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Herbicide binding to the isolated Photosystem II reaction centre

Maria T. Giardi *, Jonathan B. Marder and James Barber

AFRC Photosynthesis Research Group, Department of Pure and Applied Biology, Imperial College of Science and Technology, London (U.K.)

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The binding of several radiolabelled herbicides to the isolated higher-plant Photosystem II reaction centre was studied. The apoproteins of this chlorophyll-protein complex consist of just the D1 and D2 polypeptides and the cytochrome *b*-559 polypeptide subunits. Comigration as a band on sucrose density gradients revealed binding affinity between the reaction-centre complex and atrazine, diuron, bromoxynil, ioxynil and *i*-dinoseb. Using a variety of concentrations of diuron, ioxynil and *i*-dinoseb, the number of binding sites in the reaction centre and the herbicide binding constants were determined. This showed slightly less than one site per reaction centre for diuron and ioxynil. In each case, the binding constant was much higher than the binding constant in intact thylakoids. For *i*-dinoseb, the results indicated approx. two binding sites per reaction centre. A similar assay on the slightly larger Photosystem-II core complex indicated a single site for *i*-dinoseb, though for each complex, the binding constant for this herbicide was similar; thus, the extra 47 and 43 kDa polypeptides in the larger complex did not appear to contribute to the binding. Competition studies showed the diuron and ioxynil binding sites to be independent. However, both these herbicides partially reduced the binding of *i*-dinoseb, indicating overlapping or interacting binding sites.

Introduction

Recent progress in fractionation of the thylakoid membrane of higher-plant chloroplasts has led to isolation of the PS II reaction centre (RC II) [1]. This preparation contains just four different polypeptide chains corresponding to the α and β subunits of cytochrome *b*-559 [1], D1 (the *psb*A

* Present address: IREV-CNR, 00016 Monterotondo Scalo, Via Salaria, Km 29.300, Roma, Italy.

Abbreviations: atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; bromoxynil, 3,5-dibromo-4-hydroxybenzonitrile; D1, 32 kDa polypeptide subunit of the Photosystem II reaction centre (*psb*A gene product); D2, 30 kDa polypeptide subunit of the Photosystem II reaction centre (*psb*D gene product); dinoseb, 2,4-dinitro-6-*sec*butylphenol; *i*-dinoseb, 2,4-dinitro-6-*iso*butylphenol; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ioxynil, 3,5-diiodo-4-hydroxybenzonitrile; K_b, binding constant; Mes, 4-morpholine-ethanesulphonic acid; P-680, primary donor chlorophyll *a* of Photosystem II; Pheo, Pheophytin *a*; PS II, Photosystem II; Q_A, primary quinone acceptor of Photosystem II; Q_B, secondary quinone acceptor of Photosystem II; RC II, isolated Photosystem II reaction centre (D1/D2/cytochrome *b*-559 complex); PAGE, polyacrylamide gel electrophoresis.

Correspondence: J.B. Marder, AFRC Photosynthesis Research Group, Department of Pure and Applied Biology, Imperial College of Science and Technology, Prince Consort Road, London SW7 2BZ, U.K.

gene product) and D2 (the *psb*D gene product) [2]. During sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), these four polypeptides migrate separately in monomeric form; though some D1 and D2 also migrate as a heterologous aggregate, possibly indicating a stable heterodimer [2]. RC II contains the chromophores chlorophyll *a*, haem (of cytochrome *b*-559) and pheophytin *a* in a 4:1:2 ratio as well as some β-carotene. However, quinone appears to be absent [1].

