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New Aspects on Inhibition of Plant Acetolactate Synthase by Chlorsulfuron and Imazaquin[1]

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ABSTRACT

The sulfonylurea herbicide chlorsulfuron and the imidazolinone herbicide imazaquin were shown to be noncompetitive and uncompetitive inhibitors, respectively, of purified acetolactate synthase from barley (*Hordeum vulgare* L.) with respect to pyrvuate. From double-reciprocal plots of the time-dependent biphasic inhibition by chlorsulfuron, an initial apparent inhibition constant of 68 nanomolar was calculated (a 0 to 4 minute assay was used for the initial inhibition), and a final steady-state dissociation constant of 3 nanomolar was estimated. The corresponding constants for imazaquin were 10 and 0.55 micromolar. Specific binding of [^{14}C]chlorsulfuron and [^{14}C]imazaquin to purified acetolactate synthase from barley and partially purified enzyme from corn (*Zea mays* L.) could be demonstrated by gel filtration and equilibrium dialysis. Evidence is presented that the binding of the inhibitors to the enzyme follows the previously described mechanism of slow reversibility once excess inhibitor has been removed. However, after formation of the slowly reversible complex and subsequent dissociation, both chlorsulfuron and imazaquin seem to permanently inactivate acetolactate synthase. These results add a new feature to the mode of action of these herbicides with respect to their high herbicidal potency.

ALS[2] (EC 4.1.3.18; also referred to as acetohydroxyacid synthase) catalyzes the first common step in the plant biosynthetic pathway of the essential branched-chain amino acids. For valine and leucine biosynthesis, two molecules of pyruvate are condensed to form 2-acetolactate, while for isoleucine formation one molecule of pyruvate reacts with 2-ketobutyrate in a similar reaction. The enzyme has raised strong interest since structurally unrelated classes of modern and very potent herbicides and inhibitors, the sulfonylureas (16), the imidazolinones (18), the triazolo pyrimidines (20), and the pyrimidinyloxybenzoates (10) have been shown to specifically inhibit acetolactate synthase. With microorganisms the enzyme and the inhibitory mode of action have been well studied. In *Escherichia coli* and *Salmonella typhimurium* three isozymes (ALS I, II, and III) differing in substrate preference and feedback regulation have been identified, purified and well characterized (4, 5, 14). Isozymes are not evident with the plant enzyme (3).

Most of the enzymological and kinetic studies on inhibition of ALS by herbicides have been carried out with the ALS II from *S. typhimurium*, which resembles the plant enzyme with regard to its sensitivity to herbicides, but is different in subunit composition and feedback regulation (12, 15). In time course experiments, sulfometuron methyl, a sulfonylurea, and imazaquin, an imidazolinone, have been shown to act as biphasic, slow-binding inhibitors (8). This means that interaction with the enzyme starts with an initial inhibition, followed by a time-dependent increase in potency leading to a final, steady state level of inhibition. This biphasic feature can be explained by a slow conversion of an initial and rapidly formed ALS-inhibitor complex to a more tightly bound complex. For bacterial ALS reversal of this inhibition process has been reported (16).

An amazing feature, particularly of the sulfonylureas, is the effectiveness of exceptionally low application rates of 2 to 75 g/ha. This is thought to be due to the low ALS content in the cells as well as to the potency of the inhibitors. Limited information is available on the interaction of inhibitors and their kinetics with higher-plant ALS. Enzymological studies have been hampered by the general use of crude extracts. Only ALS from barley has been purified recently (2). Slow-binding inhibition could be demonstrated for chlorsulfuron (sulfonylurea) with the crude pea enzyme (12), and for imazapyr (imidazolinone) with ALS-containing preparations from corn (9). An unsolved question is the reversibility of inhibition and the possible herbicide-induced inactivation of ALS by sulfonylureas or imidazolinones *in vitro*, which has been reported recently (7). Imazapyr and also chlorsulfuron have been reported to be reversible inhibitors of ALS *in vitro* (8, 9). Pretreatment of plants with imazapyr caused a specific reduction in the level of extractable ALS (19). This effect, however, was reported as specific for imidazolinones, but not for sulfonylurea herbicides.

