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Interaction of the electron donor diphenylcarbazide with the herbicide-binding niche of Photosystem II

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In experiments using thylakoid membranes, it was found that the inhibitory action of atrazine and 3-(3,4-dichlorophenyl)-1,1-dimethylurea is weaker in the presence of the Photosystem II electron donor *sym*-diphenylcarbazide. The concentration dependences of *sym*-diphenylcarbazide for electron donation and the relief of inhibition by the herbicides were different, higher concentrations being required for the latter. These data could not be explained by the electron donation properties of *sym*-diphenylcarbazide. Instead, the donor was shown to displace [^{14}C]atrazine from its binding site. The photoreduction of 2,5-dichlorobenzoquinone, monitored using oxygen evolution experiments, was also strongly reduced in the presence of *sym*-diphenylcarbazide. A direct interaction of *sym*-diphenylcarbazide with the herbicide-binding niche is demonstrated.

Introduction

Various inhibitors, electron donors, and electron acceptors have been characterized and widely used in the study of photosynthesis. They constitute an important tool for the elucidation of the mechanisms implicated in electron transport and for the determination of the sequence of electron carriers serving this function. As an electron donor to PS II, DPC is routinely used to assess the photochemical activity of the photosystem when oxygen evolution is inhibited or to locate the site of action of PS II inhibitors [1–4]. Recent reports indicated that it donates electrons to Z^+, the primary donor of PS II [5–7].

However, in a previous study, we have found that inhibition of the photoreduction of DCIP by the herbicide atrazine was weaker in the presence of DPC [8]. An incomplete inhibition of electron transport from semicarbazide to potassium ferricyanide by DCMU at a concentration that fully inhibits oxygen evolution has also previously been observed [9]. These inhibitors, along with other triazine, urea and phenolic compounds, bind to a 32 kDa polypeptide on the acceptor side of the PS II core complex also known as the Q_B (secondary quinone acceptor of PS II) binding protein [10–12]. The real meaning of the weaker inhibitory action in the presence of DPC has not been clarified. Due to the respective active sites of DPC and herbicides, the above cannot be interpreted in terms of simple electron donation from DPC to PS II.

In this work, we have analysed in detail the interaction between DPC and the photosynthetic inhibitors atrazine and DCMU in thylakoid membranes. We present the first clear demonstration that DPC decreases atrazine binding and that, in addition to its active electron donation site on the donor side of PS II, it can occupy a binding site in the herbicide-binding niche.

Materials and Methods

Thylakoid membranes were isolated from deveined spinach leaves or from barley leaves as described previously [13]. The final preparation was resuspended in 50 mM Tes-NaOH (pH 7.5), 2 mM $MgCl_2$, 1 mM NH_4Cl,

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Abbreviations: PS, photosystem; DCIP, 2,6-dichlorophenolindophenol; DPC, sym-diphenylcarbazide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tes, (*N*-tris[(hydroxymethyl]methyl-2-aminoethanesulfonic acid; Chl, chlorophyll; DCBQ, 2,5-dichlorobenzoquinone.

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and 330 mM sorbitol at a Chl concentration of 2 mg/ml. Chlorophyll was determined according to Arnon [14]. The membranes were either used the same day or stored in liquid nitrogen.

Light-minus-dark rates of photoreduction of DCIP were measured at 600 nm using a UV/VIS-spectrophotometer (Perkin-Elmer, model 553) as described previously [15]. The reaction medium contained thylakoid membranes at a Chl concentration of 5 μg/ml suspended in 50 mM Tes-NaOH (pH 7.5), 2 mM $MgCl_2$, 1 mM NH_4Cl, 330 mM sorbitol and 30 μM DCIP. Oxygen evolution was monitored with a Clark-type electrode as described elsewhere [16]. The reaction medium was composed of thylakoid membranes at a Chl concentration of 11 μg/ml suspended in the above buffer but with specified concentrations of DCBQ as electron acceptor.