Isolated RC II displays only a limited range of photochemical activities which all involve charge separation to form P-680$^+$ Pheo$^-$ and then P-680$^+$ re-reduction and/or Pheo$^-$ reoxidation either by charge recombination [3,4] or with exogenous reagents [1,5]. However, the complex itself contains no stable secondary electron acceptors; in more complex PS II preparations and thylakoids, this function is served by bound quinones Q_A and Q_B. Isolated photosynthetic reaction centres from bacteria contain either one [6] or two [7] quinone molecules which can be located by X-ray crystallography. In crystals of *Rhodopseudomonas viridis* reaction centres, a single menaquinone molecule was identified as Q_A, while a Q_B-binding pocket was recognised after soaking the crystals in ubiquinone, or in the competitive inhibitors *o*-phenanthrolene or terbutryn [6]. In higher plants, there is also competitive binding between quinones and certain herbicides [8,9]. The molecular interactions of herbicides with specific components of PS II is the subject of a recent review [10] which paid particular attention to the implications of different models of PS II architecture. In view of the growing acceptance of the D1/D2/cytochrome *b*-559 model as the PS II reaction centre (materially represented by RC II), some of the controversies surrounding herbicide binding may now be further addressed. In this paper we demonstrate that isolated RC II retains a binding capacity for triazine and phenylurea herbicides. We also show that RC II can specifically bind the phenolic herbicides ioxynil, bromoxynil and *i*-dinoseb, suggesting that their binding site, as for triazines and phenylureas, is within the D1/D2/cytochrome *b*-559 complex rather than on the 47 kDa polypeptide (*psb*B gene product) or 43 kDa polypeptide (*psb*C gene product) as was previously suggested (reviewed in Ref. 10).

Materials and Methods

Photosystem II reaction centers. A purified Photosystem II reaction centre fraction (RC II) was isolated from pea seedlings using procedures described elsewhere [1,2,5] and frozen by immersion in liquid nitrogen after addition of 10% glycerol. The frozen material was stored at −80 °C until use.

Cytochrome b-559 assay. Cytochrome *b*-559 was quantified spectrophotometrically from the dithionite-induced absorbance increase at 559 nm using an absorption coefficient of 15 $cm^{-1} \cdot mM^{-1}$.

Radioactive herbicides. [ethyl-^{14}C]Atrazine (25 Ci/mol) and [methyl-^{14}C]diuron (57 Ci/mol) were purchased from Amersham. [ring-^{14}C]Ioxynil (9.5 Ci/mol) and [ring-^{14}C]bromoxynil (11.6 Ci/mol) were a gift from May and Baker Ltd. [2′,3′-^{3}H]*i*-Dinoseb (490 Ci/mol) was a gift from Dr. W. Oettmeier of the Ruhr Universität, Bochum.

Radioactivity measurement. Radioactivity was determined by scintillation counting using Optiphase Safe (LKB) as the cocktail. Counting efficiencies were determined by making similar samples containing radioactive standards.

Sucrose density gradient centrifugation. Reaction mixtures containing RC II (approx. 18 μg chlorophyll) and 1–3 nmol radioactive herbicide in 1 ml 20 mM Mes-NaOH (pH 6.3), 0.1% Triton X-100 were incubated in the dark for 10 min on ice. The reaction mixtures were then layered over 10 ml 0.1–1 M linear sucrose gradients containing 20 mM Mes-NaOH (pH 6.3) and 0.1% Triton X-100, and centrifuged at 180000 × *g* for 16 h at 4 °C. RC II was recovered as a green band about halfway down the centrifuge tube with a recovery of about 70–80%. The radioactivity in the RC II fraction and other fractions was determined.

Measurement of herbicide binding sites. Reaction mixtures of 0.5 ml were prepared in buffer containing 20 mM Mes-NaOH (pH 6.3), 0.1% Triton X-100 and 0.1 M sucrose. Each reaction contained RC II equivalent to 15 μg chlorophyll and a range of concentrations of [^{14}C]diuron or RC II equivalent to 5 μg chlorophyll and a range of concentrations of [^{14}C]ioxynil or [^{3}H]*i*-dinoseb. The mixtures were incubated in the dark for 10 min on ice and then centrifuged at 435000 × *g* for 1 h at 4 °C in a Beckman TL100 tabletop ultracentri-

fuge. The top 400 µl of supernatant was collected and the pellet resuspended in the remaining 100 µl. The radioactivity in each fraction, and in each experiment the recovery of cytochrome *b*-559 in the pellet (usually about 70%) were determined using the methods described above.