In this communication, data are presented on inhibition of purified ALS from barley by chlorsulfuron and imazaquin with an emphasis on the reversibility of the inhibition. Additional experiments with a partially purified enzyme from corn are discussed.

MATERIALS AND METHODS

Plant Material

Etiolated shoots of barley (*Hordeum vulgare* L.) were cultivated as described previously (3). Three days after germinat-

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[2] Abbreviations: ALS, acetolactate synthase; TPP, thiamine pyrophosphate; FAD, flavin adenine dinucleotide; FPLC, fast protein liquid chromatography; I_{50}, value for 50% inhibition; K_i, inhibition (association) constant.

ing, shoots were harvested, washed in distilled water, collected, and stored at 70 K for later use.

Seeds of corn (*Zea mays* L.) were soaked in water and subsequently planted in moistened vermiculite and allowed to germinate in a dark chamber at 33°C. Four days after germinating, etiolated shoots were harvested and treated as described for barley shoots.

Enzyme Assay

Unless otherwise indicated, the assay mixture contained in a final volume of 0.5 mL: 32 mM Na-phosphate (pH 6.5); Na-pyruvate in different concentrations (40 mM pyruvate for the standard assay); 0.5 mM TPP; 1 mM $MnCl_2$; 50 μM FAD; inhibitors in different concentrations, plus enzyme. After incubation for 30 min at 37°C, the reaction was stopped with 0.25 mL 6 N H_2SO_4. The tubes were heated at 60°C for 12 min and acetoin was determined by the method of Westerfeld (25). Under these conditions, 100 nmol/1.3 mL acetoin were equivalent to an absorbance of 1.55 at 530 nm. Protein was determined according to Bradford (1).

Enzyme Purification

Barley Enzyme

The purification of ALS from etiolated barley shoots was carried out as described previously (2), except for hydrophobic-interaction chromatography where the Butyl-Sepharose CL-4B was replaced by Fractogel TSK Butyl-650 M, and except for the final purification step which was carried out with an Alkyl-Superose HR 5/5 column (hydrophobic interaction). The purified enzyme preparations had specific activities of 2.4 to 3.1 μmol min^{-1} mg^{-1}.

Corn Enzyme

ALS from etiolated corn shoots was partially purified by ammonium-sulfate precipitation and the first hydrophobic-interaction chromatography step as described for barley ALS. Accordingly, the specific activity of the partially purified corn enzyme was substantially less than that of the barley preparation, namely 25 to 50 nmol min^{-1} mg^{-1}. Both the corn and the barley enzyme could be stored at 70 K until later use with negligible loss of activity.

Equilibrium Dialysis

Equilibrium dialysis was performed with a Dianorm equipment (Switzerland) equipped with double-chamber Teflon cells and standard cellulose dialysis tubings with molecular mass cutoff of 12 to 14 kD. Dialysis was carried out for 5 h at 22°C in an enzyme assay mixture containing 5 mM pyruvate. The specific activity of the corn ALS used was 25 to 50 nmol min^{-1} mg^{-1}. Equal concentrations [^{14}C]chlorsulfuron (specific activity of 5.56 mCi/mmol) were added to both chambers. After dialysis, the difference of radiolabeled inhibitor between the enzyme and the buffer compartment was detected by liquid scintillation counting (Rack-Beta II, LKB). Time-course experiments with [^{14}C]chlorsulfuron alone served as a control and proved that a 95% equilibrium had been achieved after dialysis for 1.5 h.

Fine Chemicals and Chromatographic Media

Butyl-Sepharose CL-4B, Alkyl-Superose HR 5/5 and Sephadex G-25 (PD-10) minicolumns were purchased from Pharmacia Fine Chemical Inc. FAD, TPP, DTT, and amino acids were obtained from Sigma Chemical Co., Munich. Fractogel TSK DEAE-650 S and Fractogel TSK Butyl 650 M were purchased from Merck, Darmstadt, Germany; acetoin (3-hydroxy-2-butanone) from Aldrich-Chemie, Steinheim, Germany. The HPLC column Zorbax Bio-Series GF-450 is a product of Du Pont. Imazaquin and both the unlabeled and the radiolabeled chlorsulfuron were gifts of American Cyanamid, Princeton, NJ, and of Du Pont, respectively.

