Original Article

The Mode of Action of Chlorsulfuron in Culture Cells of Tobacco and Hamster

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Chlorsulfuron inhibited the growth of tobacco cell cultures at 1 ppb, with a half inhibition (I_{50}) at 0.5 ppb. In contrast, cultured cells of hamster kidney (BHK) continued to grow even in a medium containing 100 ppm chlorsulfuron. Acetolactate synthase (ALS) from tobacco cell cultures was strongly inhibited by chlorsulfuron with an I_{50} values of 5–10 ppb (14–28 nM). Gas chromatography confirmed the production of acetolactate in the enzyme preparations from BHK cells and chick livers, but, no inhibition with 500 ppb chlorsulfuron. The properties of acetolactate-forming enzymes from tobacco cells and BHK cells were compared in cofactor requirement, pH dependence and valine inhibition. The enzyme from BHK cells strongly required thiamine pyrophosphate (TPP), it was not inhibited by valine, and the optimum pH in the enzyme reaction was 7.4. On the other hand, the tobacco enzyme did not strongly require TPP, it was inhibited by valine, and the optimum pH was 8. That chlorsulfuron does not inhibit acetolactate-forming enzymes in mammalian cells is one likely reason for its low toxicity against mammalian cells. The binding mechanism of the herbicide with the tobacco cell enzyme was noncompetitive with pyruvate as a substrate and uncompetitive with TPP as a cofactor.

INTRODUCTION

Acetolactate synthase (ALS) works as a catalyst in first step of biosynthesis of branched-chain amino acids, valine, leucine and isoleucine. ALS is inhibited by three structurally unrelated classes of herbicides, sulfonylureas,¹⁾ imidazolinone²⁾ and sulfon-anilides,³⁾ which have extremely high herbicidal potency,^{1,3)} pre- and postemergent activity and low mammalian toxicity.⁴⁾ The low mammalian toxicity may depend on the fact that they have no target enzyme in mammal because essential amino acids are not synthesized in mammals.

Acetolactate and acetohydroxybutyrate are formed from pyruvate and from 2-oxobutyrate and pyruvate respectively through nonoxidative decarboxylation as so are other thiamine pyrophosphate (TPP)-dependent enzymes.⁶⁾

One of the unique features of ALS is that it requires FAD. FAD may be essential for prevention of protonation of hydroxyethyl-TPP (HETPP) intermediates.⁷⁾ For complete activation of ALS, divalent cation is also required: Mg^{2+} is the most effective.⁸⁾

Many enzymes capable of decarboxylating pyruvate catalyze TPP-dependent condensation reactions to yield acylolins such as acetoin. Among the known TPP-dependent enzymes, α -decarboxylase is unique since free aldehyde is formed as a major decarboxylation end product of an α -keto acid and it produces acetoin as a secondary decarboxylation product of pyruvate. Studies on α -decarboxylase have been done in wheat germ⁹⁾ and yeast,¹⁰⁾ and a decarboxylation mechanism of α -keto acids have been demonstrated.¹¹⁾

A recent report says that pyruvate oxidase, one of flavoenzymes, shares homology in the

amino acid sequence with the large subunits of ALS isozymes I, II and III, which are respectively encoded by the *ilvB*, *ilvG* and *ilvI* genes in *E. coli*, and that the enzyme is very similar to the gene products by these genes in size. In pyruvate oxidase reaction, ubiquinone binds to a region of the enzyme's active site equivalent to the flavine-binding site,¹²⁾ and it also inhibits bacterial ALS activity. These observations suggest that the quinone-binding site of ALS and that of pyruvate oxidase share a common evolutionary heritage.¹³⁾

In this paper, we report of chlorsulfuron, a sulfonylurea herbicide, the low toxicity to mammals by comparing the effects on the growth of plant cells and mammalian cells, and the inhibition of acetolactate formation. The characteristics of acetolactate-forming enzymes prepared from plant cells and mammalian cells and tissues are also discussed.

MATERIALS AND METHODS

1. Chemicals

Analytical-grade chlorsulfuron: 2-chloro-*N*-(4-methoxy-6-methyl-1,3,5-triazine-2-yl)amino-carboxy benzenesulfonamide was a generous gift by Du Pont Japan Co. Ltd. TPP, FAD: flavine adenine dinucleotide, butanedione and sodium pyruvate were purchased from Wako Pure Chemical Industries Ltd. DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid), 2-PDS: 2,2'-dithio-dipyridine, DDD: 6,6'-dithio-bis-2-naphthol, DPIP: 2,6-dichlorophenol-indophenol sodium salt were purchased from Dojin Laboratories Ltd. Eagle's MEM medium was purchased from Nissui Pharmaceutical Industries Ltd.