Results

For diuron and atrazine, the binding affinities are greatly reduced by procedures involving detergent treatment of thylakoids. Since we routinely use low concentrations of Triton X-100 to prevent

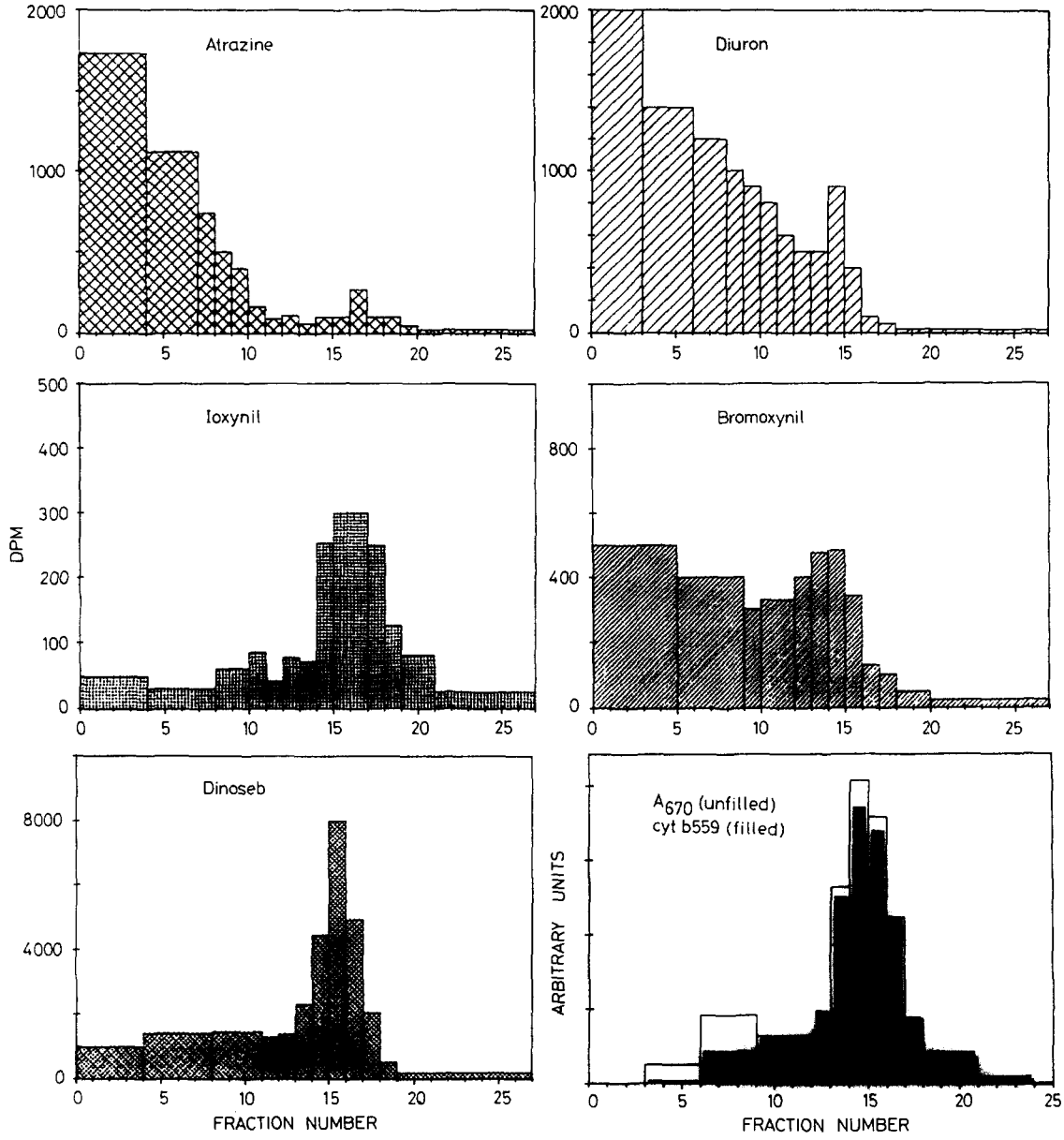

Fig. 1. Comigration of radioactive herbicide and RC II on sucrose density gradients. Preparation of herbicide RC II mixtures and sucrose density-gradient centrifugation were carried out as described in Materials and Methods. The histograms represent radioactive herbicide concentration and RC II (670 nm absorbance and cytochrome *b*-559 (cyt b559)) determined for individual 0.3 ml fractions, which are numbered from the top to the bottom of the respective gradient.

