Communication

Cyclohexanedione Herbicides Are Selective and Potent Inhibitors of Acetyl-CoA Carboxylase from Grasses

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ABSTRACT

Biochemical studies of plant species susceptible to the cyclohexanedione herbicides, alloxydim, sethoxydim, and clethodim, have demonstrated that these selective grass herbicides inhibit acetyl-coenzyme A carboxylase, the second enzyme common to both fatty acid and flavonoid biosynthetic pathways. The Ks for the cyclohexanediones tested ranged from 0.02 to 1.95 micromolar, depending on the species. The enzyme isolated from broadleaf plants was much less sensitive to inhibition by these herbicides (Ks from 53 micromolar to 2.2 millimolar). These results may explain the mechanism of action of these herbicides and their selectivity for monocotyledonous species.

Over the past 10 years a series of substituted 1,3-cyclohexanediones (alloxydim, sethoxydim, and clethodim) has been introduced as postemergence grass herbicides for use in broadleaf crops (Fig. 1). These compounds exhibit a remarkably similar spectrum of phytotoxicity on both annual and perennial grasses without affecting broadleaf plants (6, 13, 14). Studies on the uptake, translocation, and metabolic fate of these herbicides in tolerant and susceptible plants have shown that the selectivity for monocotyledonous species is probably not due to differential metabolism of the compounds to nonherbicidal forms, or to differential uptake or transport (4, 24, 25). These results suggest that the tolerance of broadleaf crops and weeds is based on the insensitivity of the target site to these compounds.

Recent studies on the mode of action of sethoxydim (2, 3, 10) have shown that de novo fatty acid biosynthesis is inhibited by this herbicide in susceptible plants. In order to elucidate the mode of action of clethodim (16), we began to examine the individual enzymes involved in the biosynthetic pathway. Both flavonoid and fatty acid biosynthetic pathways in plants share the first two steps which are catalyzed by acetyl-CoA synthetase and acetyl-CoA carboxylase. In addition, fatty acid synthesis in plants involves six separate enzymes which use ACCP-bound intermediates. In this report, we describe the potent inhibition in grasses of the second enzyme common to these pathways, acetyl-CoA carboxylase (EC 6.4.1.2), by alloxydim, sethoxydim, and clethodim, and the much weaker inhibition of this enzyme in tolerant broadleaf species.

MATERIALS AND METHODS

Chemicals. [14C]Acetate and [14C]acetyl-CoA were obtained

Abbreviations: ACP, acyl carrier protein; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDH, lactate dehydrogenase.

from NEN-Dupont. Analytical grade clethodim was synthesized at the Chevron Chemical Ortho Research Center, Richmond, CA. Alloxydim (Nippon Soda Corp.), sethoxydim (BASF Wyandotte Corp.), bentazon (BASF Wyandotte Corp.), and acifluorfen (Rohm & Haas Co.) were further purified on silica columns. All other reagents were from Sigma Chemical Co.

Plant Material. Barley (Hordeum vulgare var Briggs), wheat (Triticum aestivum L. var Anza), and corn (Zea mays L. var Dekalb) were planted in vermiculite, watered, maintained in a growth chamber in the dark for 6 to 10 d at 21°C, then illuminated for 6 to 8 h prior to harvesting the shoots. Mature spinach leaves (Spinacia oleracea) were obtained from a local retailer and used immediately. Etiolated mung bean seedlings (Phaseolus aureus) were harvested after 5 d growth in clay pots.

Preparation of Fatty Acid Biosynthesis System. Enzyme activ-

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1 Abbreviations: ACP, acyl carrier protein; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDH, lactate dehydrogenase.
ities of an ACP-dependent fatty acid synthetase system were partially purified from barley chloroplasts by osmotic lysis, ammonium sulfate precipitation, and column chromatography on Pharmacia Sephadryl® S-300 by a modification of the procedure of Hoj and Mikkelsen (11). Chloroplasts were lysed by suspension for 30 min in 10 mM Tricine (pH 9) containing 1 mM MgCl₂ and 2 mM DTT. Enzymes were precipitated with ammonium sulfate between 35 and 65% saturation (at 0°C). Other steps were the same as in (11).