2. Plant and Animal Materials

A tobacco (*Nicotiana tabacum* L cv SAMSUN) cell suspension culture was a gift from Dr. C. Nakamura, Faculty of Agriculture, Kobe University, and a hamster kidney (BHK) cell culture from Dr. M. Takehara, Faculty of Medicine, Kobe University.

The tobacco cell suspension culture was weekly transferred to a Murashige-Skoog medium containing 2,4-D at a final concentration of 1.5 μ M. The culture was placed on a gyratory shaker at 120 rpm and incubated at

27°C under fluorescent lamps.

The BHK cell culture was weekly transferred to Eagle's MEM medium containing calf serum and incubated at 37°C. Chick livers were taken from 4 week-old chicks, frozen in liquid nitrogen and stored in a deep freezer before use.

3. Extraction of ALS

ALS was extracted as described by Ray.¹⁾ The tobacco cells, BHK cells and chick livers were homogenized in five volumes of extraction buffer containing 0.1 M K_2HPO_4 (pH 7.5), 1 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM TPP, 10 μ M FAD and 10% (v/v) glycerol. The homogenate was centrifuged at 12,000 rpm for 20 min. The enzyme was collected at 20–50% saturation with ammonium sulfate in centrifugation at 10,000 rpm for 15 min. After centrifugation, the pellet was dissolved in a buffer containing 0.1 M K_2HPO_4 (pH 7.5) and 0.1 mM $MgCl_2$, and desalted on a column of Sephadex G-25 equilibrated with the same buffer.

4. Assay for ALS

The reaction mixture containing 20 mM phosphate buffer (pH 7.0), 20 mM sodium pyruvate, 0.5 mM TPP, 0.5 mM $MgCl_2$ and 10 μ M FAD in a total volume of 0.5 ml and was incubated at 30°C for 1 hr. Two aliquots of the reaction mixture were terminated by adding 50 μ l of 6 N H_2SO_4 or 2.5 N NaOH. The ALS activity was determined by measuring the amount of acetoin formed in the ALS assay and converting through acid decarboxylation of acetolactate. The acetoin was measured based on the Westerfeld test for carbinols.¹⁴⁾ The acetolactate produced during the ALS assay was converted into acetoin by heating at 60°C for 15 min. The acetoin formed was incubated with 0.5 ml creatine (0.3%) and 0.5 ml 1-naphthol (5% in 2.5% NaOH) at 60°C for 15 min and kept at room temperature for another 15 min. A absorption of the colored product was measured at 525 nm with a spectrophotometer (Hitachi U-3200) and the net ALS assay activity was calculated by subtracting the value of undecarboxylated sample from that of decarboxylated one.

5. Detection of Acetolactate by Gas Chromatography

Acetolactate was detected by gas chromatography as described by Gollop.¹⁵⁾ The enzyme reaction mixture was diluted 20 times with FeCl_3 and FeSO_4 solution (iron salts, 0.15 mM each for final). This solution was incubated in a large test tube (30×210 mm), tightly sealed with a Teflon stopper at 80°C for 10 min, to convert acetolactate into diketone (2,3-butanedione). Diketone was distilled by passing air through the solution in a test tube held in a bath at 60°C and collected in a small tube containing 2 ml dry methanol in an ice bath. The methanol solution of diketone was analyzed by gas chromatography using a 3 mm×4.3 mm glass column of 5% polyethyleneglycol 20 M on Gaschrom Q at 95°C at a nitrogen flow rate of 1 kg/cm³. The injector was held at 140°C. A Shimadzu Model GC-8A gas chromatograph equipped with a ⁶³Ni electron capture detector was used.

6. Determination of Protein

Protein concentration was determined according to Bradford with crystalline bovine serum albumin as standard.¹⁶⁾

RESULTS

1. Chlorsulfuron Inhibition of Growth of Tobacco and BHK Cells

Chlorsulfuron was added 7 days after the initiation of cell cultures. Seven days later, the growth of tobacco cells was measured by weighing dried cells, while the growth of BHK cells by counting the number of living cells. The growth of the tobacco cells was inhibited almost completely by 1 ppb chlorsulfuron with a half inhibition (I_{50}) at 0.5 ppb (Fig. 1). The growth of BHK cells was, however, not inhibited even by 100 ppm chlorsulfuron (Fig. 2). The complete inhibition was achieved by 10 ppm actinomycin D, a DNA synthesis inhibitor.

