Competitive interaction of three peroxidizing herbicides with the binding of [^{3}H]acifluorfen to corn etioplast membranes

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The specific binding of the herbicide acifluorfen 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid to corn etioplast membranes is competitively inhibited by protoporphyrinogen IX, the substrate of protoporphyrinogen oxidase. Three other peroxidizing molecules, oxadiazon [5-ter-butyl-3-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazol-2-one], LS 82556 [(*S*)3-*N*-(methylbenzyl)carbamoyl-5-propionyl-2,6-lutidine], and M&B 39279 [5-amino-4-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)pyrazol], also compete with acifluorfen for its binding site. The four herbicides thus bind to the same site, or to closely located sites, on the enzyme protoporphyrinogen oxidase.

Binding; Diphenylether; Herbicide; Protoporphyrinogen oxidase

1. INTRODUCTION

It has been recently shown that diphenylether (DPE) herbicides exert their light-dependent phytotoxic effects by inducing an accumulation of protoporphyrin IX (PP IX) in treated plants ([1-4]; see [5] for a review). It has also been demonstrated that the accumulation of PP IX can be explained by the capacity of DPE to inhibit protoporphyrinogen oxidase, which is the last enzyme of the common branch of the heme and chlorophyll biosynthetic pathways in plants [6,7]. Furthermore, we have shown that three other peroxidizing herbicides: oxadiazon [5-ter-butyl-3-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazol-2-one], LS 82556 [(*S*)3-*N*-(methylbenzyl)carbamoyl-5-propionyl-2,6-lutidine], and M&B 39279 [5-amino-4-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)pyrazol], which all induce PP IX accumulation in treated plants [8-11], are also powerful inhibitors of protoporphyrinogen oxidase activity [12].

One could thus wonder why the four types of molecules all inhibit the same enzyme activity, although they are chemically unrelated. In order to gain an insight into this question, we have examined the possibility that these molecules share the same binding site on the enzyme.

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Abbreviations: DPE, diphenylether(s); AF, acifluorfen 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

2. MATERIALS AND METHODS

2.1. *Chemicals*

LS 82-556 and oxadiazon were provided by Rhône Poulenc Agrochimie, France, and M&B 39279 by Rhône Poulenc Agriculture (formerly May and Baker Agrochemicals, UK). [^{3}H]Acifluorfen ([^{3}H]AF), was labeled on position 3 of the nitro-benzene ring with a specific activity of 4.95 Ci/nmol, (the method of the synthesis will be published elsewhere).

2.2. *Binding experiments*

All experiments presented in this study were done with corn etioplasts prepared as previously described [6].

Binding of [^{3}H]AF was estimated according to [13]. Experiments were performed in Eppendorf tubes, with a final reaction volume of 1 ml. Etioplasts (0.6 mg protein) were suspended in 0.1 M, pH 7.2 phosphate buffer containing various concentrations of [^{3}H]AF, and a competitor herbicide (oxadiazon, LS 82556, or M&B 39279) or the substrate of the enzyme, protoporphyrinogen IX. The suspensions were thoroughly mixed, then incubated at 30°C for 10 min. That time was reduced to 1 min for experiments with protoporphyrinogen IX, to avoid too much depletion of that molecule by the enzymatic reaction. The samples were then centrifuged for 6 min in a Beckman Microfuge B. The clear supernatants (0.5 ml) were taken out and mixed with 10 ml of Dynagel (Baker) for radioactivity measurements. After wiping the tube walls with absorbing paper, the pellets were solubilized in 0.5 ml of Solulyte (Baker), mixed with 10 ml of Lipofluor (Baker), and their radioactivity estimated.

Amounts of free inhibitor were calculated from the supernatant radioactivity, and amounts of bound inhibitor from the radioactivity of the pellets. Double reciprocal plots (bound AF versus free AF) were used to determine the specific binding constant K, and the number X_t of binding sites/mg of protein [13].