**Preparation of Acetyl-CoA Carboxylase.** The enzyme preparations for the grasses and spinach were partially purified using the steps outlined above and in (11). Mung bean enzyme was obtained from etiolated mung bean seedlings which were frozen, homogenized in liquid N₂, suspended in buffer, filtered and centrifuged to remove cell debris, then loaded onto a DEAE ion exchange column. Fractions containing the carboxylase activity were pooled and further purified by chromatography on Sephadryl® S-300.

**Assay of Fatty Acid Biosynthesis and Transacylase Activities.** Assays used in Table IA contained 6 mM NaP, (pH 7.9), 12 mM NaHCO₃, 100 μM [²¹⁴]acetate (5 μCi), 95 μM CoA, 9.5 mM ATP, 0.6 mM NADH, 1.2 mM NADPH, 50 μg Escherichia coli ACP, 5 mM MgCl₂, 5 mM MnCl₂, 0.6 mg enzyme, and inhibitor in a total volume of 0.84 ml. Reactions were initiated by addition of enzyme and were terminated after a 30 min incubation at 25°C by addition of potassium hydroxide. Details of the workup conditions are described in (11) for fatty acid synthetase assays. For Table IB, the assay contained 7 μM [²¹⁴]acetyl-CoA (0.1 μCi), 0.07 mM unlabeled malonyl-CoA, 50 μg E. coli ACP, 1.4 mM NADPH, 0.7 mM NADH, 0.2 mg enzyme and inhibitor in 0.9 ml of 0.09 M Tris (pH 7.6). After 15 min the assays were quenched and treated the same as above. The assays used in Table IC contained 80 μM [²¹⁴]acetate (2 μCi), 5 mM ATP, 10 mM MgCl₂, 2.5 mM MnCl₂, 12.5 mM NaHCO₃, 50 μg E. coli ACP, 80 μg yeast S-acetyl-CoA synthetase (Sigma), 0.6 mg enzyme and inhibitor in a total volume of 0.4 ml of 6 mM NaP, (pH 7.9). After 20 min at 25°C, the assay was terminated by addition of cold perchloric acid and worked up as described in (11) for the transacylase assays. Background controls were run in the absence of ACP and were subtracted from the results of each of the above assays.

**Assay of Acetyl-CoA Carboxylase.** The grass enzymes were assayed using a modification of the continuous spectrophotometric assay described in (9). The reaction mixture contained in a total volume of 1.0 ml 0.1 M K-Taps (pH 8.5) at 25°C, 2.5 mM ATP, 5 mM MgSO₄, 15 mM NaHCO₃, 0.2 mM PEP, 10 μg LDH (7 units), 15 μg PK (6 units), and 10 μl of the barley carboxylase fraction. Due to the small yields of enzyme and a very high level of contaminating phosphatase background activity, a fixed time radiochemical assay (9) was used with the carboxylases from broadleaves. A typical assay contained in a total volume of 0.2 ml 0.1 M Tris (pH 7.5), 3 mM potassium citrate, 0.2 mg BSA, 7 mM MgCl₂, 2.5 mM ATP, 8 mM [¹⁴C]NaHCO₃, (15 μCi), 1 mM DTT, carboxylase, and variable amounts of acetyl-CoA and inhibitor. Assays were linear for 10 min at 37°C with the spinach enzyme and for 5 min with the mung bean enzyme. The reaction was quenched by addition of 40 μl of 6 mM HCl, protein removed by centrifugation, and a 200 μl aliquot dried in a scintillation vial which removed any unreacted [¹⁴C]bicarbonate. The remaining radioactivity is a measure of product formed and was quantitated by liquid scintillation counting after the addition of 1 ml H₂O and 15 ml Atomlight® (NEN-DuPont). Assays were initiated by the addition of acetyl-CoA. The background rate determined in the absence of acetyl-CoA was subtracted from each assay.

**Data Processing.** Concentrations of inhibitors causing 50% inhibition (I₅₀ values) were calculated by linear regression analysis of Dixon plots of the data (21). Lineweaver-Burk plots and complete inhibition patterns were analyzed using the FORTRAN programs of Cleland (7).

