Studies on the Herbicide Binding Site in Isolated Photosystem II Core Complexes from a Flat-Bed Isoelectrofocusing Method

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Z. Naturforsch. **45c,** 366–372 (1990); received November 3, 1989

Isoelectrofocusing, Photosystem Two Core Complexes, Different Populations of PS II, Herbicide Binding, Atrazine-Resistant Mutant *Senecio vulgaris*

Isoelectrofocusing has been used to separate various chlorophyll-protein complexes of photosystem two (PS II). Light-harvesting complexes containing chlorophyll *a* and chlorophyll *b* (LHC II) were located in bands having p*I*s in the region of 4.5. At slightly higher pH other light-harvesting complexes containing little or no chlorophyll *b* were found. In the most basic region of the isoelectrofocusing gel, were located PS II core complexes characterized by containing the proteins of CP47, CP43, D1, D2 and α-subunit of cytochrome b_{559}. The number of PS II core bands depended on the particular conditions employed for the separation procedure and in some cases were contaminated by CP29. It is suggested that this heterogeneity resulting from different protonation states of the PS II.

The least-acidic PS II core complex (p*I* 5.5) was found to bind the herbicides atrazine, diuron and dinoseb. In contrast, a PS II core complex with a p*I* of 4.9 bound only diuron. Its inability to bind atrazine was shown to be due to the low pH but no such explanation could be found for dinoseb.

When atrazine-resistant mutant *Senecio vulgaris* was used, no binding of radioactive atrazine was observed with the PS II cores having a p*I* of 5.5. It is therefore suggested that the normal atrazine binding observed with PS II cores involves the high affinity site detected with intact membranes. With the PS II cores, however, this site has a reduced affinity probably due to structural modification in the D1-polypeptide resulting from the isolation procedures.

Introduction

A large number of herbicides inhibit photosynthesis by blocking electron transfer at the reducing side of photosystem II. They interfere with the electron flow between the primary Q_A and secondary Q_B plastoquinone acceptor of photosystem II.

Although chemically different, the inhibitors displace each other from the membrane, indicating a common binding domain. In higher plants, there is also competitive binding between quinones and certain herbicides, leading to the hypothesis that they act either by direct or allosteric displacement of plastoquinone from its binding site. However a kinetic model has not been excluded on the basis of the experimental data available so far [1, 2].

The studies of Tischer and Strotmann [3] have shown that thylakoid membranes are able to bind photosynthetic herbicides with a biphasic relationship and by extrapolation of the linear components of double reciprocal plots, a high and low affinity binding processes may be separated. They called the high affinity process "specific binding" while the low affinity binding was suggested to be an association with lipids or pigments of the membrane.

The correlation of the high binding affinity sites with the inhibition of photosynthetic electron transport became clear with the appearance of mutants resistant to atrazine which show a greatly reduced binding affinity for atrazine [4, 5]. The atrazine resistance seems to result from a change in the microenvironment of the D1 (32 KD) protein which is a key component of the reaction centre II [6, 7]. In contrast the low affinity binding reaction observed for herbicides in PS II particles (BBY) was attributed by Carpentier *et al.* [8] to the exist-

Abbreviations: Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; i-dinoseb, 2,4-dinitro-6-isobutylphenol; diuron, 3-(3,3-dichlorophenyl)-1,1-dimethylurea; DM, dodecyl-β-D-maltoside; hydroxy-atrazine, 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine; IEF, isoelectrofocusing; SDS, sodium dodecyl sulphate; PS II, photosystem two.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0500–0366 $ 01.30/0

ence of a second site for herbicide binding on the oxidizing side of PS II.

In recent studies we demonstrated that the isolated reaction centre II of PS II and also the isolated PS II core complex still retain a binding capacity for herbicides. We showed that the isolated reaction centre II can bind not only diuron and atrazine but also the phenolic herbicides suggesting the binding site for this class of compounds is also on the D1/D2/cyt b_{559} complex and not on CP47. Indeed these herbicides were found to reduce the binding affinity of each other in a way similar to that observed with intact thylakoids [9, 10]. A noticeable difference, however, was that the herbicide binding affinities were much lower with the isolated reaction centre compared with the natural membrane system. The question arises, therefore, as to whether the binding sites on the isolated PS II reaction centre are the low affinity sites previously reported or are due to a modification of the high affinity site following detergent extraction. In this paper we investigate further the binding of herbicides to populations of PS core complexes which have been obtained using a new method of isoelectrofocusing. The work has been aided by the use of both herbicide susceptible and resistant material.

