Herbicide/Quinone Binding Interactions in Photosystem II

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Many inhibitors prevent the oxidation of the primary electron-accepting quinone (Q_A) by the secondary quinone (Q_B) in photosystem II by displacement of Q_B from its binding site. On the other hand, plastoquinone-1 and 6-azido-5-decyl-2,3-dimethoxy-*p*-benzoquinone displace herbicides. Binding studies show the herbicide/quinone interaction to be (apparently) competitive.

The herbicide binding is influenced differentially by various treatments. In this paper it is shown that the affinity of, for example, bromoxynil is decreased by thylakoid unstacking or by light- or reductant-induced reduction of certain thylakoid components, whereas atrazine affinity remains unchanged. Furthermore, absence of HCO_3^- in the presence of formate leads to an affinity decrease of bromoxynil and atrazine, but to an increase in i-dinoseb affinity. Other differential photosystem II herbicide effects are known from the literature.

Since different and unrelated groups of Q_A^- oxidation inhibitors have been found, and because of the above-mentioned dissimilarities in binding characteristics for different inhibitor groups, the hypothesis of non-identical, but "overlapping" binding sites for different herbicide groups and the native quinone must be more extensively defined. In this manuscript we evaluate both the competitive herbicide/quinone binding model, and a model in which binding of one ligand alters the protein conformation resulting in a dramatic decrease in the binding affinity of ligands from other chemical groups; in this model ligands from the same or related chemical groups bind competitively. Thus, the latter model proposes that only one herbicide or quinone molecule can be bound with high affinity to the herbicide/quinone binding environment, but it depends on the chemical structure of the ligands whether the binding interaction between two ligands is truly competitive or more indirect (allosteric), mediated through the protein conformation.

Introduction

A large variety of different chemicals, acting as powerful herbicides, are known to block the electron transport between the primary and secondary electron-accepting plastoquinone (referred to as Q_A and Q_B, respectively) at the Photosystem II (PS II) acceptor side (for a recent review, see [1]). Q_A and Q_B are inferred to be bound to functional protein constituents of PS II [2]. Q_B and PS II herbicides were hypothesized to bind competitively to the same binding environment [3, 4]. Using the method of binding radioactively labelled PS II herbicides to thylakoid membranes [5], all PS II inhibitors tested, as well as an electron accepting quinone [6], were shown to replace each other (with the exception of 2-bromo-3-methyl-1,4-naphthoquinone [7]). These results and recent data analyzing the competition between herbicides and quinones [8–10] support the hypothesis of a common binding environment for Q_B and PS II herbicides. This conclusion, however, does not necessarily indicate that all PS II herbicides and quinones have certain binding determinants in common as was implicated in previous models of overlapping binding sites [11, 12].

The construction of a comprehensive model for the binding of PS II inhibitors must consider various ways in which different chemical classes of inhibitors bind (apparently) competitive with

Abbreviations: Atrazine, 2-chloro-4-(ethylamino)-6-isopropylamino-*s*-triazine; bromoxynil, 3,5-dibromo-4-hydroxybenzonitrile; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; i-dinoseb, 2,4-dinitro-6-isobutylphenol; durohydroquinone, tetramethyl- -benzohydroquinone; ioxynil, 3,5-diiodo-4-hydroxybenzonitrile; metamitron, 4-amino-4,5-dihydro-6-phenyl-3-methyl-1,2,4-triazin-5-one; PQ, plastoquinone; PS, photosystem; Q_A, primary electron-accepting quinone; Q_B, secondary electron-accepting quinone; Q_B protein, rapidly turned over protein ($M_r \sim 32000$ Da) that is specifically labelled by azido-atrazine.

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respect to quinone and herbicide binding. Two primary mechanisms exist:

1) binding to physically overlapping sites so that the two compounds cannot simultaneously exist in the common site, or
2) binding to distinctly different sites such that binding of one compound causes a conformational change in the binding protein(s), thereby dramatically reducing the affinity of the second compound.

The latter possibility, obviously, could involve different binding sites either in close or distant proximity. The idea of allosteric herbicide binding is consistent with the observation that the herbicide binding affinity is affected by protein phosphorylation [13], CO_2-depletion [14, 15], and covalent linkage of an azidoquinone [6].

Due to space limitations, only part of the results shown at our presentation in Wageningen can be presented here. For further details the reader is referred to [9].