**RESULTS AND DISCUSSION**

**Initial Observations.** Experiments with partially purified fractions from barley chloroplasts indicated that an early step in fatty acid biosynthesis was inhibited by clethodim, since incorporation of label from [¹⁴C]acetate into lipids was blocked while incorporation from [¹⁴C]acetyl-CoA was not affected (Table I). Three different radiochemical assays were used to pinpoint the biochemical site of action of the cyclohexanedione grass herbicides. The first assay followed the incorporation of label into lipids (measured as hexane soluble radioactivity) from [¹⁴C]acetate in the presence of MgATP, HCO₃⁻, CoA, ACP, NAD⁺, and NADPH. Our results (Table IA) confirmed earlier work (2, 3, 10) which showed that fatty acid biosynthesis in susceptible species was blocked by sethoxydim. A second pathway assay followed incorporation of label into lipids starting from a later intermediate, [¹⁴C]acetyl-CoA, in the presence of malonyl-CoA, ACP, NAD⁺, and NADPH. This assay bypasses the first two steps in the biosynthetic pathways since both acetyl- and malonyl-CoA were present in the assay. Cerulenin, a known antibiotic inhibitor of the condensing enzyme step for plant, animal, bacterial, and fungal fatty acid biosynthesis (20), completely inhibited the first two pathway assays, as expected for an inhibitor of a later step in the pathway. The results shown in Table IB indicate that these herbicides affect a step prior to the first committed steps in fatty acid biosynthesis, *i.e.* before the loading of ACP.

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**Table 1. Inhibition of Fatty Acid Biosynthesis in Barley by Clethodim and Sethoxydim**

<table>
<thead>
<tr>
<th>Assay Contents</th>
<th>Net dpm</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Complete ([¹⁴C]acetate → lipid)</strong> + 0.43 mM cerulenin</td>
<td>9660</td>
<td>0</td>
</tr>
<tr>
<td>+ 5.3 mM sotmydium</td>
<td>92</td>
<td>99</td>
</tr>
<tr>
<td>+ 3.8 mM clethodim</td>
<td>29</td>
<td>99.7</td>
</tr>
<tr>
<td><strong>B. Complete ([¹⁴C]acetyl-CoA → lipid)</strong> + 0.73 mM cerulenin</td>
<td>6160</td>
<td>0</td>
</tr>
<tr>
<td>+ 8.9 mM sotmydium</td>
<td>5310</td>
<td>19</td>
</tr>
<tr>
<td>+ 8.9 mM clethodim</td>
<td>5020</td>
<td>19</td>
</tr>
<tr>
<td><strong>C. Complete ([¹⁴C]acetate → protein)</strong> + 1 mM clethodim</td>
<td>109,760</td>
<td>0</td>
</tr>
</tbody>
</table>

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with acetate or malonate via specific transacylases. Table IC provides further confirmation that the susceptible enzyme is an early one in the pathway. Label was followed from [14C]acetate into an acid-precipitable component, a measure of protein labeling. This assay includes the first two steps of the pathway plus the transacylase steps (acyt-cOA:ACP transacylase and malonyl-COA:ACP transacylase), in which the label becomes protein-bound. Incorporation of label into protein was only partially blocked by clethodim (86%), since only the pathway acetate \(\rightarrow\) acet-cOA \(\rightarrow\) malonyl-COA \(\rightarrow\) malonyl-ACP would be affected by inhibition of the second enzyme. Label could still be incorporated into protein via acetate \(\rightarrow\) acet-cOA \(\rightarrow\) acet-c APC.

The above assays suggest that only the second enzyme is the herbicide target. Inhibition of the first enzyme, acet-cOA synthetase, should block label incorporation into protein completely rather than partially. To test this hypothesis further, both acet-cOA synthetase and acet-cOA:ACP transacylase (the third enzyme) were partially purified from barley chloroplasts and assayed for inhibition by clethodim. Neither enzyme was significantly inhibited by this grass herbicide (data not shown), whereas acet-cOA carboxylase was strongly inhibited \((K_i = 10^{-7} \text{ M})\), (Table II)

### Inhibition of Acyl-CoA Carboxylase

All three cyclohexanedione herbicides tested, alloxodim, sexitoxodim, and clethodim, were potent inhibitors of barley chloroplast acet-cOA carboxylase (Table II). These inhibitors were from 10 to 100 times more tightly bound compared to any of the substrates under the conditions described in Table II. Since acet-cOA carboxylases from all sources contain covalently bound biotin (23), it is not surprising that avidin, a protein which binds free biotin extremely tightly (18), is also a potent inhibitor of the barley enzyme. Also, as expected, several other postemergence herbicides which have a different mode of action, bentazon, acifluorfen, and 2,4-D, were found to be poor inhibitors of acet-cOA carboxylase.