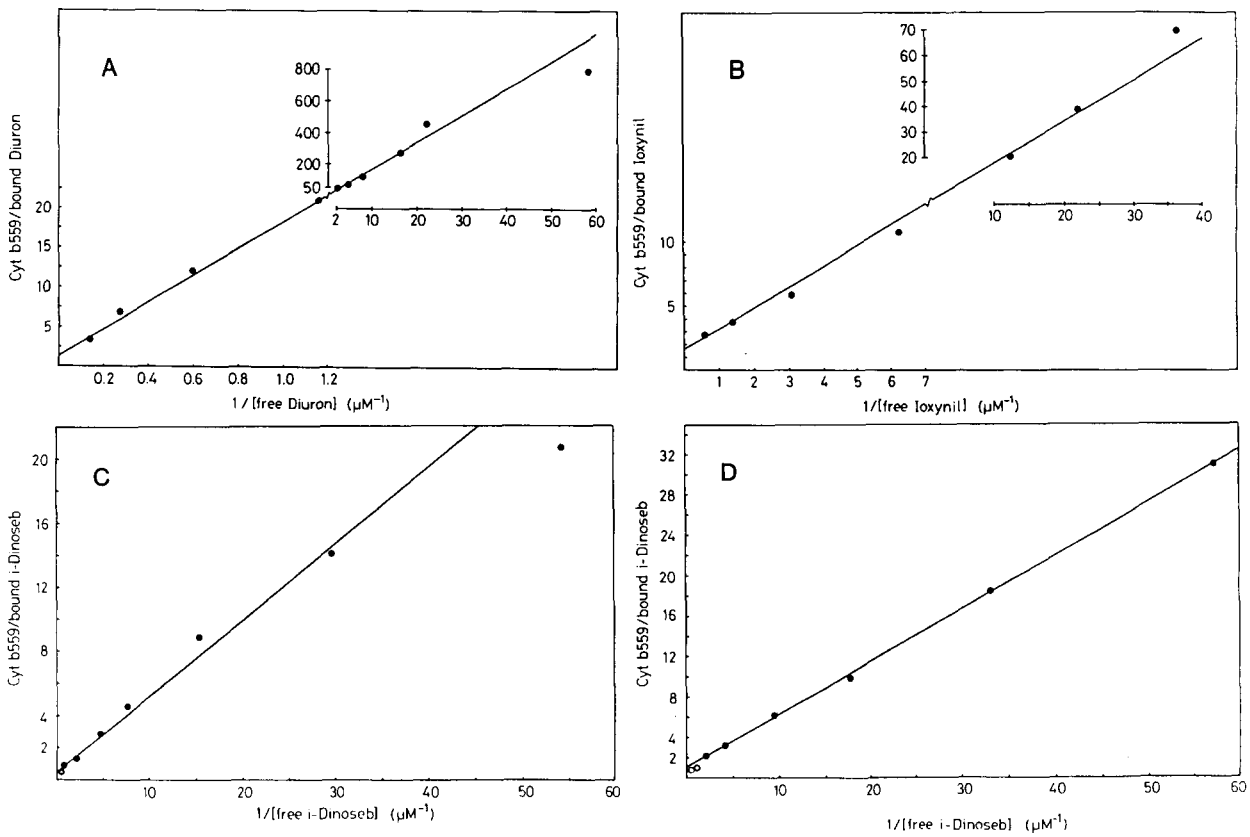

Fig. 2. Measurement of herbicide binding kinetics. Binding assays were performed on three different batches of RC II (A, B and C) or on PS II cores (D), the latter being prepared from pea leaves using a published method [11]. Free and bound herbicide measurements were made as described in Materials and Methods. The straight lines were calculated from non-linear 'least-squares' fitting of the data to a standard binding equation. The closed symbols represent data used in the statistical analysis. The open symbols were judged to constitute non-specific binding and were omitted from the calculation. For diuron and ioxynil, the data are presented on two sets of axes; the fitted lines have the same gradient and intercept on each set. Cyt b559, cytochrome *b*-559.

sedimentation of RC II, we examined the effect of 0.1% detergent on the binding displayed by thylakoid membranes. At pH 7.2, specific binding became undetectable. However, this problem could be overcome by using pH 6.3, and this was therefore used for all further experiments.

Sucrose density gradient centrifugation was used to detect association (specific or non-specific) of radiolabelled herbicides with RC II. Fig. 1 shows the results of density gradient fractionation of RC II herbicide mixtures. In each case, a peak in herbicide concentration coincides with the band containing RC II. In similar experiments using non-PS II-containing chlorophyll-protein complexes (e.g., purified light-harvesting antenna com-

TABLE I

HERBICIDE BINDING SITES AND BINDING CONSTANTS IN ISOLATED RC II AND PS II CORE PARTICLES

The values were calculated by fitting the data in Fig. 2 to a standard binding equation. Each value is presented ±95% confidence limits.

Herbicide	Particle	K_b (μM)	Sites/ cytochrome *b*-559
Diuron	RC II	12.59 ±1.34	0.742±0.078
Ioxynil	RC II	1.013±0.122	0.622±0.039
i-Dinoseb	RC II	0.972±0.454	2.048±0.225
i-Dinoseb	PS II cores	0.478±0.046	0.914±0.024

plex LHC II), the green bands from the gradients contained no peak of radioactive herbicide (not shown). Our evaluation of the results of the experiment in Fig. 1 and similar experiments, is that RC II is capable of binding *i*-dinoseb, ioxynil, bromoxynil, diuron and atrazine in approximate order of decreasing affinity.