Materials and Methods

Photosystem II preparations and herbicide binding experiments

PS II enriched particles (BBYs) were prepared by a method similar to that of Berthold *et al.* [11] except for small modifications. For peas the Triton X-100:chlorophyll ratio was 25:1 and the incubation time 20 min, for *Senecio vulgaris* and its wild type that ratio was 18:1 and the incubation time 10 min [11]. By means of centrifugation the resulting PS II particles (1 mg chlorophyll) were washed with 50 mM Tris pH 9, resuspended in 2 mM EDTA, washed with water, centrifugated at $3000 \times g$ for 10 min. They were incubated with radiolabelled herbicide (0.5–100 μM) after solubilization in 1% dodecyl-β-D-maltoside (DM) (0.5 ml) and applied on a flat-bed of granulated gel. The bed (2.5 × 22 cm) of Ultrodex (LKB) in glycine 0.1 M, 0.08% dodecyl-β-D-maltoside, and 2% ampholine carrier ampholytes of the appropriate pH range, was prepared on a special tray keeping at 4 °C in the dark (LKB Multiphor Apparatus). Isoelectrofocusing (IEF) was run with a constant power of 3 W/tray for 16 h. The bed was fractionated into 28 fractions 0.5 g each. Elution of the proteins and radiolabelled herbicide was carried out 4 times, each time with 0.5 ml Hepes 50 mM (pH 7), 0.5% dodecyl-β-D-maltoside in small plastic columns. Photosystem II core complex was also isolated with a sucrose gradient method from peas [12].

Radiolabelled herbicides

[Ethyl-^{14}C]atrazine (25 Ci/mol) and [ethyl-^{14}C]-hydroxy-atrazine were a kind gift from Ciba-Geigy, [methyl-^{14}C]diuron (57 Ci/mol) was purchased from Amersham. [2′,3′-^{3}H]i-dinoseb (490 Ci/mol) was a kind gift from Dr. W. Oettmeier.

Results and Discussion

In a previous study [9] we attempted to use sucrose density gradients to separate PS II complexes and investigate herbicide binding. This approach, however, was hampered by the diffusion of the radiolabelled herbicides, particular atrazine, within the gradient which tended to mask the specific binding. A recent trend in the field of protein purification is the use of isoelectrofocusing in a flat-bed of granulated gel. This technique has already proved useful for the purification of light-harvesting complexes from PS II enriched particles [13]. Using the protocol given in the Materials and Methods we have found that isoelectrofocusing (IEF) fractionation of PS II enriched particles after solubilization with non-ionic detergent results in several green bands which are schematically shown in Fig. 1. Table I gives the chlorophyll and carotenoids composition of the green bands. Most of the chlorophyll *b* containing complexes migrate as sharp bands at more acidic pH (4–4.9), while bands containing mainly chlorophyll *a* form diffuse green area at higher pH values (4.9–6). The complexes in the green bands were identified from their absorption spectra and their polypeptides composition judged by 6 M urea SDS-PAGE. Denaturing gels were also blotted and assayed with polyclonal antibodies to identify PS II components (Fig. 2).

Table I. Characteristics of the green fractions obtained by IEF of PS II particles solubilized in DM. pH range 4–6. The complexes were resuspended in 0.2% DM, 50 mM Hepes pH 6.5.

Green bands	p*I*	Chl *a*/*b*	Chl/carot.	% Chl	Complex
1	5.8	4.9	4.0	1.4	PS II particles
2	5.5	>10	6.6	3.5	PS II core
3	4.9	>10	5.8	4.7	PS II core + CP29
4	4.8	4.0	5.3	7.0	LHC
5	4.7	2.9	6.5	8.5	LHC
6	4.5	1.4	8.2	52.0	LHC II
7	4.4	1.3	6.8	22.9	LHC II

LHC II, light-harvesting chlorophyll *a*/*b* complexes of PS II; LHC, light-harvesting complexes of PS II which contain no, or little, chlorophyll *b*; CP29, chlorophyll-binding protein having apoprotein of apparent molecular weights of approximately 30 KD. Chlorophyll and carotenoids were determined following the procedure of Wellburn and Lichtenthaler [14].