Herbicide-binding experiments were performed under dim light using a method similar to that described in Ref. 17. [^{14}C]Atrazine having a specific activity of 19.5 μCi/mg was generously provided by Ciba Geigy, Greensboro, NC. The reaction mixture, adjusted to a final volume of 1 ml, contained 50 mM Tes-NaOH pH 7.5, 2 mM $MgCl_2$, 1 mM NH_4Cl, 330 mM sorbitol, and thylakoid membranes equivalent to 50 μM Chl. Radiolabelled atrazine was added to obtain between 90 and 2000 disintegrations/min in the counting vials. The samples were centrifuged 4 min in an Eppendorf centrifuge after a 10-min incubation at room temperature with [^{14}C]atrazine and other additives (DCMU or DPC) when appropriate. Aliquots of 0.75 ml were taken from the supernatants for the determination of free atrazine by scintillation counting. The data were analysed as described by Tischer and Strotmann [18]. Herbicides were added in ethanol solutions. For all experiments the final ethanol concentration in the reaction medium was less than 1%, which did not modify the photosynthetic activity of the thylakoid membranes.

Results and Discussion

Atrazine and DCMU are two well-known inhibitors having an active binding site on the acceptor side of PS II [19]. They strongly inhibit PS II electron transport even at very low concentration. In Fig. 1, the inhibitory effect of these compounds on the photoreduction of DCIP in thylakoid membranes isolated from spinach leaves is demonstrated. Also shown in Fig. 1 is the effect of the electron donor DPC on the strength of this inhibition. From these data, it can be calculated that 1.6 mM DPC relieved between 10 and 100% of the inhibition, depending on the concentration of inhibitor. Similar data were obtained with thylakoids isolated from barley (not shown). The above is also in agreement with the results of experiments previously performed with other plant materials [8].

In Fig. 2A, the rate of photoreduction of DCIP is shown as a function of various concentrations of DPC. When DPC is added, to serve in conjunction with water as electron donor in the absence of inhibitor, there is an increased electron donation capacity which results is an increased rate of photoreduction of DCIP. This increase is probably due to the presence of PS II centres inactive in oxygen evolution that can reduce

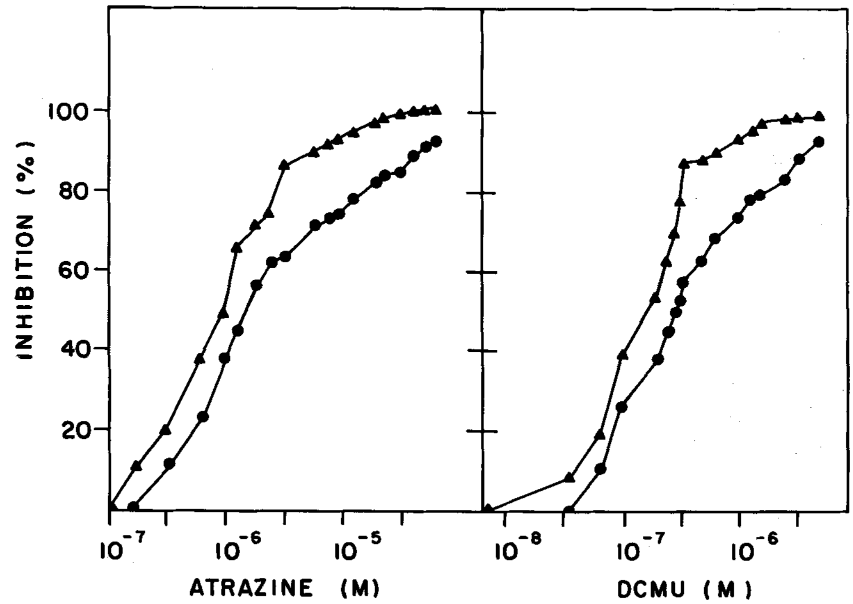

Fig. 1. Inhibition of PSII-dependent electron transport (▲, $H_2O \rightarrow$ DCIP; ●, H_2O + DPC $\rightarrow$ DCIP) in spinach thylakoid membranes inhibited by atrazine (left) and DCMU (right). DPC was added at 1.6 mM. The control (uninhibited) rates with and without DPC were 300 and 270 nmol DCIP/mg Chl per h, respectively.