Protein concentrations were determined by the method of Bradford [14].

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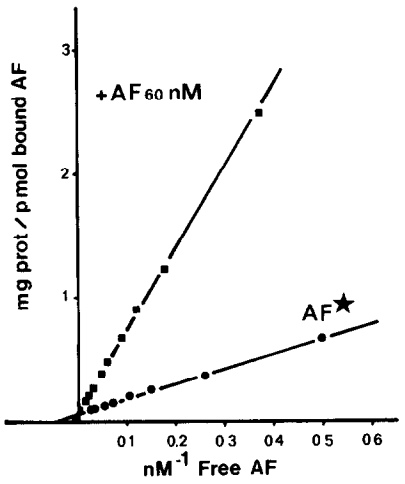

Fig. 1. Double-reciprocal plots of the binding of [^{3}H]acifluorfen (AF*) alone, or in the presence of 60 nM AF, to corn etioplast membrane fractions.

All experiments were done at least twice, with not less than two replicates. Differences between replicates from the same experiment were always below 10%.

3. RESULTS

3.1. *Binding of AF to corn etioplast membranes*

In order to minimize the importance of unspecific binding to corn etioplast membranes, [^{3}H]AF, i.e. the free acid form of the molecule, was used instead of its methyl ester (acifluorfen methyl). The latter is more lipophilic, and thus more prone to unspecifically binding to membrane lipids. As shown in Fig. 1, the double reciprocal plot, mg protein/nmol bound [^{3}H]AF vs 1/free [^{3}H]AF concentration [13] gave a linear relation. From the result of 11 experiments, graphical determination led to the following estimates of the specific binding constant.

$K = 13.6 \pm 4.1$ nM.

and the number of binding sites:

$X_t = 15.8 \pm 1.8$ pmol/mg prot.

The slight variation between the binding curves of AF obtained from various experiments (compare Figs. 2A and C, for instance) can probably be explained by the use of different batches of corn etioplasts.

Moreover, as expected for specific binding, cold AF competitively displaced [^{3}H]AF (Fig. 1).

3.2. *Inhibition of [^{3}H]AF binding by three other peroxidizing herbicides*

Since oxadiazon, LS 82556, and M&B 39279 are all potent inhibitors of protoporphyrinogen oxidase activity [12], it is of great importance to determine whether

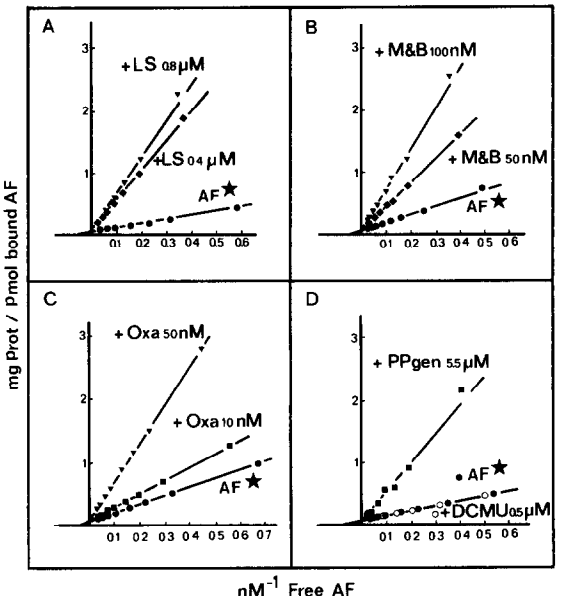

Fig. 2. Double-reciprocal plots of the binding of [^{3}H]acifluorfen (AF*) in the presence of LS 82556 (A), M&B 39279 (B), oxadiazon (C), protoporphyrinogen IX and DCMU (open circles) (D). LS = LS 82556; Oxa = oxadiazon; PPgen = protoporphyrinogen IX.

they all compete for the same binding site on the enzyme. In order to answer that question, we have incubated corn etioplast suspension with [^{3}H]AF in the presence of various concentrations of the other molecules.