RESULTS

ALS of different purification grades from barley and corn has been assayed for inhibition by chlorsulfuron and imazaquin (Table I). In this study purified enzyme does not imply a homogenous preparation of ALS by the criteria of SDS-PAGE as has been published previously (2), but indicates a more than 2000-fold enrichment with ALS as the major band on SDS-PAGE representing more than 55% of the total protein. To allow direct comparison of our results with previously published data (12) the standard assay mixture contained 40 mM Na-pyruvate and the incubation time was 30 min. Protein in the assay was 1.2 mg for the crude extract of barley, 0.5 μg for the purified enzyme, and 100 μg for the partially purified enzyme from corn. In all experiments imazaquin and especially chlorsulfuron were found to be potent inhibitors of ALS both from barley and corn with I_{50} values ranging from 18 to 34 nM for chlorsulfuron and from 3.8 to 12.0 μM for imazaquin. Because highly active inhibitors such as chlorsulfuron are utilized at low concentrations in enzyme assays, part of the compound may be inactivated by nonspecific binding to contaminating proteins. As checked with different concentrations of our enzyme preparation the same degree of inhibition was obtained indicating that nonspecific binding was negligible.

A more detailed analysis of inhibition of purified barley

Table I. *I_{50} Values for Inhibition of Acetolactate Synthase from Barley and Corn by Chlorsulfuron and Imazaquin*

Specific activities were 1.2 nmol acetoin min^{-1} mg^{-1} for the crude extract and 2.9 μmol min^{-1} mg^{-1} for the purified enzyme from barley. The specific activity of the partial purified enzyme from corn was 25 nmol min^{-1} mg^{-1}. The I_{50} value for inhibition is defined as the concentration of inhibitor which inhibits acetolactate synthase activity 50% in a 30 min standard assay. Calculation was done by regression lines of double-reciprocal plots ($r^2 > 0.99$).

Species	Chlorsulfuron	Imazaquin
	nM	μM
Barley		
Crude extract	34[a]	6.0
Purified enzyme	33	3.8
Corn		
Partially purified enzyme	18	12.0

[a] Values are the average of plots from at least two separate experiments.

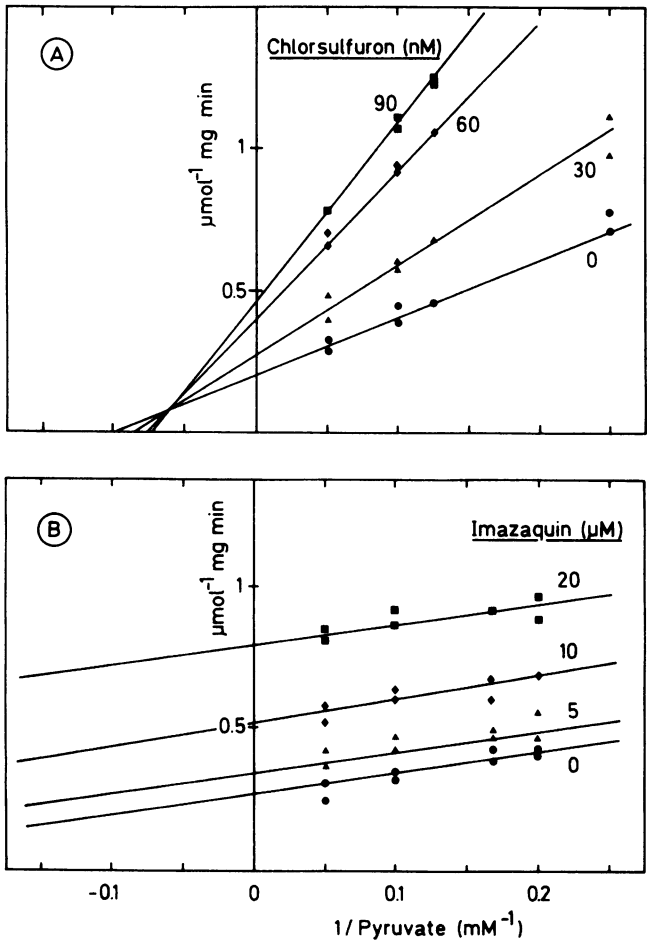

Figure 1. Initial inhibition of purified barley ALS by chlorsulfuron (A) and imazaquin (B) in presence of different pyruvate concentrations. Data refer to a 0 to 4 min assay with specific activities of the controls of 2.8 μmol min^{-1} mg^{-1} (regression coefficient > 0.99).