2. Inhibition of Acetolactate Formation by Chlorsulfuron in the ALS Assay with Tobacco and Mammalian ALS

ALS from plants, bacteria and fungi form acetolactate in a standard enzyme assay, but no acetolactate-forming enzyme system has

Fig. 1 Effect of chlorsulfuron on the growth of tobacco cell cultures.

Fig. 2 Effects of chlorsulfuron on the growth of BHK cell cultures.

●: 100 ppm chlorsulfuron, ○: control, △: 10 ppm actinomycin D.

been found in animals yet. We examined if acetolactate is formed by animal enzymes with the same mechanism as by plant enzymes, since acetolactate and acetoin are reported to be formed from pyruvate in a cell free enzyme preparation from animal tissues. Formation of acetolactate and acetoin by animal enzyme in fact was detected by the method of Wester-

feld.¹⁴⁾ Effects of chlorsulfuron on the formation of acetolactate were then examined using enzyme preparations from tobacco and mammalian cells. The enzyme from tobacco cells was completely inhibited by 40 ppb chlorsulfuron with a half inhibition at 5–10 ppb (14–28 nM) (Fig. 3). This result agreed well with the study done by Ray¹⁸⁾ using peas. On the other hand, the enzymes extracted from BHK cells and chick livers were not inhibited even

Fig. 3 A dose response for inhibition of the ALS activity of tobacco cell cultures by chlorsulfuron.

Fig. 4 Effects of chlorsulfuron on the ALS activity of chick livers and BHK cell cultures.

○: chick livers, ●: BHK cell cultures.

by 500 ppb chlorsulfuron (Fig. 4). These results clearly show that chlorsulfuron remarkably inhibited plant ALS activity but not the activity of enzymes from mammalian cells and tissues.

3. Identification of Enzymatic Products Formed in the ALS Assay by Gas Chromatography

Since valine and isoleucine are essential amino acids for animals, animals may not have any ALS. The activity of ALS from mammalian sources was therefore assayed by measuring the activity of acetoin conversion from enzymatic products by acid decarboxylation. Gas chromatography confirmed that acetolactate was formed not only in the tobacco enzyme but also in the enzymes from BHK cells and chick livers.

After one-hour incubation of three enzyme assay solutions, products were converted into diketone (butanedione) by oxidation with iron salts. The resultant diketone was injected into the gas chromatograph. The products in the three enzyme assays showed the peaks corresponding to those of authentic diketone at a retention time of 0.8 min, but an unoxidized check sample did not show any peak at that time (Fig. 5).

It thus became evident that the enzyme systems of BHK cells and chick livers produced the same product, acetolactate, as did the

Fig. 5 Gas chromatograms of diketone derived from ALS assay products by three enzyme sources.

A: tobacco cell cultures, B: chick livers, C: BHK cell cultures.

Fig. 6 Effects of pH on the ALS activity of tobacco and BHK cell cultures.

○: tobacco cell cultures, ●: BHK cell cultures.

tobacco enzyme system.

4. Optimum for ALS from Tobacco and BHK Cells

Although it was shown that mammalian cells and tissues had an ability to produce acetolactate, their acetolactate-forming mechanism might be different from that of plant ALS. The enzymes from tobacco and BHK cells were assayed at different pH. The tobacco cell enzyme had its optimum pH at 8.0 and its activity was much higher on the alkaline side, showing a broad peak. On the other hand, the BHK cell enzyme showed its optimum pH at around 7.0–7.5 and the curve had a sharper peak (Fig. 6).

5. Effects of Cofactors on Tobacco and Mammalian ALS Activities

The assay system of ALS required TPP, FAD and Mg^{2+} as cofactors. Effects of these cofactors added to each enzyme assay solution were examined on the enzyme preparations from tobacco cells, BHK cells and chick livers.

The activity of the tobacco cell enzyme decreased only when TPP was excluded, and the other two cofactors gave no effect. The activity of the BHK cell enzyme also decreased greatly when TPP was excluded. The

Table 1 Cofactor requirement of ALS of tobacco and BHK cell cultures and chick liver.

Component	Acetoin nmol/hr/mg protein		
	Tobacco cells	BHK cells	Chick liver
Complete	2894 (100)	592 (100)	1208 (100)
—TPP	1925 (67) ^{a)}	290 (49) ^{a)}	351 (29) ^{a)}
—FAD	2637 (91)	516 (87)	1527 (126)
— Mg^{2+}	2567 (89)	742 (125)	1225 (101)
—TPP, FAD	1936 (67)	167 (28)	355 (29)
—TPP, Mg^{2+}	2289 (79)	845 (142)	1425 (118)
—TPP, FAD, Mg^{2+}	1976 (68)	22 (4)	683 (57)

—: Omitted from complete reaction mixture.