Using ampholytes for the pH range 4–6, two PS II core complexes are obtained (see Fig. 2). The first complex (p*I* 5.5) resolved by 6 M urea SDS-PAGE into 6 polypeptide bands with apparent molecular masses of 10, 32, 34, 43, 47, 55 KD and seems to be similar to that isolated by sucrose gradient procedures [12]. These polypeptides have been previously attributed to functional components of the PS II [12] and respectively identified as the apoprotein of cyt b_{559}, D1 , D2, CP43, CP47 and the D1/D2 heterodimer. The second PS II complex (p*I* 4.9) contained the minor antenna CP29. When this complex was subjected to sucrose density centrifugation it gave two bands at 0.9 M and 0.5 M sucrose. The former contained the PS II core complex similar to that obtained at p*I* 5.5 by IEF while the latter was due to CP29. Therefore it seems that CP29 was a contaminant.

If ampholytes for the pH range 4–7 are used, PS II cores are recovered in a single large green band with p*I* 5.9. In contrast, if ampholytes for an acidic pH range are used (3.5–5), IEF leads to five PS II core bands. SDS-PAGE indicated that all these complexes contain the same polypeptide profile as given above. We observed that the number of PS II core bands separated by IEF depended on the initial pH of solubilization. In fact an increased number of bands is observed when the sol-

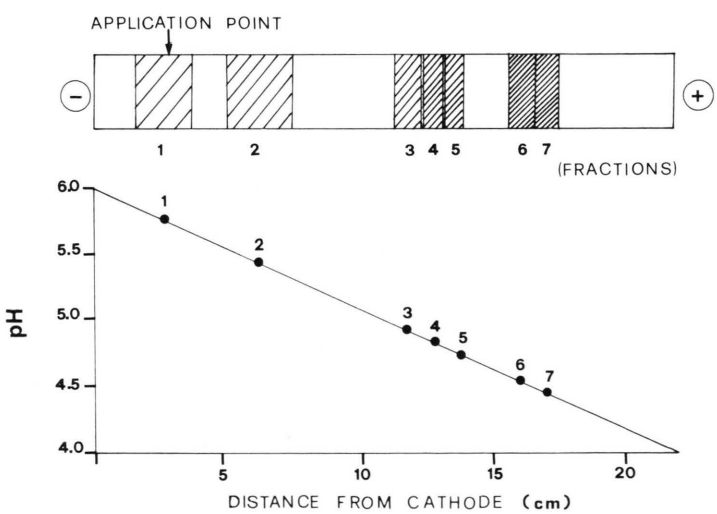

Fig. 1. Scheme of the IEF separation of the solubilized PS II membranes (peas and *Senecio vulgaris*) in the presence of 1% DM in the pH range 4–6. From the drawing p*I* of each complex can be obtained.

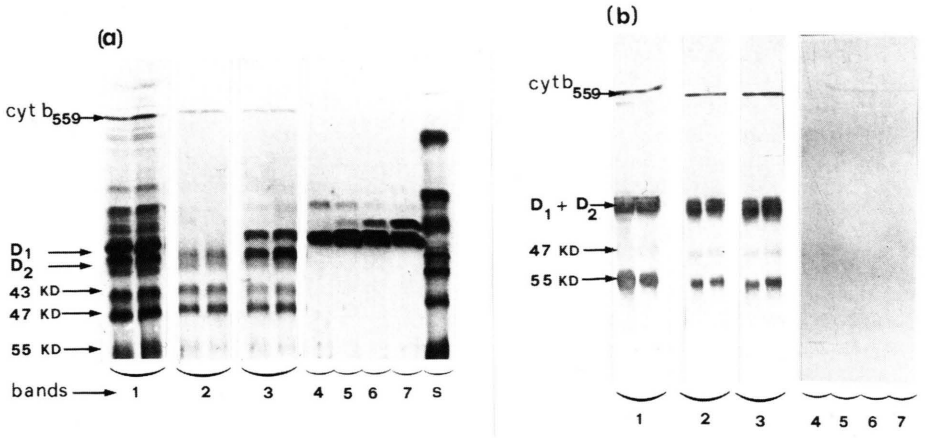

Fig. 2. a–b. Polypeptides composition (a) and western blotting analysis (b) of the complexes from IEF of *Senecio vulgaris* PS II enriched membranes in the pH range 4–6. The IEF complexes were run in 6 M urea SDS-PAGE as previously described [15], then coloured or transferred to nitrocellulose sheet. The blots were assayed with polyclonal antibodies. The binding of the antibody was detected with a secondary *anti*-rabbit IgG coupled with phosphatase [16].

ubilized PS II enriched membranes (BBYs) are applied on a more acidic position of the bed, keeping the same ampholytes pH range (data not shown). Thus we suggest that the PS II core complexes showing different p*I* but similar polypeptides composition differ in their protonated state.

