [33,34]. In Fig. 4 it can be seen that concentration-dependent DPC inhibition of both DCIP reduction and oxygen evolution is similar, although the amount of inhibition is small. In the presence of 50 nM chlorotoluron, which inhibits electron transport from water to DCIP by 40% at zero DPC, the inhibition of electron transport by DPC is less pronounced.

The interaction of chlorotoluron and DPC was also investigated in Tris-treated thylakoids, conditions under which DPC is the sole donor for PS II [6,7]. The Tris-treated thylakoids did not evolve oxygen when illuminated in the presence of an acceptor (data not shown). With these thylakoids DPC behaved as an electron donor, photo-reducing DCIP with a K_m of 90 μM (Fig. 5). The reaction was chlorotoluron sensitive and the inhibition was of a non-competitive type (Fig. 5). This could be the consequence either of the presence of two independent sites, one for electron donation and one for chlorotoluron inhibition, or the result of a very large difference in K_d for the donor and inhibitor. The dissociation of [^{14}C]chlorotoluron in the presence of DPC in Tris-treated thylakoids (Fig. 6) is better explained by the presence of independent sites. Even at 6 mM DPC less than 50% of chlorotoluron

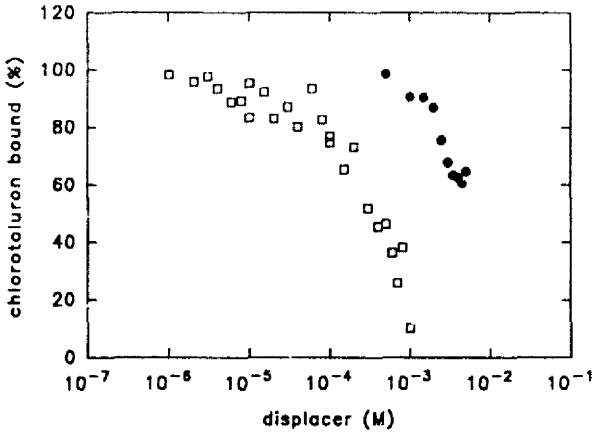

Fig. 6. Dissociation of [^{14}C]chlorotoluron by the presence of hydrazobenzene (□) and diphenylcarbazide (●) from Tris-treated thylakoids. Each sample contained 50 nM [^{14}C]chlorotoluron. The maximal amount of [^{14}C]chlorotoluron bound was 19 pmol for experiments with HB as displacers, and 9 pmol for the experiments with DPC due to the presence of 5% ethanol throughout.

was dissociated, indicating that the apparent ED_{50} is two orders of magnitude higher than the K_m for DPC electron donation. For both HB and DPC the dissociation curves of chlorotoluron in normal and Tris-treated thylakoids were alike although the electron transport capacities for these two types of thylakoids are quite different.

Discussion

The results of two types of binding experiment, saturation and displacement by unlabelled ligand, demonstrate that there is only one binding site for chlorotoluron, a DCMU-like herbicide, in intact thylakoids. Nevertheless, the presence of DPC can decrease the inhibition of electron transport by the herbicide, as seen in PS II particles [19], indicating that the interaction of the donor and inhibitor is at the Q_B site. The existence of this interaction is confirmed by the dissociation of chlorotoluron from thylakoids in the presence of DPC or HB, but both dissociation and saturation experiments indicate that this is not a simple displacement by competition for the binding site. Purcell et al. [35] in a recent report of atrazine "displacement" by DPC proposed that it could be the consequence of a transmembrane conformational change influencing the Q_B site after DPC has bound to the donor side, or a direct competition between the donor and atrazine at the acceptor side. Our results support the conformational change. Indeed they are best explained on the basis of an indirect chaotropic effect of DPC and HB on the conformation of the Q_B niche resulting in an increase in the K_d of the herbicide. This indirect effect of donors on the Q_B site is supported by the fact that the dissociation of chlorotoluron in the presence of donors is similar in normal and Tris-treated thylakoids.

Our conclusion that there is only one site for herbicide binding on PS II is in variance with the proposals that Q_B site herbicides can bind to the donor side of PS II [16,19]. Furthermore, our results does not support the possibility of specific binding of DPC to the acceptor side of PS II [35] or electron donation at the Q_B site [23]. The decreased sensitivity of electron transport to herbicides in the presence of donors [9,10,17,19,35] would be the consequence of the decrease in the affinity of herbicides for the Q_B site; the presence of donors would somehow change the conformation of the Q_B niche. The same changes at the level of Q_B were interpreted by some authors as an action of the herbicides on the donor side of PS II [8,9,16–22] solely because of the involvement of donors. The effect is more pronounced in PS II particles, where lower concentrations of donors are needed, due to the already much higher K_d of herbicides under these conditions [19,22]. This is probably due to the modification of the Q_B niche by the extraction procedure rather than the exposure of a new binding site on the donor side (as proposed by Carpentier et al. [19]).

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