Additional evidence that acet-cOA carboxylase is the site of action of these grass specific herbicides is that the enzyme from broadleaf plants is much less sensitive to the compounds, which is consistent with the whole plant data. The inhibition constants of several representative herbicides were determined and compared for the enzyme from susceptible grasses and tolerant broadleaves (Table III). It is clear that the enzyme from broadleaves is much less sensitive to inhibition by these herbicides \((K_i\) values are 400 to 60,000 times higher depending on which species are compared). These data also suggest an explanation for the selective control of grassy species by these herbicides.

The covalently bound biotin in acet-cOA carboxylase acts as the carboxyl carrier between an ATP-dependent biotin carboxylation site and a carboxyltransferase site (18, 23). In characterizing the inhibition of barley chloroplast acet-cOA carboxylase by these compounds, we found that the inhibition patterns of clethodim versus any of the substrates, MgATP, HCO3−, or acet-cOA, fit best to the equation for linear noncompetitive inhibition (7). However, the apparent inhibition constant was most sensitive to the level of acet-cOA \((K_i\) rather than MgATP or HCO3− \((K_i)\) suggesting that these compounds interfere with the acet-cOA \(\rightarrow\) malonyl-CoA transferase site. The apparent inhibition by clethodim did not increase over a 4 h period indicating that the cyclohexanediolones are irreversible inhibitors. Additional experiments are needed to further characterize the binding site of the herbicide and to determine whether the catalytic functions are directly affected or whether regulatory sites are involved.

Inhibitors of lipid biosynthesis, especially of plants, are relatively rare. These include only two antibiotic classes represented by cerulenin, which was discussed previously (20), and thiocamycin, which was reported to inhibit plant and bacterial acet-cOA:ACP transacylase (19). Other reported inhibitors of lipid biosynthesis are the thiocarbamate (8, 26) and phenoxypenterypropionate (12) herbicides whose sites of action in the pathway are unknown. To our knowledge the selective and potent inhibition of acet-cOA carboxylase by the cyclohexanedione grass herbicides represents the first demonstration of inhibition of the enzyme from higher plants by xenobiotic compounds. It appears that inhibition of acet-cOA carboxylase may be the mechanism of action of the cyclohexanediolone herbicides, and that selectivity for monocotyledenous species is achieved by differences in sensitivity at the target enzyme level. Thus, lipid and/or flavonoid biosynthesis may be added to plant metabolic processes such as photosynthesis (8), carotenoid biosynthesis (8), microtubule assembly (17), and essential amino acid biosynthesis (1, 5, 15, 22), whose inhibition is herbicidal.

**Note Added in Proof.** After submission of this manuscript, we have been made aware of two papers citing acet-cOA carboxylase as the site of action for the cyclohexanediolone herbicides:
Table III. Species Specificity of the Inhibition of Plant Chloroplast Acetyl-CoA Carboxylase

Assay conditions are described in "Materials and Methods." The $K_a$ values for acetyl-CoA are averages from the fits for each inhibitor. Inhibition data were fitted to the equation for linear noncompetitive inhibition (7). Only the $K_a$ values are shown.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Grasses</th>
<th>Wheat</th>
<th>Corn</th>
<th>Spinach</th>
<th>Broadleaves</th>
<th>Mung bean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibition Constants</td>
<td>µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA ($K_a$)</td>
<td>11.8 ± 4.6</td>
<td>10.7 ± 3.0</td>
<td>135 ± 39</td>
<td>49 ± 25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clethodim</td>
<td>0.14 ± 0.3</td>
<td>0.02 ± 0.003</td>
<td>1240 ± 280</td>
<td>53 ± 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sethoxydim</td>
<td>0.96 ± 0.19</td>
<td>0.47 ± 0.11</td>
<td>2160 ± 500</td>
<td>1880 ± 620</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alloxydim</td>
<td>1.95 ± 0.34</td>
<td>0.88 ± 0.17</td>
<td>ND*</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Not determined.

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