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Herbicide Resistance in *Datura innoxia*[1]

Kinetic Characterization of Acetolactate Synthase from Wild-Type and Sulfonylurea-Resistant Cell Variants

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ABSTRACT

Acetolactate synthase (ALS, EC 4.1.3.18), the first enzyme in the biosynthesis of branched-chain amino acids, was isolated from wild-type and sulfonylurea-resistant *Datura innoxia* cell variants and characterized. Apparent K_m values of the ALS for pyruvate from three sulfonylurea-resistant variants (CSR2, CSR6, and CSR10) were manyfold greater than that of the wild type. The inhibition of wild-type and herbicide-resistant ALS activity by chlorsulfuron (CS), a sulfonylurea herbicide, and L-leucine (L-Leu), one of the feedback inhibitors of the enzyme, was examined. ALS from two CS-resistant variants exhibited severalfold greater resistance to CS than did the wild-type enzyme. Inhibition of ALS by L-Leu fitted a partially competitive pattern most closely. It is proposed that the herbicide resistance mutation accentuated the partial inhibition characteristics of ALS by L-Leu. ALS from one of the two CS-resistant variants (CSR6) had a K_i for L-Leu an order of magnitude greater than that of the wild-type enzyme. The alterations in kinetic properties observed in the ALS from sulfonylurea-resistant variants are discussed in relation to the possible evolutionary significance of the herbicide binding site of this enzyme, the physiological effects of such biochemical alterations, and their practical utility in genetic studies.

ALS[3] (EC 4.1.3.18, also known as acetohydroxy acid synthase) is the first enzyme in the biosynthesis of Val, Leu, and Ile. This enzyme catalyzes the condensation of an acetaldehyde moiety derived from pyruvate either with another molecule of pyruvate to form 2-acetolactate or with 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate.

ALS is feedback inhibited by the end products Val and Leu in both micro-organisms and in plants (5, 11, 26). Recently, it has been discovered that ALS is the target of several structurally diverse herbicidal compounds such as SUs, IZs, and TPs (9, 16, 22, 24). The SU herbicides have been found to act as slow-binding inhibitors of the enzyme (9). A large number of herbicide-resistant mutants have been isolated and characterized from a wide range of organisms such as bacteria, yeast, and lower and higher plants (see ref. 8 for review). Despite these studies, the interaction of herbicides and feedback inhibitors with ALS is not fully understood. In particular, knowledge about the alterations in kinetics of ALS resistant to herbicides is very limited. Such information would be valuable in assessing herbicide-resistant mutants for use in gene transfer and in somatic hybridization. It could also aid the rational design of new ALS inhibitors as herbicides.

In our laboratory, a number of SU-resistant and IZ-resistant variants of *Datura innoxia* have been isolated, and ALS from these variants exhibits different degrees of cross-resistance to SUs and IZs. The lack of cross-resistance in some of the variants has been used to support the hypothesis that there are two separable binding domains for SUs and IZs on the ALS molecule (18, 19). ALS from several of these variants has also been found to be altered in feedback sensitivity to Val, Leu, and Ile (14). In this report, the properties of ALS from SU-resistant variants are compared with that of the wild type. We report the mechanism of inhibition of ALS from *D. innoxia* by the feedback inhibitor, Leu. The implications of the altered kinetic constants of the ALS from SU-resistant variants are discussed in relation to our current understanding of the herbicide binding site of ALS and the regulation of branched-chain amino acid biosynthesis in higher plants.

MATERIALS AND METHODS

Cell Suspension Cultures

Maintenance and isolation of SU-resistant cell variants of *Datura innoxia* from a predominantly haploid (>90%) cell culture were described previously (18).

Assay of ALS

ALS was extracted from 3-d-old *D. innoxia* cell suspension cultures using methods of enzyme extraction and assay modified from those of Chaleff and Mauvais (2) and Ray (16). Cells were collected over Miracloth by suction and washed twice with 100 mL of glass-distilled water, collected again, and suspended in 2 volumes of an extraction solution (buffer A) containing 0.1 M potassium phosphate buffer (pH 7.5), 5 mM sodium pyruvate, 0.5 mM $MgCl_2 \cdot 6H_2O$, 10 μM FAD, 2.5

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[3] Abbreviations: ALS, acetolactate synthase; CS, chlorsulfuron; IZ, imidazolinone; SU, sulfonylurea; TP, triazolopyrimidine sulfonanilide; FAD, flavine adenine dinucleotide; $K_{m_{app}}$, apparent K_m values.