We next sought to determine the stoichiometry between herbicide binding sites and RC II. For this, we used a table-top ultracentrifuge capable of achieving very high centrifugal forces to sediment RC II from uniform herbicide-containing reaction mixtures. After centrifugation, most of the chlorophyll was at the bottom of the centrifuge tube, partly in a loose pellet. The absorption spectrum of this material indicated it to be intact RC II. Using cytochrome *b*-559 as a measure of RC II amount, approx. 70% of the sample is recovered at the bottom of the tube. The remainder is in the upper portion of the tube and apparently represents RC II breakdown. The exact yield varied between RC II preparations and was therefore determined for each binding assay. Using measurements of the herbicide concentration at the top (free) and at the bottom (bound + free) of the tubes, the data shown in Fig. 2) were derived. The figure shows double-reciprocal plots relating free herbicide concentration to the amount bound. The linear relationships in the data indicate a single predominant binding constant. Fig. 2 also shows data for *i*-dinoseb binding to PS II cores [11], which contain 47 and 43 kDa polypeptides not present in RC II. Table I shows the values for K_b and the stoichiometry between the binding sites and cytochrome *b*-559. Previous studies to determine the binding parameters of herbicides in thylakoids revealed K_b values of 34 nM for diuron [12], 3–30 nM for ioxynil [12,13], and 69 nM for *i*-dinoseb [14]. Although we have used relatively high herbicide concentrations because of the much reduced binding affinities, we have not observed the same degree of non-specific binding that has appeared in similar studies on thylakoids. This may be because many of the lipid and protein species which are probably involved in non-specific binding in thylakoids are absent in RC II. Alternatively, the experimental methods used in this work may improve the apparent specificity. In previously reported work, 'bound' herbicide was estimated by subtraction of the final 'free' herbicide concentration from the starting concentration (e.g., see Ref. 15), so binding to the centrifuge tubes would indicate additional (non-specific) sites. However, we have measured the amount of herbicide in the RC II fraction directly, so any herbicide 'lost' by binding to the apparatus has no effect on the estimates of binding.