^{a)} Percent of complete reaction mixture.

lack of FAD or Mg^{2+} did not affect the enzyme activity, but the omission of three cofactors greatly reduced it. The activity of the chick enzyme was reduced only when TPP was omitted (Table 1).

These results suggested that tobacco ALS bound TPP tightly in the native cells or in the enzyme-extracting buffer solution. On the other hand, the mammalian enzymes required TPP as an essential cofactor during the enzyme assay.

6. Effects of Valine on the Assay of ALS from Tobacco Cells and Chick Livers

ALS is subjected to feedback inhibition by

Fig. 7 Effects of valine on the ALS activity of tobacco cell cultures (○) and chick livers (Δ).

Fig. 8 Effects of SH-group inhibitors on the ALS activity of tobacco cells (A) and chick livers (B).

DTNB (Δ), 2-PDS (●), DDD (□), DPIP (○).

valine, the end product of its biosynthetic pathway. Therefore, the valine sensitivity of ALS from tobacco cells and chick livers were examined. The tobacco ALS was inhibited by 40% with 5 mM valine and by 80% at 50 mM. The chick liver enzyme was not inhibited with 10 mM valine but inhibited by 30% at 50 mM and by 60% at 100 mM, the concentration of solubility limit (Fig. 7). A half-inhibition (I_{50}) of the tobacco cell enzyme by valine, thus was estimated to be one-fifth of that of the chick liver enzyme.

7. Effects of Sulfhydryl (SH)-Group Inhibitors on ALS Activity of Tobacco Cells and Chick Livers

SH-Group inhibitors severally inhibit enzymes by binding themselves at their active sites. Four SH-group inhibitors were tested for enzymes from tobacco cells and chick livers. Their effects appeared to be the same on both enzymes: DTNB, 2-PDS and DDD stimulated the enzyme activity, whereas DPIP inhibited (Fig. 8). The result suggested that the enzymes from both tobacco cells and chick livers did not have SH groups at their active sites, and that the inhibition by DPIP might be due to its direct binding at the active sites of the enzymes.

8. Inhibition of Tobacco Cell ALS by Chlorsulfuron

Inhibition of tobacco cell ALS by chlorsul-

Fig. 9 Inhibitory properties of chlorsulfuron with pyruvate on the ALS activity of tobacco cell cultures.

Levels of chlorsulfuron: 0 (○), 4 ppb (Δ), 6 ppb (□) and 8 ppb (●).

Fig. 10 Inhibitory properties of chlorsulfuron with TPP on the ALS activity of tobacco cell cultures.

Levels of chlorsulfuron: 0 (●) and 5 ppb (○).

furon was examined at different concentrations of the substrate, pyruvate, and the cofactor, TPP. As already mentioned, tobacco ALS activity was detected without adding TPP to the reaction mixture. Under the experimental conditions at different concentrations of pyruvate without TPP, the Hanes-Woolf plot showed that the inhibition was noncompeti-

tive (Fig. 9). At some lower concentrations of chlorsulfuron, uncompetitive inhibition was observed. Inhibition of tobacco ALS activity by chlorsulfuron was further examined at different concentrations of TPP with 20 mM pyruvate. The Hanes-Woolf plot showed inhibition was uncompetitive between TPP and chlorsulfuron (Fig. 10).

These results indicated that chlorsulfuron inhibited the enzyme activity by binding the complexes of enzyme, cofactor and substrate.

DISCUSSION

In a simple biological system of cell cultures, the herbicide chlorsulfuron severely inhibited the growth of plant cells (tobacco), but not the growth of animal cells (BHK). A biochemical background for such difference in the herbicide sensitivity to plant and animal cells is probably that acetolactate synthase (ALS) from tobacco is inhibited at an extremely low concentration while the herbicide has no effect on the enzyme activity from animal cells.

Chlorsulfuron inhibited the growth of tobacco cells at lower concentration than those inhibitory to the ALS activity. This is probably because in plant cells treated with chlorsulfuron the growth is inhibited immediately after cell division and DNA synthesis are inhibited. However, chlorsulfuron does not directly inhibit either mitosis or DNA synthesis, as already shown by non-inhibition of mitotic entry by added valine and isoleucine,¹⁷⁾ and it does not inhibit DNA polymerase either.¹⁸⁾

We showed by gas chromatography that the enzyme from mammalian cells and tissues formed acetolactate in the ALS assay identical to that formed by tobacco cell enzyme. The ALSs from tobacco cells, BHK cells and chick livers were different in chlorsulfuron susceptibility as well as in pH dependence, cofactor requirement and valine inhibition.