mM DTT, and 1 mM each of L-Val and L-Leu. For each experiment, 50 to 100 g of cells were used. Glass beads (1 mm) were then added at a ratio of 2 g per 10 mL of buffer A and the mixture homogenized in a Braun homogenizer (type 2876) cooled with CO_2. Glass beads were then removed by filtration through two layers of Miracloth. Debris from the resulting filtrate were then removed by centrifugation at 20,000*g* for 20 min at 4°C. Saturated ammonium sulfate was added to the supernatant to a final concentration of 50%, and the mixture was stirred and kept in an ice bath for 10 min. The protein pelleting at 50% ammonium sulfate was collected by centrifugation at 20,000*g* for 20 min at 4°C and redissolved in a small volume of enzyme buffer (buffer B) containing 0.1 M potassium phosphate buffer (pH 7.5), 0.5 mM $MgCl_2 \cdot 6H_2O$, and 10 μM FAD. The mixture was then desalted on a Sephadex G-25 column equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) at 4°C. The fractions containing protein were pooled and diluted with buffer B to a final concentration of protein between 1 and 1.5 mg/mL.

The assay of ALS was carried out at 30°C for 90 min in a 0.5 mL final volume of the reaction mixture containing varying levels of sodium pyruvate, 0.5 mM thiamine pyrophosphate, 10 μM FAD, 1 mM $MgCl_2 \cdot 6H_2O$, varying levels of the inhibitors CS or L-Leu in 20 mM potassium phosphate buffer (pH 7.0), and 100 μL of the extracted enzyme. The pH of the final reaction mixture was not affected by the addition of the inhibitors and the enzyme extract.

The conversion of acetolactate to acetoin by H_2SO_4 and NaOH and the determination of acetoin were carried out at 65°C according to the procedure of Westerfeld (28) as modified by Ray (16). The reaction was stopped by the addition of 50 μL of 6 N H_2SO_4 and followed by incubating the tubes at 65°C for 15 min. Then, 0.5 mL each of freshly made creatine (0.5% w/v in water) and 1-naphthol (5% in 2.5 N NaOH) were rapidly added to the reaction mixtures, which were then shaken and incubated for another 15 min at 65°C. The tubes were then cooled in an ice bath and shaken periodically. Full color development was attained within 15 min of incubation in the ice bath and was read in a spectrophotometer (DU-7 UV-Visible Spectrophotometer; Beckman Instruments, Inc., Irvine, CA) at 525 nM. Spectrophotometric measurements were completed well before 2 h after full color development since preliminary experiments showed that beyond 2 h the $A_{525\ nM}$ began declining slowly. For each treatment, there were three or five replicate tubes as well as one control tube similar to the treatment but that was acidified before the addition of the enzyme extract. The o.d. in this control tube was subtracted from the values for treatment reactions. Other controls were: (a) boiled enzyme extract; and (b) assay mixture lacking substrate. There was no detectable interference either from pyruvate decarboxylase (13) or from direct acetoin formation.

The stock solution of CS was prepared using acetone as solvent. The latter was removed by taking the stock solutions to dryness under nitrogen and solutions prepared by redissolving the compound in 5 mM potassium phosphate buffer (pH 7.5). A stock solution of L-Leu was prepared in water. All stock solutions were made on the day of their use.

The concentration of protein in the extracts was determined by the method of Bradford (1). Reaction velocities were calculated by using the micromolar extinction coefficient of pure acetoin (7.06×10^{-3} μmol^{-1} cm^{-1}) and were expressed as μmol acetoin (mg protein)$^{-1}$ h^{-1}.

Design of Enzyme Kinetic Experiments and Analysis of Data

The range of substrate concentration used for determination of K_m values was chosen based on preliminary experiments. The range of pyruvate concentration was from 0.2 to 15 mM for the wild-type enzyme, from 1 to 50 mM for variant CSR2, and from 0.2 to 40 mM for other variants. Types of inhibition and calculation of inhibitor constants (3) were performed using graphical methods described by Webb (27). Experiments were repeated two to six times using different extracts. The data were analyzed using an SAS computer program (SAS Institute Inc., Cary NC), and linear regression of data for double reciprocal plots was calculated by the method of least squares. The $K_{m_{app}}$ and V_{max} values were calculated by fitting the data to the equation

$$v = V_{max}[S]/K_m + [S]$$

using the nonlinear regression program by the method of least squares.

Chemicals

CS (95% pure) was obtained from DuPont Co., Wilmington, DE. Sodium pyruvate and 1-naphthol were from BDH Ltd., Poole, England. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

RESULTS

SU Resistance of *D. innoxia* Variants

The variants used in this investigation were previously isolated in our laboratory from cell suspension cultures of *D. innoxia* and represented a collection of independently isolated, stable mutations for ALS-based SU resistance (18). Variants CSR2, CSR6, CSR10, and CSR15 were selected for resistance to CS, whereas SMR1 was selected for resistance to sulfometuron methyl (18). ALS from these variants was compared with ALS from a wild-type cell line, Px4.