Fig. 3 shows the results of experiments to determine the extent of competition between radioactive *i*-dinoseb and non-radioactive diuron. At

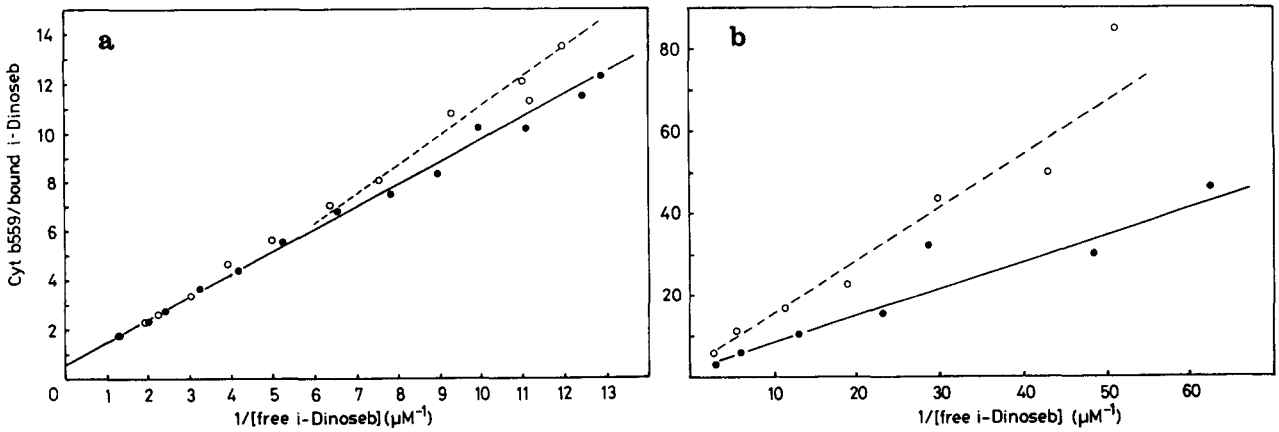

Fig. 3. Effect of diuron on the binding of [^{14}C]*i*-dinoseb. Closed symbols represent data obtained in the absence of diuron. Open symbols represent data obtained in assay solutions containing 20 μM unlabelled diuron. Measurements of free and bound *i*-dinoseb were made as described in Materials and Methods. The figure shows two double-reciprocal plots covering different concentration ranges of *i*-dinoseb. These were obtained using two separate batches of RC II, though within each plot all measurements were made using the same batch. Cyt b559, cytochrome *b*-559.

TABLE II

BINDING OF LABELLED HERBICIDE IN THE PRESENCE AND ABSENCE OF UNLABELLED HERBICIDE

Radiolabelled ioxynil or *i*-dinoseb was added as indicated to concentrations of 200 nM or 40 nM, respectively. Unlabelled ioxynil and dinoseb were used at 200 μM. The amount of (radiolabelled) herbicide binding was determined as described in Materials and Methods. Displacement values represent the loss of binding in the presence vs. absence of unlabelled herbicide.