Materials and Methods

The thylakoid isolation from pea leaves and from leaves of triazine-susceptible or resistant biotypes of *Amaranthus hybridus* L. was as in [16]. After isolation, the thylakoids were suspended in a medium consisting of 50 mM tricine/NaOH, 10 mM NaCl, 5 mM $MgCl_2$ and 0.3 M sorbitol, at pH = 7.6. This medium was used for the experiments unless indicated otherwise.

The herbicide binding experiments performed as described in [6] were done under controlled light intensity conditions (virtually in the dark unless indicated otherwise). In order to ensure equilibrium conditions, it was necessary to incubate the thylakoids with [^{14}C]bromoxynil or ioxynil for at least 10 min. For atrazine, shorter incubation times were sufficient.

For determining the binding affinity of i-dinoseb, the [^{14}C]atrazine or [^{14}C]ioxynil binding at ten different labelled herbicide concentrations was monitored in the absence and presence of three different concentrations of unlabelled i-dinoseb. Using the equations derived for competitive herbicide binding [5, 9], the dissociation constant of [^{12}C]i-dinoseb was calculated. Direct measurements of radioactive i-dinoseb binding to the inhibitor/quinone binding environment is very tricky because of the large contribution of i-dinoseb binding to thylakoid membrane components unrelated to inhibition of electron transport (see, for example, [17]).

HCO_3^--depleted thylakoids were obtained by suspending thylakoids in a CO_2-free medium consisting of 50 mM MES/NaOH, 10 mM NaCl, 5 mM $MgCl_2$, 25 mM HCOONa and 0.2 M sorbitol at pH = 6.0. The thylakoid suspension was allowed to incubate for 1 h at room temperature in a capped tube, which was flushed previously with N_2. After this incubation period, 5 mM $NaHCO_3$ was added to certain samples ("reconstituted thylakoids").

Results

I. Quinone reduction by light or dithionite

Reduction of the plastoquinone pool and Q_B by durohydroquinone or dithionite leads to a marked decrease in the affinity of ioxynil, but not in that of atrazine [9]. This effect was attributed to a reduction-induced conformational change of a special region of the inhibitor/quinone binding environment. We also observed that the bromoxynil affinity was decreased upon reduction by dithionite (Fig. 1). The same affinity changes appear to be inducible by light (Fig. 1); these changes are reversible in the dark (data not shown). Therefore, the reduction-induced affinity change is unlikely to be due to a chemical reduction of bromoxynil because this reaction is expected to be irreversible. An affinity decrease upon illumination comparable to that of bromoxynil was also observed for ioxynil and i-dinoseb, whereas the binding of atrazine (and also of metamitron, W. Vermaas, W. Draber, and G. Renger, unpublished) was only slightly affected (see Table I).

II. Divalent cations

Grana unstacking caused by omission of Mg^{2+} from the solution used for thylakoid suspension and subsequent restacking by $MgCl_2$ addition also appears to result in affinity changes of bromoxynil (Fig. 2A) and ioxynil (data not shown), whereas atrazine binding remains almost unaffected (Fig. 2B). As could be expected, the total number of herbicide binding sites is insensitive to stacking and unstacking.

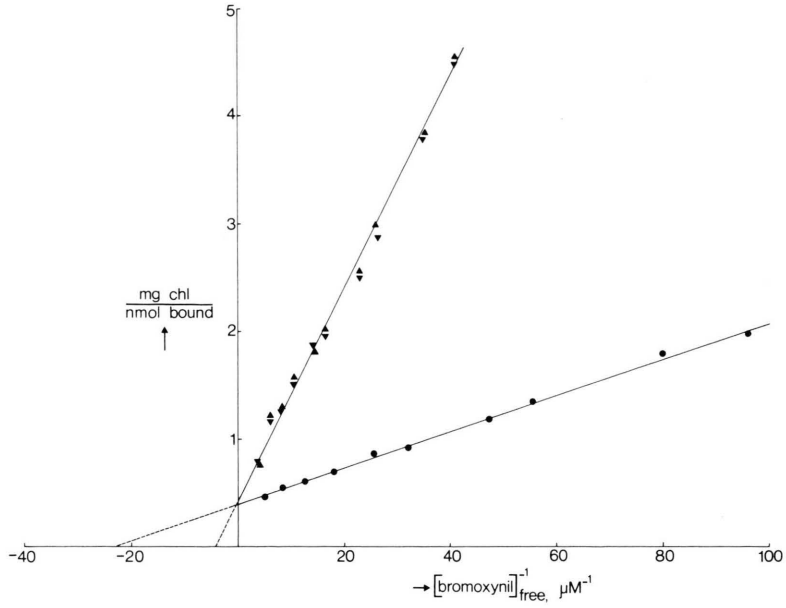

Fig. 1. Double-reciprocal plot of [^{14}C]bromoxynil binding to pea thylakoids (50 μg · ml^{-1} chlorophyll) in the dark in the absence (●) and presence (▼) of 2 mM sodium dithionite and in bright room light without dithionite (▲). K_d = 45 nM in oxidized and 220 nM in reduced conditions. One bromoxynil binding site per ~400 Chl molecules.