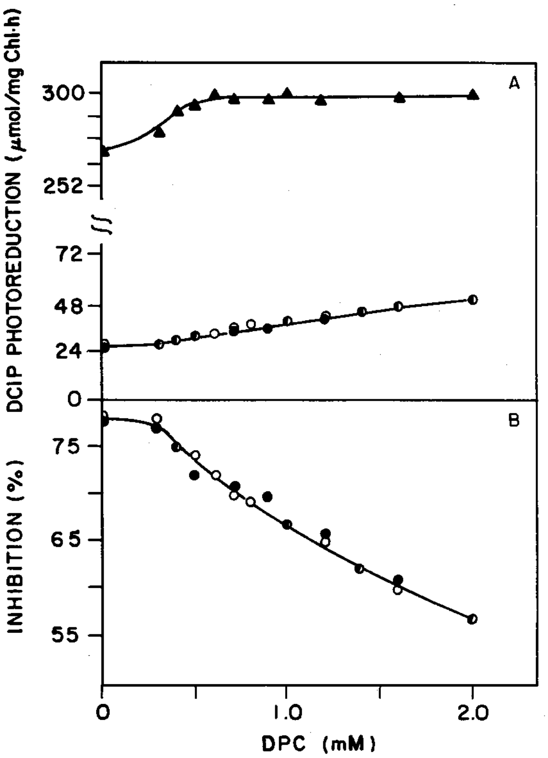

Fig. 2. (A) Effect of DPC on the rates of photoreduction of DCIP in spinach thylakoid membranes: ▲, control; ●, $2.3 \cdot 10^{-6}$ M atrazine added; ○, $2 \cdot 10^{-7}$ M DCMU added. (B) Percentage of inhibition caused by atrazine (●) and DCMU (○) calculated from the data in (A). Similar data were obtained with thylakoid membranes isolated from barley.

DCIP in the presence of DPC. A maximal value is obtained when DPC reaches a concentration where it saturates the photosystem (about 1 mM). Lower rates of photoreduction of DCIP were obtained following the addition of atrazine or DCMU (Fig. 2A). In these cases, though the photoreduction activity did increase with increasing concentrations of DPC, the highest concentration of DPC used (2 mM) still did not produce an optimal activity.

The percentage of inhibition of the photoreduction by atrazine and DCMU was calculated at various concentrations of DPC from the data of Fig. 2A and is shown in Fig. 2B. At the fixed atrazine and DCMU concentrations used (causing about 80% inhibition in the absence of DPC) increasing concentrations of DPC produced a decreased inhibitory effect. From the different concentration dependences of the two effects of DPC (e.g., electron donation (Fig. 2A) and release of inhibitory action (Fig. 2B)), it can be concluded that these are separate and independent actions of DPC.

Diphenylcarbazide is known as an electron donor specific for PS II [2,20]. When both water and DPC served as electron donors, the decreased inhibitory action of atrazine and DCMU was not explained by a simple electron donation effect of DPC. Electron donation to the oxidizing side of PS II would not overcome the inhibition on the acceptor side of the photosystem. Further, in an experiment using methylviologen as electron acceptor and $DCIPH_2$ as electron donor in samples inhibited with 1.2 mM DCMU, our preparations had an oxygen uptake rate of 550 µmol O_2/mg Chl per h. However, when DPC (0.5–2 mM) was used instead of $DCIPH_2$, there was no oxygen uptake at all, which clearly demonstrated that DPC is not an electron donor to PS I. Thus, the above data point toward a decreased inhibitor binding at the Q_B site following the interaction of DPC with the PS II complex. The possibility of the displacement of the inhibitors from their binding site was tested by following the binding of radiolabelled atrazine. Increasing concentration of DPC caused a proportional reduction in bound [^{14}C]atrazine. Even at the DPC concentrations usually used for electron donation (between 0.5 and 1 mM), atrazine binding was decreased by about 25% (not shown). The nature of the interaction between DPC and the herbicide-binding site is analysed using the double reciprocal plots of Fig. 3. The effect of unlabelled DCMU on [^{14}C]atrazine binding is shown as a comparison with the effect of DPC. Both produced a decrease in the binding constant, *K*, via a direct competitive action against atrazine binding.

The probability of a direct interaction of DPC with the Q_B site prompted us to verify the effect of DPC on oxygen evolution measurements with DCBQ as artificial electron acceptor. As seen in Fig. 4, the initial rate of oxygen evolution with either 100 µM or 600 µM DCBQ declined with increasing concentration of DPC. This result indicates that DPC greatly limits the photoreduction of the acceptor by the photosystem. Only 45 µM DPC was required to reduced oxygen evolution by 50% when 100 µM DCBQ was used. This concentra-