Overall we found that the best conditions for separation and purity of PS II core complexes were obtained with ampholytes mixture for the pH range 4–6 (see Fig. 1 and 2 and Table I) and consequently all the experiments with radiolabelled herbicides were made in this pH range.

The pattern of the bands obtained with solubilized PS II enriched membranes of *Senecio vulgaris* and its atrazine-resistant mutant was found to be very similar to that obtained with peas (Fig. 2).

In our experiments PS II enriched membranes were incubated in the presence of radiolabelled herbicide after solubilization with 1% DM and then applied on the cathode region of the flat-bed at pH 5.8. Fig. 3 to 6 show the analyses of chlorophyll and radioactivity recovery for various fractions throughout the bed. The total radioactive herbicide recovery was about 60% of initial value due to some loss by binding to the surfaces of the apparatus. With the three herbicides used (diuron, atrazine and dinoseb) peaks of radioactivity coincided with the PS II bands. In contrast no such peaks were observed with the light-harvesting II complexes, despite the fact that these contained the majority of the chlorophyll. In similar control

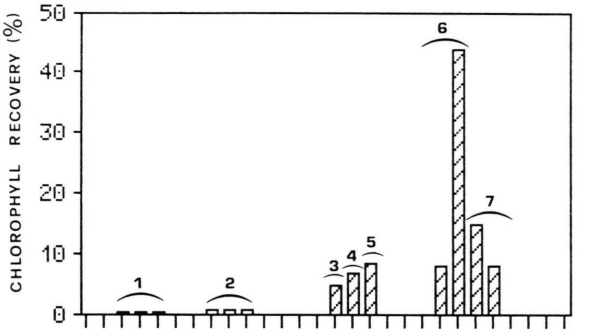

Fig. 3. Chlorophyll *a* + *b* recovery in IEF separated bands of solubilized PS II enriched membranes of wild type *Senecio vulgaris*. The histogram represent chlorophyll recovery for individual 0.5 g fractions. The fractions correspond to the different bands as indicated in Fig. 1.

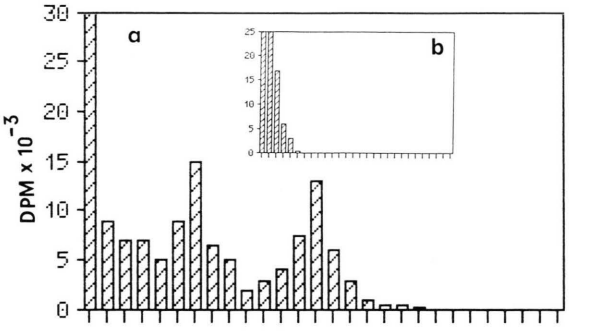

Fig. 4. Histogram of radioactive diuron distribution in 0.5 g fractions of IEF gel in the pH range 4–6 (see Fig. 1). Herbicide concentration 4 μM; a) with solubilized PS II enriched membranes; b) control without solubilized membranes.

experiments using no solubilized PS II enriched membranes, atrazine and diuron were recovered near the negative electrode, while dinoseb was found near the positive electrode due to its migration within the applied field as a consequence of protonation and/or binding to ampholytes. This could be in accordance with the observation reported by Trebst *et al.* [17–18] that the first two compounds have electrophilic characteristics while the last is a nucleophile.

As Fig. 5c (insert) shows, the PS II core complexes isolated from the atrazine-resistant mutant *Senecio vulgaris* does not bind atrazine even at the highest concentrations used.

The two PS II complexes showing different p*I* (5.5 and 4.9) do not bind the different classes of herbicides in a similar way even if they are present on the bed in a similar concentration. In fact the complex with p*I* 5.5 binds all three types of herbicides while the complex with p*I* 4.9 binds diuron and to some extent atrazine but does not bind dinoseb at all. Due to the different pH at which the herbicides are kept on the flat bed, we examined the effect of pH on the herbicide binding to thylakoid membranes. As can be seen in Fig. 7 diuron binding is not affected by pH. However atrazine binding affinity decreases particularly below pH 5.5. On the contrary dinoseb affinity binding is increased at lower pH. Thus the lack of bound atrazine at pH 4.9 can easily be explained as a pH effect. Indeed it is known that the atrazine molecule is not stable at this pH, being easily hydrolyzed to hydroxy-atrazine. Hydroxy-atrazine is a non-toxic atrazine metabolite that does not bind to thylakoids (unpublished) and can be found in microorganisms plants and soil as a degradation product of atrazine [20]. Thus in our conditions we would not expect atrazine to bind efficiently to the PS II complex with p*I* 4.9. On the contrary the lack of bound dinoseb to this complex cannot be explained directly as a pH effect.