ALS as a function of pyruvate concentration is shown by Figure 1, A and B. Double-reciprocal plots (Lineweaver-Burk plots) proved to be noncompetitive[3] and uncompetitive with respect to pyruvate for the initial inhibition by chlorsulfuron and imazaquin, respectively. Initial inhibition was estimated approximately by use of a 4-min assay. By secondary plots of the intercepts of the lines in Figure 1, A and B (fitted by regression analysis) with the ordinate against the applied inhibitor concentrations, the substrate-independent K_i values were obtained, namely 68 nM for chlorsulfuron and 10 μM for imazaquin. Corresponding experiments have been carried out to determine inhibition (association) constants for the final, steady-state inhibition using the time interval from 2.5 to 3 h. ALS activity of the controls without inhibitor was linear up to 2 h and decreased slightly (less than 5%) between 2 and 3.5 h. The K_i values for the steady state were determined as 3 nM for chlorsulfuron and 0.55 μM for imazaquin, respectively (data not shown). The maximum rate constant for the (time-dependent) transition between the initial and the final, steady-state inhibition by chlorsulfuron was estimated to be approximately 0.074 min^{-1}. This rate constant is approximate because it was extrapolated on the basis of two concentrations of chlorsulfuron (5 and 50 nM). Calculation was done by the data modeling program as described by LaRossa and Schloss (8). For imazaquin this value has not been calculated.

The criteria of Williams and Morrison (26) were applied to barley and corn ALS to establish data whether the binding of chlorsulfuron or imazaquin to ALS is reversible or not. Purified barley ALS (8 μg) was preincubated with 2.5 μM [^{14}C] chlorsulfuron in a 0.5 mL enzyme assay mixture with the pyruvate concentration reduced to 5 mM to facilitate the binding of the inhibitor to ALS. After incubation, unbound inhibitor was removed by desalting on a PD-10 column equilibrated with identical assay buffer. Complete separation was only achieved and the presence of free herbicide avoided by pooling a smaller volume (3 mL) of the eluate, thereby only using about 80% of the protein eluted from the PD-10 column (see protocol of Pharmacia). Subsequently, the de-