III. Effects of HCO_3^-/CO_2

It is known that, in the presence of formate, absence of HCO_3^- leads to a decrease in the affinity of atrazine [14] and of ioxynil [15]. We observed a considerable decrease in bromoxynil affinity upon CO_2-depletion as well: in the absence of HCO_3^- the bromoxynil dissociation constant K_d was 125 nM, whereas after addition of 5 mM HCO_3^- K_d decreased to 58 nM. The number of bromoxynil binding sites did not change upon HCO_3^- addition. In our hands the atrazine affinity was lower (K_d = 225 nM and 58 nM in the absence and presence of HCO_3^-, respectively) than reported by Khanna *et al.* [14]. Preliminary results indicate that the affinity dependence of i-dinoseb on the presence of CO_2 is opposite to that of atrazine, ioxynil or bromoxynil.

Table I. Herbicide affinity in light and in darkness. Dissociation constants (K_d) in nM of three herbicides in darkness and in bright room light. 50 μg · ml^{-1} chlorophyll. Atrazine and ioxynil affinity were determined directly by binding experiments with these ^{14}C-labelled herbicides; i-dinoseb affinity was determined by competition experiments of [^{12}C]i-dinoseb with [^{14}C]atrazine (see [9]).

	Light	Dark
Atrazine	63	57
i-Dinoseb	450	220
Ioxynil	22	6

At pH = 6.0, the K_d of i-dinoseb is 30 nM in the absence, and 105 nM in the presence of HCO_3^-. This behaviour may partially explain the seemingly competitive interaction between HCO_3^- and 4,6-dinitro-*o*-cresol [18] and i-dinoseb [19].

IV. Quinone addition in triazine-resistant and -susceptible thylakoids

As could be expected from the hypothesis of a common quinone/inhibitor binding environment, PQ-1 competes with PS II herbicides for binding. Based on competition studies between PQ-1 and [^{14}C]bromoxynil, the apparent dissociation constant of PQ-1 was found to be 15 μM in triazine-susceptible (not shown), and 20 μM in triazine-resistant thylakoids (Fig. 3). Since most PQ-1 may partition into the thylakoids, it is better to express the PQ-concentration in terms of a PQ-1/chlorophyll (Chl) ratio. The Chl concentration was 100 μg · ml^{-1}; thus, the PQ-1/Chl ratio necessary for decreasing the bromoxynil affinity by a factor of 2 was 0.15 for triazine-susceptible and 0.20 for triazine-resistant thylakoids. On the basis of 400 chlorophylls per PS II chain and neglecting the binding of the native plastoquinone molecules, a number of 60–80 PQ-1 molecules per PS II chain is calculated to be necessary in order to occupy the herbicide binding