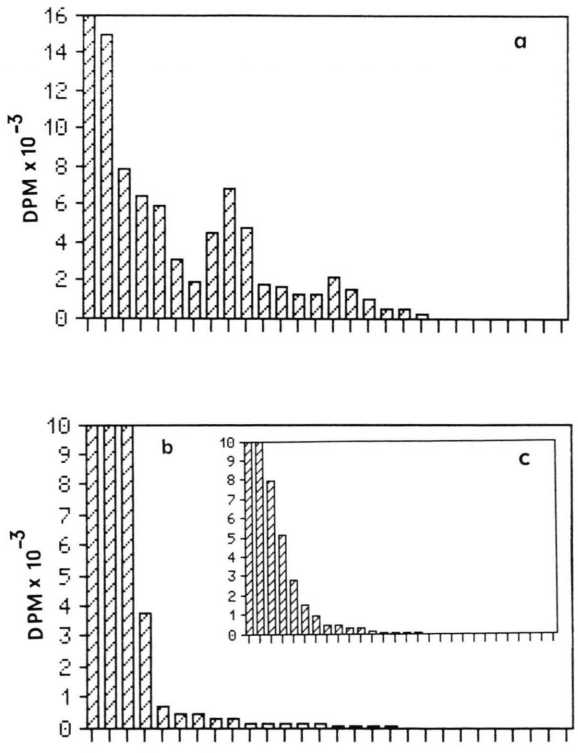

Fig. 5. As in Fig. 4 but with radioactive atrazine; a) with solubilized PS II enriched membranes from wild type *Senecio vulgaris;* b) control without solubilized membranes and c) insert with solubilized PS II enriched membranes from atrazine-resistant mutant of *Senecio vulgaris.*

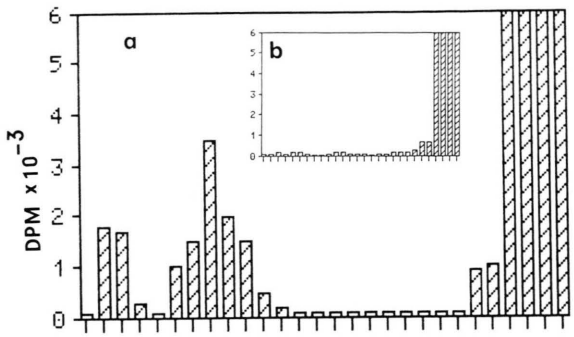

Fig. 6. As in Fig. 4 but with radioactive dinoseb.

We tried to determine the number of binding sites and the binding constant for atrazine in PS II core complexes after extraction from the flat-bed with 0.2% DM, 50 mM Hepes pH 7 [9]. The procedure is problematical because of the non-uniform distribution of free atrazine in the supernatant which lead to an approximate 5% contamination of the pellet. In a previous study [9] we neglected this small effect for other herbicides which showed a greater binding to the isolated PS II reaction centre. For atrazine, however, our results showed too much scatter to allow estimation of the number of binding sites. Despite this a crude analysis of the data suggests a ratio of 1 herbicide-binding

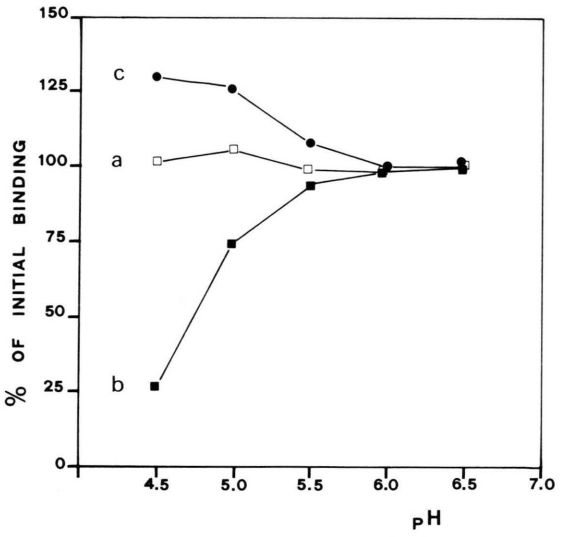

Fig. 7. pH effect on the herbicides binding affinity of *Senecio vulgaris* thylakoids. The values represent the herbicide nmol bound at each pH compared with the bound nmol at pH 6.5 (100%). Herbicide concentration 0.05 μM. a) Diuron; b) atrazine; c) dinoseb.