Labelled	Unlabelled	Bound/ cytochrome *b*-559	Displacement (%)
Ioxynil	none	0.190	–
Ioxynil	dinoseb	0.043	77
i-Dinoseb	none	0.021	–
i-Dinoseb	ioxynil	0.012	43

high *i*-dinoseb concentrations, the degree of binding is not detectably changed by the presence of a relatively high background concentration (20 μM) of diuron (Fig. 3a). The same diuron concentration does reduce *i*-dinoseb binding at lower concentrations (Fig. 3b). This is seen in the increased gradient of the double-reciprocal plot. This behaviour is not consistent with simple competition between the two herbicides. Furthermore, the effect probably involves at least two distinct types of *i*-dinoseb binding site. Similar results have been obtained using 2.5 μM unlabelled ioxynil (data not shown). However, the presence of unlabelled diuron (20 μM) showed no effect whatsoever on the binding of radioactive ioxynil. Additional experiments were performed using fixed concentrations of labelled herbicide and varying concentrations of unlabelled herbicide. Table II shows the dinoseb is very effective in eliminating most of the ioxynil binding, while ioxynil, even at high concentration, eliminates *i*-dinoseb binding only partially.

Discussion

Specific herbicide binding is characterised by a unique binding constant (K_b) rather than the range of binding affinities which characterises non-specific binding. The detection of specific binding depends on a unique K_b with a lower value (indicating higher affinity) than the range for the non-specific binding. Our results clearly show that specific binding of herbicides to RC II occurs, but not necessarily that this is the same specific binding that is seen in thylakoid membranes. The binding of a molecule in a site is a function of its interactions with structural elements of that site. Each interaction contributes quantitatively to the binding. Some of the structural elements may be able to form a binding site for other types of molecule; thus several molecular species may bind competitively in the same structural domain. This is probably the case for several PS II herbicides which bind in the Q_B site [16]. The elimination of some interactions occurs when some structural elements are removed or rearranged, and this may alter the binding affinity for some molecular species. In studying herbicide binding to isolated Photosystem II reaction centres, we have quantified the number of binding sites and the binding constant for each of three herbicides. Thylakoids bind these herbicides at sites which are approximately equimolar with the PS II reaction centre [15]. Allowing for some degeneration of binding sites in our samples, RC II appears to contain single binding sites for diuron and ioxynil, and two sites for *i*-dinoseb (Table I). This suggests that major structural elements of the native herbicide sites are present and functional in RC II, though the reduced binding affinities indicate that some elements of the sites are removed or altered. We cannot eliminate the possibility that the herbicide binding sites detected in this work are low-affinity sites considered 'non-specific' in the thylakoid assays, or newly created sites which arise as an artefact of the RC II preparation. It is therefore important to establish biochemical functions of RC II which show herbicide sensitivity and quantitatively correlate the inhibition of these functions with herbicide binding as was done for thylakoids [15]. Indeed, recent experiments in this laboratory have identified a photochemical reaction of RC II which is inhibited by diuron with an I_{50} value very similar to the K_b for diuron in Table I [17].

A number of studies have suggested a primary role for the D1 protein in the binding of triazine and phenylurea herbicides. This contention is most strongly supported by two lines of evidence: (a)

gene sequences of several different herbicide-resistant mutants reveal in each case a single amino-acid substitution in the D1 protein [18–20], these mutation points all being clustered in a restricted segment of the protein; and (2) the photoaffinity probes azido-atrazine [16] and azido-monuron [21] specifically label the D1 protein. Renger and co-workers [22] have investigated the effects of proteolysis of D1 and other proteins. Trypsination of thylakoids under conditions which cleave D1 leads to a reduction in the binding of these herbicides [22]. However, a similar result obtained using a lysine-specific protease instead of trypsin implicates other proteins, since in the plant material used (pea) and in several other species D1 completely lacks lysine residues and is resistant to digestion by this enzyme [22]. Our results showing diuron and atrazine binding to RC II suggest that polypeptides outside this complex play at most a peripheral role in the binding site. It is likely that at least some of the lysine-specific digestion effects can be attributed to the susceptibility of D2 to cleavage [2].