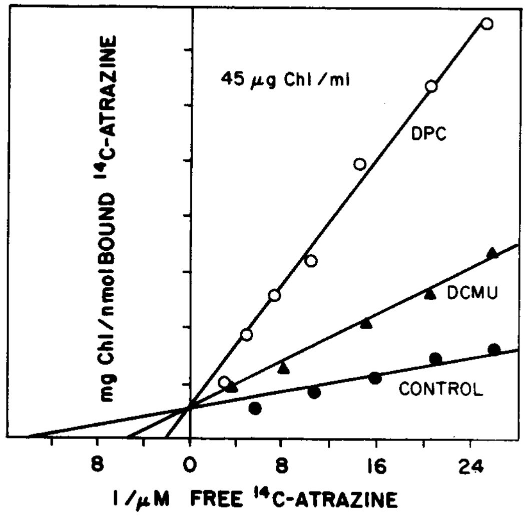

Fig. 3. Double reciprocal plots for binding of [^{14}C]atrazine to spinach thylakoids. The binding constant, *K*, is given in parentheses: ●, control (0.20 µM); ▲, 0.04 µM DCMU added (0.47 µM); ○, 0.5 mM DPC added (1.25 µM).

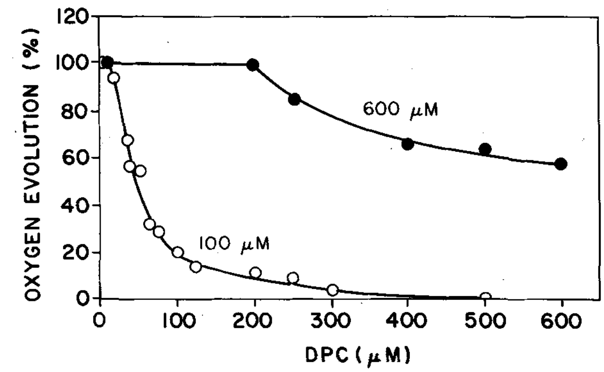

Fig. 4. Effect of DPC on the initial rates of oxygen evolution obtained in the presence of DCBQ as artificial electron acceptor: ●, 100 μM; ○, 600 μM. The control rate obtained without DPC was 320 μmol O_2/mg Chl per h.

tion of DCBQ was optimal for oxygen evolution in the absence of DPC (result not shown). However, higher concentrations of DPC were required to inhibit oxygen evolution in the presence of a higher concentration of DCBQ. To this point, it is not clear whether DPC interacts with the binding site of DCBQ or if it directly reduces DCBQ in solution. Though no experimental evidence could be gathered to support the latter mode of interaction we still have to consider it as a possibility. Diphenylcarbazide is currently used as a donor with DCIP as final electron acceptor [1] and, considering DCIP has been shown to accept electrons from the plastoquinol molecules [2,25], DPC should not interfere very much with the photoreduction of plastoquinone.

Though the electron donation site of DPC is located on the lumenal side of the thylakoid membrane and the herbicide-binding domain is on the stroma-exposed portion, the actual idea of the structure of the PS II complex would suppose that DPC and herbicides both interact with the same protein complex (see Dismukes et al. for a recent model [21]). The above data could indicate that the binding of DPC at its electron donation site modifies the structure of the PS II complex through a transmembranous conformational change that will affect atrazine binding. In fact, there are several examples of experiments were a modification of the donor side of PS II also affected the acceptor side [22–24]. Alternatively, however, DPC could bind directly to the acceptor side of PS II and compete with the binding of herbicides. This second hypothesis is better sustained by the competitive displacement of [^{14}C]atrazine by DPC demonstrated in Fig. 3.

Our finding does not constitute the first report of the displacement of herbicides acting at the Q_B site by a compound in the family of artificial electron donors and acceptors. Similar conclusions were reached concerning the PS II acceptor silicomolybdate [26,27], which was initially though to accept electrons at a DCMU insensitive site [28,29]. Competitive binding between herbicides is a well-known phenomenon [18]. It is explained by the possibility that each herbicide possesses a common area of binding with other herbicides which implicates only a portion of the molecule, with the remaining part being bound to a more specific domain in the herbicide niche [30,31]. This concept can be used to support the idea of an overlapping binding area between DPC, DCBQ, atrazine, DCMU and plastoquinone in the herbicide binding niche. Further studies will be necessary to determine whether DPC can alter the binding of all triazine and urea compounds. However, the use of DPC as electron donor should be with caution, since the displacement of the herbicides and the corresponding reduction of inhibitory action was observed at concentrations at which DPC is generally used for its electron donation abilities.

Acknowledgements

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