site per 1 cyt b_{559}, and an affinity constant in the micromolar range.

In a separate set of experiments, PS II core complexes were isolated by the sucrose gradient procedures of Gounaris and Barber [12] and used to investigate the sensitivity of atrazine and diuron binding to various treatments. Even though it was difficult to obtain quantitative data we were able to show that the binding of these herbicides is inhibited by heating, by the addition of 0.5% SDS and by proteolytic digestion with 5 μg/ml trypsin. These sensitivities are similar to those found for herbicide binding to thylakoids [1, 2, 19].

Previous published results clearly showed that the binding of herbicides to the isolated reaction centre and core complex of PS II occurs [9]. These results, however, did not prove that this is the same specific binding that is seen in thylakoid membranes. PS II core complexes with p*I* 5.5 identified in the present study also bind herbicides and in the case of atrazine there is probably a single binding site with a low affinity. This suggests that major structural elements of the native herbicide sites are still present and functional in isolated PS II complexes though the reduced affinity indicates that some elements of the site are removed or altered during the isolation, as we previously shown for D1/D2/cyt b_{559} reaction centre complex. The reduction of binding affinity of detergent prepared PS II enriched membranes (BBY particles) has previously been reported [21]. Furthermore the herbicide binding site in the isolated PS II core complexes shows the same behaviour as the thylakoids with regard to pH dependence, inhibition by heating, SDS and trypsin treatment [1, 19, 22].

It is generally accepted that triazine resistance in weeds usually originates from a single amino-acid change, modifying the structure and binding properties of the high affinity site on the D1 protein [3–5]. We have shown that unlike the wild type, PS II core complexes isolated from triazine-resistant *Senecio vulgaris* do not bind atrazine. Thus this result suggests that the herbicide binding site present in the PS II core preparations is the original high affinity site present in thylakoids and acting at reducing side of PS II. Apparently it is this site which has undergone structural modifications leading to a reduction in its herbicide binding affinity.

The low affinity herbicide binding observed in PS II enriched membranes (BBY) was suggested to be due to a second site acting on the oxidizing side of PS II [8]. It was argued that this site was masked in thylakoids by the presence of the high affinity site on the reducing side. However, this interpretation is open to question because recent data provide a new perspective on the role of the D1 protein by implying that it affects the oxidizing side of PS II in addition to performing its well established function on the reducing side [6, 23, 24].

Since the bonds between the herbicide and the protein(s) are relatively weak, probably hydrogen and/or π bonds [1, 2], we suggest that IEF method is a useful technique to study the herbicide binding. The drawback with this technique, unfortunately, is that it does not lend itself quantitative analysis. This is because the herbicide bound by the PS II complex in IEF is less than expected in an equilibrium condition due to its migration within the applied electrical field.

The IEF technique can lead to many bands of PS II core complexes which show similar polypeptides composition but different p*I* values. For the pH range 4–6 two PS II bands are obtained which show different capacities to bind dinoseb. We sug-

gest that these differences may represent different protonated states so that herbicides bind to them differently in relation to their nucleophilic and electrophilic characteristics. The modification leading to different protonated states of PS II complex has yet to be elucidated. An alternative explanation is that there has been a selective alteration of the dinoseb binding site.

The isoelectrofocusing technique used in the present study has proved useful for separating PS II core complexes from their antenna complexes and for studying herbicide binding. Whether the heterogeneity of the PS II complexes detected by IEF has any relationship with the known heterogeneity of PS II in intact membranes [25, 26] has yet to be shown.

Acknowledgements

We are very grateful for the assistance Dr. L. Di Paolo and Dr. P. Dainese. We are grateful to Prof. Giacometti and members of his group for useful discussion. We wish to thank the British Council and Italian Council of the Research for financial support according to the terms of their agreement "Cooperation between countries".

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