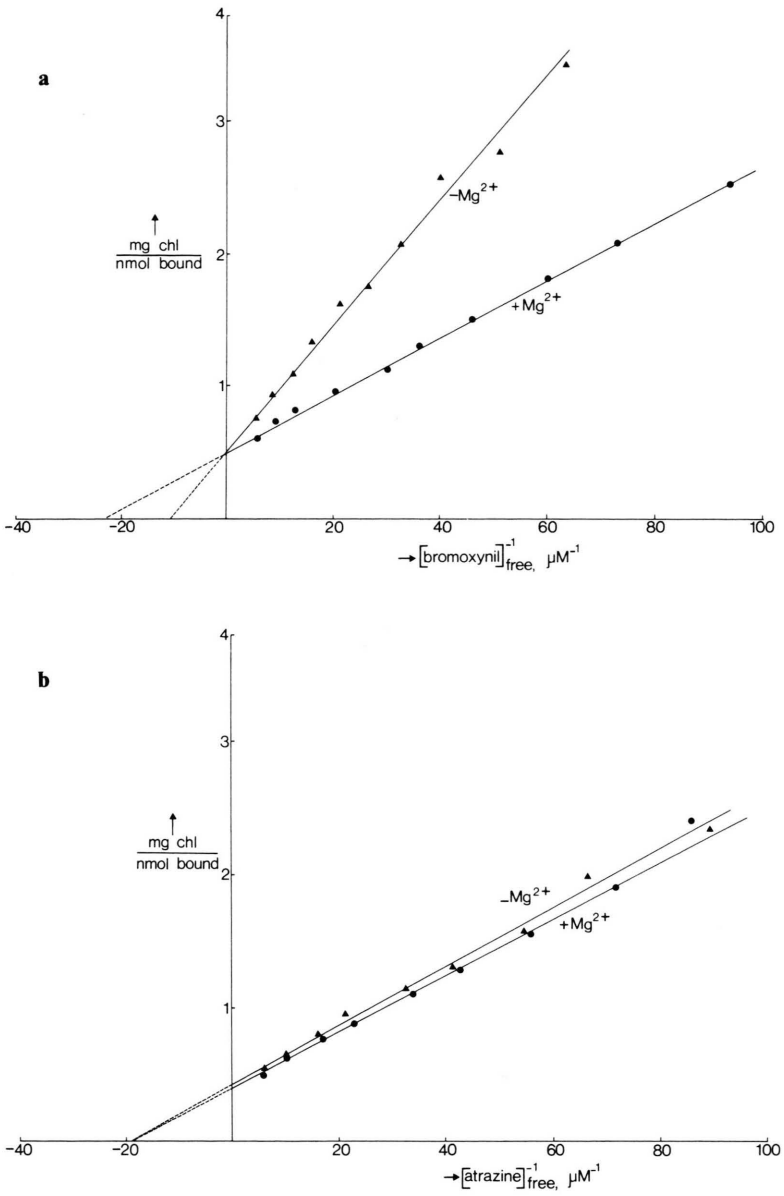

Fig. 2. Double-reciprocal plot of [^{14}C]bromoxynil (A) and [^{14}C]atrazine (B) binding to pea thylakoids in the dark in the presence (●) and the absence (▲) of 5 mM Mg^{2+}. The reaction medium used (pH = 7.6) did not contain Mg^{2+}. When necessary, 5 mM $MgCl_2$ was added. For bromoxynil, K_d = 45 nM in the presence and 93 nM in the absence of Mg^{2+}. For atrazine, K_d = 53 nM irrespective the Mg^{2+}. One herbicide binding site per 400–500 chlorophyll. 50 μg · ml^{-1} Chl.

environment in 50% of the reaction chains, assuming a uniform PQ-1 distribution.

Under similar conditions, 2.5 and 5 μM of the synthetic electron-accepting quinone 6-azido-5-decyl-2,3-dimethoxy-*p*-benzoquinone were required to increase the bromoxynil dissociation constant by a factor of 2 in triazine-susceptible and -resistant thylakoids, respectively. Thus, the azidoquinone has a higher affinity for the inhibitor/quinone binding environment than PQ-1.

Discussion

The results show that the affinity of PS II-directed inhibitors to their binding environment at the PS II acceptor side is changed by various treatments. A specific treatment does not result in a similar change for different inhibitors. Under conditions leading to reduction of the plastoquinone pool (either chemically or photochemically), the binding affinity of ioxynil, bromoxynil and i-dinoseb

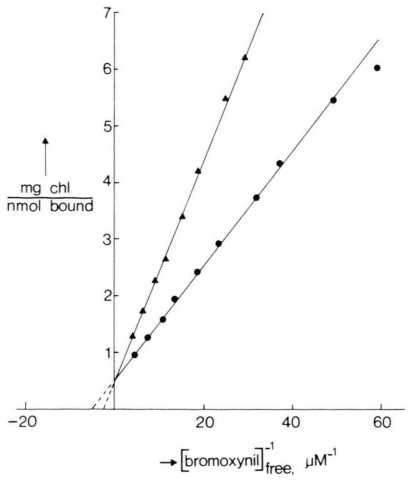

Fig. 3. Double-reciprocal plot of [^{14}C]bromoxynil binding to triazine-resistant thylakoids from *Amaranthus hybridus* in the absence (●) and the presence (▲) of 20 μM PQ-1; 100 μg chlorophyll per ml. One bromoxynil binding site per ~ 500 Chl molecules; K_d = 193 nM in the absence and 403 nM in the presence of PQ-1.

decreases, while that of atrazine or metamitron remains almost unchanged. Analogous effects are observed after grana unstacking. The modification of the binding properties can be understood by conformational changes in parts of the herbicide binding environment induced by different treatments. In the case of a reduction-induced herbicide affinity change, the conformation of the binding environment could be affected by the reduction of amino-acid side chains in the binding protein. Mg^{2+} can directly modify the structure of the binding environment or indirectly via grana stacking. Since the herbicide binding protein(s) is expected to be rather surface-exposed (for example, the 32 kDa polypeptide that can be labelled by azidoatrazine (the "Q_B protein") is attacked very easily by the protease trypsin) micro-environments in this protein complex could be altered by grana unstacking. The results obtained with Mg^{2+} omission and readdition are in line with previous findings that show significant effects of Mg^{2+} on the trypsinization patterns of PS II electron transport and its blockage by PS II herbicides [20].