As shown in Fig. 2, addition of peroxidizing herbicides to the reaction medium affected the specific binding constant of AF but had no effect on the number of binding sites. The fact that all binding curves intersect on the *y*-axis indicates a competitive displacement of [^{3}H]AF by all the other peroxidizing herbicides. The specificity of that competition was checked by a control experiment with DCMU, a well-characterized photosystem II inhibitor which has no effect on protoporphyrinogen oxidase activity. As shown in Fig. 2D, the binding of [^{3}H]AF was not affected by the addition of 0.5 μM DCMU. This result thus confirms that the competitive inhibition of the binding of [^{3}H]AF by peroxidizing herbicides is closely related to their capacity to inhibit corn etioplast protoporphyrinogen oxidase.

We have shown recently that inhibition of corn etioplast protoporphyrinogen oxidase activity by AF is competitive with respect to protoporphyrinogen, the substrate of this enzyme (Camadro et al., submitted). We have therefore examined if, as can be expected, protoporphyrinogen IX also competitively affects the binding of [^{3}H]AF. As shown in Fig. 2D, this was effectively the case.

4. DISCUSSION

The recent demonstration that protoporphyrinogen oxidase is a cellular target for DPE and four chemically

unrelated peroxidizing herbicides [6,7,12], raised the necessity to provide more information on the mode of action of these molecules. In that connection, we have recently found that the inhibition of corn etioplast protoporphyrinogen oxidase by four related DPE is competitive with respect to protoporphyrinogen IX, the substrate of this enzyme (Camadro et al., submitted).

From a mechanistic point of view, it is also of great importance to understand how so many chemically unrelated molecules can inhibit the same enzyme activity. In order to gain an insight into this question, we have investigated if these inhibitors share the same binding site on protoporphyrinogen oxidase. Following the method published for photosystem II inhibitors [13], we have examined the influence of three different protophyrinogen oxidase inhibitors (oxadiazon, LS 82556, and M&B 39279), on the specific binding of [^{3}H]AF.

The data presented here indicate that [^{3}H]AF binds specifically, with a high affinity, to the corn etioplastic membrane fractions, since the specific binding constant K is 13.6 ± 4.1 nM. AF was recently shown to be a competitive inhibitor of corn etioplast protoporphyrinogen oxidase activity (Camadro et al., submitted), and in consequence the K value should be close to the apparent K_i which was previously found equal to 120 nM. We have no explanation for the discrepancy between these two constants, which are usually closely related. Further kinetics studies of AF inhibition on purified protoporphyrinogen oxidase will be necessary to elucidate this point.

From double reciprocal plots of mg protein/bound AF vs 1/free AF concentrations, the number X_t of binding sites per mg of protein was graphically determined. For AF, X_t was found equal to 15.8 ± 1.8 pmol/mg of protein. If we consider this value to be in relation to the apparent maximum velocity previously found with corn etioplast enzyme (Camadro et al., submitted), which was 6 nmol of PPIX/h per mg protein, we obtain a specific activity of 380 mol of PP IX/h per mol of protoporphyrinogen oxidase.

Assays of binding competition between [^{3}H]AF and the other herbicides clearly indicate that AF is competitively displaced by all the protoporphyrinogen oxidase inhibitors. By contrast, the lack of effect of DCMU, which did not affect the binding of [^{3}H]AF, confirms that the effects of peroxidizing molecules on the binding of [^{3}H]AF are specifically related to their capacity to inhibit protoporphyrinogen oxidase. All these results provide evidence that although chemically unrelated, the four peroxidizing herbicides share the same binding site on the enzyme.

The competitive displacement of [^{3}H]AF by protoporphyrinogen IX, the substrate of the enzyme, was expected since AF competitively inhibits corn etioplast protoporphyrinogen oxidase. This displacement confirms first that AF actually binds to the enzyme, and secondly that the binding site of this inhibitor is probably close to the catalytic site.

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