Juni & Heym¹¹⁾ have showed that both acetolactate and acetoin were formed in a cell-free system by pyruvate oxidase preparations from pigeon breast muscle. Acetolactate and acetoin were also formed nonenzymatically through catalysis of pyruvate decarboxylation by a thiamine molecule itself.¹⁹⁾ It is not

likely that nonenzymic reaction occurs in animal ALS assay condition, because the optimum condition for nonenzymic reaction by thiamine molecule is pH 9 and 40°C.

No direct evidence has supported acetolactate formation in animal cells and tissues. However, the present study demonstrated that even in animal cells, for which branched amino acids are essential, there are acetolactate-forming enzymes tolerant to chlorsulfuron (Fig. 4).

It has been reported that one of the two ALSs isolated from *Aerobacter aerogenes* was insensitive to valine inhibition, not involved in amino acid synthesis, but consumed pyruvate under overproduction of valine.²⁰⁾ Moreover, in our earlier study acetoin was formed by the enzyme from seeds and cotyledons of soybean and this acetoin formation was involved in regulating the supply of amino acids for storage protein synthesis.²¹⁾ Thus, it is presumed that the formation of acetolactate and acetoin plays a role in consuming pyruvate accumulated in animal tissues as in microorganisms and plants.

Chlorsulfuron only inhibits plant ALS and does not affect acetolactate-forming enzyme and other TPP-dependent pyruvate decarboxylases²¹⁾ including the acetoin-forming enzyme from the animal cells. In the second step of branched amino acid biosynthesis, ketoacid reductoisomerase and dihydroxyacid dehydrase catalyzing the reaction of 2-oxoisovalerate from acetolactate are not present in animals (these enzymes are only found in plants and microorganisms). Therefore, acetolactate produced in animals is not used for further biosynthesis but degrades to other compound (acetoin). It seems that low mammalian toxicity of chlorsulfuron can be explained by chlorsulfuron-insensitive acetolactate-forming enzyme and the lack of branched amino acid biosynthesis in animals.

The mechanism of chlorsulfuron binding to ALS from tobacco cells was noncompetitive with pyruvate and uncompetitive with TTP. A noncompetitive binding mechanism of the herbicide to ALS was also reported in the bacterial enzyme,²²⁾ while the binding of imidazolinone herbicide was uncompetitive.²³⁾

The binding of sulfometuron-methyl herbi-

cide to bacterial ALS has been reported. Assay progress curves of bacterial ALS with herbicide are biphasic, showing an initial weak level and a final more potent steady state of inhibition. The herbicide-binding site overlaps with the second pyruvate-binding site.²⁴⁾ The term "extraneous site inhibitor" has been proposed for such herbicides,²⁵⁾ whose binding sites are outside the enzyme active sites and not equivalent to sites for the allosteric regulation of the enzymes. These compounds bind most tightly to the enzyme during catalytic turnover, thus blocking the completion of the catalytic cycle and releasing products.

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要 約

除草剤クロルスルフロンのタバコおよびハムスターの培養細胞における作用機構

中田昌伸

クロルスルフロン (CS) はハムスター腎臓 (BHK) 培養細胞の生育をまったく阻害しなかったが、タバコ培養細胞には 1 ppb の低濃度で阻害した。タバコおよび BHK 培養細胞とニワトリヒナ肝臓から抽出したアセトラクテート合成酵素 (ALS) の阻害を調べた結果、タバコでは 5~10 ppb CS で 50% 阻害されたが、動物からの酵素は 500 ppb でも阻害されなかった。植物および動物由来の酵素の反応生成物はアセトラクテートと確認された。しかし動物生体内において実際にこの反応が起こるかどうか明らかでないが、両酵素の最適反応条件での pH, 温度, コファクター要求性およびバリンによる阻害が異なっていた。このように CS は植物 ALS のみを阻害することにより動物毒性が低いと考えられる。タバコ培養細胞 ALS に対する CS の阻害様式を調べた結果、基質ピルビン酸に対して非拮抗作用を示し、コファクター thiamine pyrophosphate に対して拮抗作用を示した。このことから CS は酵素, 基質, コファクター複合体に結合することによって, ALS を阻害すると考えられる。