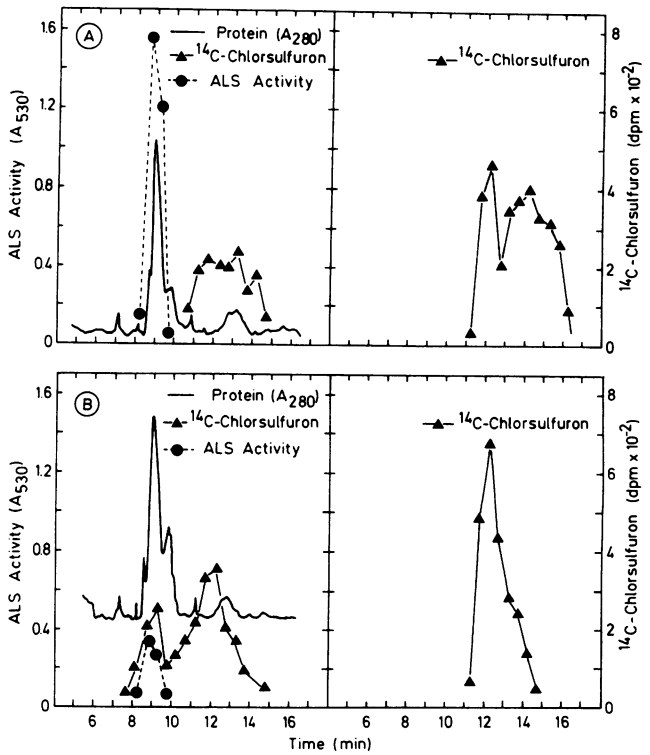

Figure 2. (A) Left side, gel filtration of purified barley ALS after incubation with [^{14}C]chlorsulfuron. Incubation was performed at 4°C for 2 h in the presence of 2.5 μM of 5.56 mCi/mmol [^{14}C]chlorsulfuron and 8 μg protein in the sample. Column and buffer temperature was 4°C. The column was a Zorbax Bio-Series GF-450 (HPLC). Desalting buffer and gel filtration buffer were identical to the incubation media without inhibitor. Right side, distribution of [^{14}C]chlorsulfuron chromatographed alone (identical conditions). (B) Left side, corresponding experiment to (A) except that the incubation was performed at 37°C and temperature of the column and the elution buffer was 22°C. Protein in the sample: 6.5 μg. Right part, as in Figure 2A.

[3] This inhibition is also called "mixed-type." It includes uncompetitive and competitive components (11).

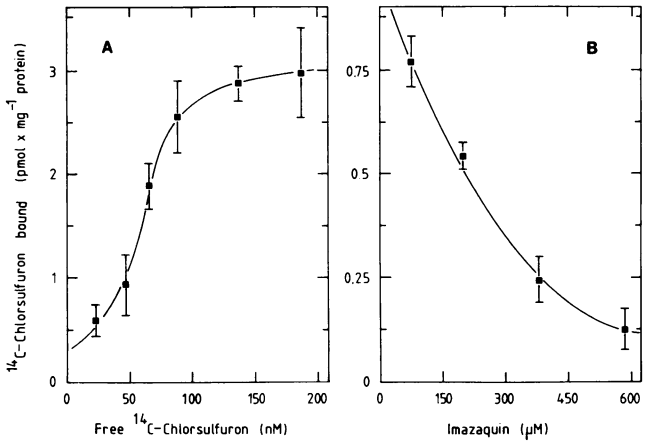

Figure 3. (A) Binding of [^{14}C]chlorsulfuron to partially purified corn ALS determined by equilibrium dialysis. Activity was 25 to 50 nmol min^{-1} mg^{-1}. (B) Replacement of bound [^{14}C]chlorsulfuron by increasing concentrations of unlabeled imazaquin. The concentration of [^{14}C] chlorsulfuron was 50 nM.

salted sample was chromatographed on an analytical gel filtration column (Zorbax Bio-Series) by HPLC (Fig. 2A, left side). Barley ALS eluted as the high mol wt form of M_r = 440,000 in the presence or absence of the inhibitor, as was reported previously (3). The [^{14}C]chlorsulfuron initially bound to the enzyme eluted much later, in a position similar to that of [^{14}C]chlorsulfuron subjected to chromatography alone (Fig. 2A, right side). The elution pattern of this control run, however, indicates that some interactions between the inhibitor and the gel filtration support can not be excluded.

The results of a corresponding preincubation experiment of the enzyme with [^{14}C]chlorsulfuron at 37°C, again with desalting and gel filtration as described, is shown in Figure 2B. Now, coelution of a part of the radiolabeled chlorsulfuron with ALS was observed, and 0.69 mol of radiolabel/mol of protomeric unit (58 kD) eluted concurrently with the enzyme (left side). Specific binding of [^{14}C]chlorsulfuron to ALS could be proved by replacing it with unlabeled inhibitor and using different concentrations of radiolabeled chlorsulfuron (data not shown, compare Figure 3). Experiments performed with only partially purified enzyme (55 nmol min^{-1} mg^{-1}) and using a semipreparative Superose 12 column also showed coelution of [^{14}C]chlorsulfuron with the high mol wt form of ALS indicating specific association of the inhibitor to the enzyme. Complete recovery of enzyme activity after gel filtration failed due to a severe loss of enzyme activity during chromatography. Both experiments shown by Figure 2, A and B, have been repeated with [^{14}C]imazaquin and led to quite similar results (data not shown).

As shown by equilibrium dialysis, specific binding of [^{14}C] chlorsulfuron to corn ALS seemingly has sigmoidal character saturating between 100 and 125 nM (Fig. 3A). A specific binding of [^{14}C]chlorsulfuron to corn ALS could also be demonstrated by displacing the inhibitor with increasing concentrations of unlabeled imazaquin (Fig. 3B).