The picture is less clear for phenolic herbicides. The photoaffinity probe azido-*i*-dinoseb labels polypeptides in the 41–53 kDa range [23]. It was therefore suggested that the 43 kDa and possibly the 47 kDa chlorophyll binding proteins of the Photosystem II core form a major part of the binding site for phenolic herbicides. This suggestion conflicts with our data, showing *i*-dinoseb, ioxynil and bromoxynil binding sites in the D1/D2/cytochrome *b*-559 complex, RC II. Moreover, if the 43 kDa and 47 kDa proteins surround the PS II reaction centre in a light-harvesting capacity (see Ref. 10), it is easier to explain the inhibitory effect of phenolic herbicides if the binding site is on neither of these proteins, but rather on the reaction centre apoproteins. Indeed, azido-*i*-dinoseb does also label a 32 kDa band in thylakoids, but this band is not apparent in the detergent-solubilised PS II-core [23]. Since it is now clear that D1/D2 aggregates may form as a result of certain detergent treatments [2], we suggest that at least some of the azido-*i*-dinoseb label observed in the 41–53 kDa range was in fact attached to aggregated D1 rather than to just the 43 or 47 kDa polypeptides, as was previously assumed [23]. We cannot rule out a peripheral role for the 43 kDa, 47 kDa and other non-RC II proteins in the native herbicide binding site, since the affinity we detect is somewhat reduced. However, the affinity for *i*-dinoseb is reduced to a similar extent in PS II cores, indicating that the 43 and 47 kDa proteins, although present, make little or no contribution to binding in this particular PS II preparation.

The occurrence of binding sites for different herbicide classes within RC II raises the possibility that the sites might be overlapping or interacting in some way. Oettmeier and co-workers [24] have compared the binding of different classes of herbicides and concluded that the binding site for the phenolic herbicides probably involves different proteins to the site for the triazines and phenylureas. Some evidence for this is the differential sensitivities of the sites to various perturbations, e.g., proteolytic digestion, detergent extraction and mutations in the D1 protein. Competition studies involving pairs of herbicides have produced conflicting results. Vermaas [25] has produced results showing competitive binding between atrazine and ioxynil, thus indicating a common site. On the other hand, diuron and ioxynil interact non-competitively in their binding to intact thylakoids [24], indicating non-overlapping but interdependent sites. This interdependence is apparently lost in RC II, since ioxynil and diuron bind independently rather than competitively or non-competitively (see Results). Competition studies involving *i*-dinoseb binding to RC II are complicated by the presence of two sites per reaction centre. The results presented in Fig. 3 and Table II suggest that diuron or ioxynil binding can interfere with some, but not all, the *i*-dinoseb sites. Because these effects involve only some of the sites, we cannot specify whether or not the interactions are competitive. A second problem is that the results do not show whether the *i*-dinoseb sites affected by diuron are the same ones affected by ioxynil. However, their equivalence is suggested from previous work [24], which demonstrated a single site for the *i*-dinoseb analogue 2-iodo-4-nitro-6-*iso*-butylphenol in intact thylakoids, which exhibited both competitive interaction with ioxynil and non-competitive interaction with diuron. It is tempting to speculate that the second (diuron/ioxynil-independent) *i*-dinoseb binding site in RC

II corresponds to the Q_A site which is devoid of plastoquinone in this particle [1]; in PS II-core preparations, which contain tightly bound plastoquinone [11], only one site was observed (Table I).

We suggest that the approach we have adopted in this work can be extended to test the binding of other molecular species, e.g., various quinones. Such experiments would be useful in characterising the Q_A and Q_B sites and possibly other sites in RC II, and comparing them to the native sites which exist in intact thylakoid membranes. This would help identify conformational changes induced by the isolation of RC II, providing objective criteria for assessing the reconstitution of PS II.

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