Since replacement of HCO_3^- by $HCOO^-$ is known to cause a large impairment of electron transport between Q_A and Q_B [21], it is not surprising that HCO_3^- also affects herbicide binding. The observation that i-dinoseb and, for example, atrazine affinity behave qualitatively different upon CO_2-depletion and readdition is in line with many other observations (also, see above) that the direct environments of the binding sites for atrazine and i-dinoseb do not behave similarly. The most striking difference in behaviour of atrazine and phenol-type inhibitors is found in triazine-resistant thylakoids in which the atrazine affinity is decreased by 3 orders of magnitude whereas the affinity of phenol-type inhibitors is increased considerably [12]. These effects are caused by a change in only one amino acid in the 32 kDa "Q_B protein" (J. Hirschberg *et al.*, these proceedings).

Triazine-resistance also appears to differentially modify quinone affinity: the results presented here indicate that in triazine-resistant thylakoids the affinity of some quinones was decreased slightly whereas the tetrachloro-*p*-benzoquinone affinity is known to be increased considerably [10]. Assuming that, because of the large structural similarities between the different quinones, the binding sites for these quinones are (partially) overlapping, other determinants than only the quinone head group appear to modulate the quinone binding [10]. Not only quinone binding is modified in triazine-resistant thylakoids, but also the Q_B^- stabilization appears to be changed, resulting in a shifted equilibrium between $Q_A^- \cdot Q_B$ and $Q_A \cdot Q_B^-$ [10, 22].

The amount of PQ-1 needed to occupy half of the binding sites (PQ-1/PS II chain = 60 in triazine-susceptible thylakoids) is higher than might be expected because the PQ pool that is filled by PS II consists of about 7 molecules per PS II chain in thylakoids [23]. Oettmeier and Soll very recently even reported that they could not observe herbicide displacement by PQ-1 addition without previous extraction of the native PQ [24]. The relative inefficiency of PQ-1 in herbicide displacement may indicate that added quinones do not partition uniformly into thylakoid membranes. Alternatively, the quinone binding site may be occupied by Q_B in only part of the reaction chains [25], or the PQ-1 affinity for the site may be considerably lower than that of the native PQ-9.

The selective changes in inhibitor binding to the thylakoid suggest that the binding domains which regulate inhibitor binding can be selectively altered. There are two mechanisms by which these selective effects can occur. First, there may be multiple

"allosteric" binding sites on the Q_B protein (or even on adjacent polypeptides), such that binding at any of these sites causes a conformational change in the protein complex that limits the subsequent binding of unrelated inhibitors or the native PQ. The binding interaction between two related inhibitors or quinones, however, is expected to be competitive. The alternative model is that all inhibitors and PQ bind to a common domain, and thereby bind competitively. This "common site" model must imply the participation of many polypeptide side chain determinants in a binding site, such that these determinants can be selectively altered to give rise to the affinity changes summarized in this paper. However, the "common site" model cannot accommodate the observation that [^{14}C]ioxynil and, to a lesser extent, [^{14}C]atrazine binding is still possible after covalent linkage of an azidoquinone to the binding environment [6], unless it is assumed that the covalent quinone binding has changed the binding environment such that herbicide binding becomes possible, although with decreased affinity. No additional assumptions are required, however, to explain the herbicide binding data in the presence of covalently bound azidoquinone [6] and non-covalently bound 2-bromo-3-methyl-1,4-naphthoquinone [7] by the "allosteric" model. Again, it should be stressed that also in this model allosteric interactions may only play a role between two different types of herbicides or between a herbicide and a quinone. The binding interaction between related herbicides or quinones is assumed to be truly competitive.

The final elucidation of a PS II inhibitor binding model will depend on a combined effort using protein chemistry coupled to both physiological assays and the use of a wide chemical range of inhibitors. Recent rapid advances in these fields offer encouragement that critical experiments can be conducted in the near future.

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