Studies on a possible recovery of ALS activity after preincubation with chlorsulfuron or imazaquin have been carried out using partially purified corn ALS. Again, preincubation of corn ALS was performed with radiolabeled inhibitors present in concentrations higher than the steady-state K_i values. Table II shows the degree of inhibition after incubation for 2 h. Conditions of incubation were identical to those described for the experiments with the barley enzyme except that FAD was reduced to 5 μM. After desalting on PD-10 columns aliquots were measured both for ALS activity and for coeluted radiolabeled inhibitor using liquid scintillation counting. As shown by Table II, activity of the eluted enzyme could not be restored, although the inhibitor was substantially removed by the desalting steps (columns 2 and 3). In control experiments with inhibitors alone (no enzyme), no radioactivity was eluted after desalting. Thus, based on the binding kinetics obtained by equilibrium dialysis, we calculated an amount of [^{14}C] chlorsulfuron equivalent to 640 ± 50 dpm should be specifically bound in the sample incubated at 22°C. The dpm value

Table II. *Recovery of Corn Acetolactate Activity after Incubation with Chlorsulfuron or Imazaquin Present and after Removal of Excess Inhibitor*

Inhibitor and Temperature	(1) After a 2 h Incubation		(2) After First Desalting		(3) After Second Desalting	
	activity (%)[b]	*dpm*[c]	*activity* (%)	*dpm*	*activity* (%)	*dpm*
[^{14}C]Chlorsulfuron[a]						
22°C	32 ± 3	640 ± 50[d]	31 ± 3	355 ± 10	32 ± 6	90 ± 8
4°C	79 ± 6	ND[e]	71 ± 2	275 ± 12	81 ± 4	91 ± 10
[^{14}C]Imazaquin						
22°C	42 ± 9	ND	46 ± 6	1750 ± 50	40 ± 12	995 ± 40

[a] Inhibitor concentrations were 100 nM of [^{14}C]chlorsulfuron and 10 μM [^{14}C]imazaquin, respectively. Incubation was carried out for 2 h. (For details on conditions see "Results.") [b] ALS activity in percent of control (control rate: 20–30 nmol min^{-1} mg^{-1}) without inhibitor, determined in a standard assay. [c] Radiolabeled inhibitors were detected by liquid scintillation counting (LKB, Rack-Beta II). Values represent the total amount of radioactivity in the sample (3 mL). [d] Values after incubation were determined by equilibrium dialysis and represent the enzyme-bound inhibitor. Data variations are given in terms of the standard error of the mean of at least three determinations. Equilibrium dialysis of column 1 had to be repeated six to seven times; due to the small supply of labeled inhibitors only the figure for chlorsulfuron at 22°C could be determined. [e] Not determined.

of 355 ± 10 indicates that 55% of the ALS present had lost the previously bound inhibitor. In a second step all desalted samples of column 2 have been incubated for additional 2 h and then desalted a second time on freshly equilibrated PD-10 columns as described above (column 3). In all experiments ALS activity remained at the low level measured either directly after incubation with inhibitor or after the desalting steps. Inactivation of ALS by the inhibitors was temperature-dependent, because incubation at 4°C yields more activity than at 22°C. Finally, it should be noted that the control activity (minus inhibitor) was reduced by about 15% only by incubation and desalting treatments. Both [^{14}C]chlorsulfuron and [^{14}C]imazaquin were substantially removed by desalting (columns 2 and 3), indicating a dissociation of the enzyme-inhibitor complex has occurred.

DISCUSSION

Inhibition of ALS from enteric bacteria (8) as well from higher plants by sulfonylureas (12) and imidazolinones (7, 9) has been described to be time-dependent and biphasic indicating that the mode of action of these herbicides should be examined under particular conditions.

As shown by our data, both the initial and the final (steady-state) inhibition of purified barley enzyme by chlorsulfuron were shown to be noncompetitive. For pea extract, assayed with sulfometuron methyl, inhibition also was reported to be noncompetitive (6), while for a bacterial ALS II isozyme, sulfometuron methyl has been proved to be a nearly competitive inhibitor (13). Our data with imazaquin confirm an uncompetitive behavior, as has previously been reported for inhibition of crude corn ALS (18). Nevertheless, contrasting results showing imidazolinones to act as noncompetitive inhibitors of ALS from pea and bacteria have been published (6, 15). Our findings of noncompetitive and uncompetitive mode of action fit well with the assumption that the binding of the first pyruvate molecule results in an enzyme form to which the inhibitor can be tightly bound (17). In case of chlorsulfuron the inhibitor binding site seems to overlap with the domain of the second pyruvate leading to a somewhat different kinetics for chlorsulfuron than that observed for imazaqin. On the other hand, competition between chlorsulfuron and imazaquin as shown here by equilibrium dialysis indicates at least overlapping inhibitor binding sites. This conclusion has also been drawn for bacterial ALS II (15). Additionally, cross-resistance pattern of mutants of tobacco with respect to sulfonylureas and imidazolinones provide further evidence for overlapping binding sites (21). The inhibitor molecules do not show similar and possibly essential structural groups. This fact has been already described for "photosynthetic" herbicides. In this case, inhibitors with a wide chemical variety apparently are attached to the same binding region of the D-1 peptide (22, 23).

Analysis of the time-dependent inhibition by chlorsulfuron was hampered by small quantitites of purified enzyme and by the use of fixed-time instead of continuous assays. Nevertheless, a maximum rate constant of 0.074 min^{-1} can be estimated for the transition between initial and steady-state inhibition which is close to the previously reported rate constant of 0.05 min^{-1} for inhibition of pea ALS by sulfometuron methyl (6), and is somewhat less than the corresponding rate constant for bacterial ALS II (0.15 min^{-1}). The substrate-independent inhibition constants for bacterial ALS assayed with sulfometuron methyl are substantially higher than those for the plant enzyme and chlorsulfuron (350 and 39 nM for initial and final inhibition compared to 67.5 and 3 nM determined with our barley ALS). For a detailed review on slow-binding inhibition of ALS, see Schloss and Aulabaugh (17).

The results presented in Figure 2A indicate that under reduced turnover conditions (low temperature) no radiolabel by [^{14}C]chlorsulfuron took place. This is in line with findings on bacterial ALS II which indicated that in the absence of pyrvuate no slowly reversible complex is formed (8).

Reversal of inhibition has been examined with partially purified ALS from corn. For both bacterial ALS II and corn ALS a fully active enzyme could be restored after prolonged exposure to sulfometuron methyl and imazapyr (9, 17). However, in case of corn ALS these authors did not examine turnover conditions, because no pyruvate or TPP were present, preventing the inhibition to form the slow-reversible complex. The results presented in Table II indicate a permanent inactivation of ALS after incubation in the presence of chlorsulfuron or imazaquin and after their subsequent removal. ALS inhibition measured with imazapyr was reported to depend on the dose and period of exposure (6), a finding which is inline with the data reported in this study. We assume this inhibitor-induced enzyme inactivation represents a substantial contribution to the effectiveness of low application rates observed under field conditions.

Nevertheless, neither imazaquin nor chlorsulfuron seem to meet the criteria of irreversible inhibitors or mechanism-based inactivators (24). Although inactivation of corn ALS seems to be permanent after removal of excess inhibitor, the data of Table II suggest a dissociation of the enzyme-inhibitor complex, which occurred faster than anticipated. For barley ALS a calculation based on the initial and final dissocition constants (67.5 and 3 nM) and the maximum rate of transition (0.074 min^{-1}) yields a dissociation half-time of 3.5 h. For bacterial ALS II a 1.6-h period has been calculated (17).

Summarizing the presented data, only partial agreement between plant ALS and bacterial ALS II is evident. While both the time-dependent inhibition and the slow dissociation of the enzyme-inhibitor complex are in accordance, there are decisive differences with regard to reversal of inhibition, *i.e.* recovery of enzyme activity. Apparently, there are different physical and catalytic properties of plant and bacterial ALS, emphasizing the need for further studies on the plant enzyme.

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