As low a concentration as 10 nM SU herbicide CS inhibited 50% of wild-type ALS activity in a fixed time assay for the product in the presence of excess substrate. On the other hand, 2,000 nM CS was required to inhibit 50% of CSR2 ALS activity. ALS from CSR6 was highly resistant to CS in that 70% of ALS activity remained uninhibited even in the presence of 50,000 nM CS.

Determination of $K_{m_{app}}$ for Pyruvate

A strict Michaelis-Menten kinetics of ALS with respect to pyruvate was observed when extracts from wild-type (Px4, Fig. 1A), and SU-resistant variants (CSR2 for example, Fig. 1B) were examined. The kinetic constants determined for ALS from Px4 and SU-resistant variants are listed in Table I. $K_{m_{app}}$ value for ALS from CSR2 was an order of magnitude higher than that of wild-type ALS. The Km_{app} values of ALS from other variants CSR6, CSR10, CSR15, and SMR1 were also severalfold higher than the $K_{m_{app}}$ of the wild-type enzyme

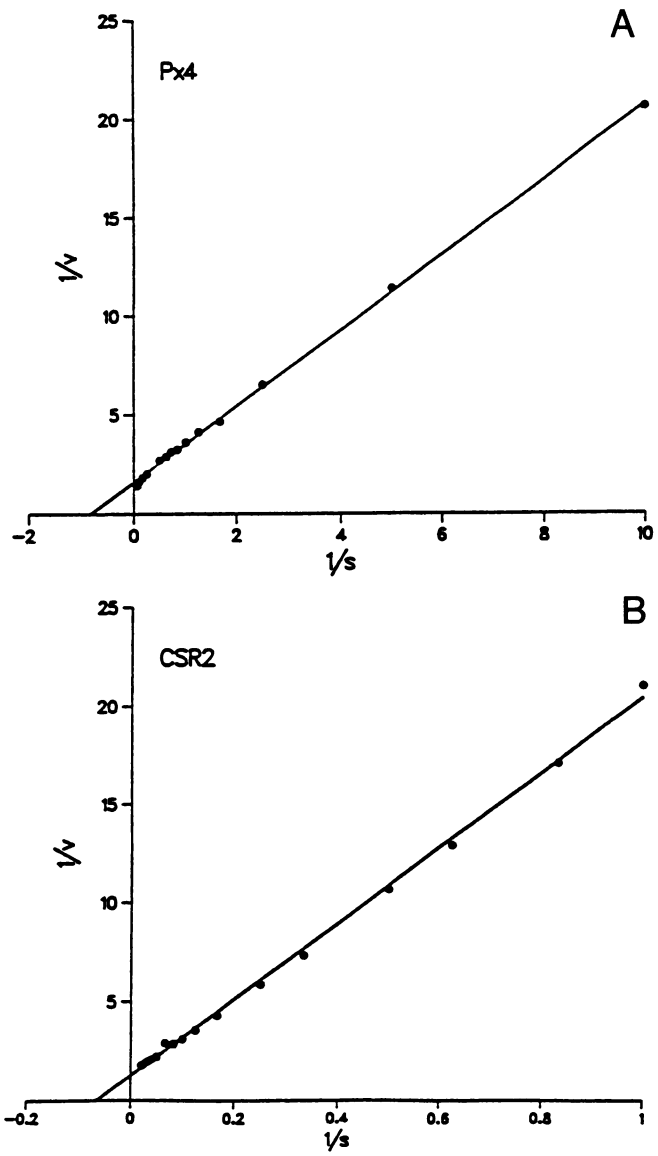

Figure 1. Double reciprocal Lineweaver-Burk plot of ALS activity from wild-type cells, Px4 (A), and CS-resistant variant CSR2 (B). The substrate (s) pyruvate concentration was varied and other assay conditions were as described in "Materials and Methods."

(Table I). However, V_{max} in all cases remained largely unchanged.

Among the variants studied here, CSR2 was resistant to SUs but more sensitive to IZs than the wild type, whereas CSR6 exhibited the highest level of resistance to SUs compared with all other SU-resistant variants of *D. innoxia* isolated (18). These interesting properties of CSR2 and CSR6 prompted us to choose these two variants for further characterization.

Inhibition of ALS from *D. innoxia* by L-Leu and Determination of K_i Values of ALS from Px4, CSR2, and CSR6

A double reciprocal plot of velocity *versus* pyruvate concentration indicated that inhibition of *D. innoxia* ALS by L-Leu approximated to a competitive inhibition pattern (Fig. 2). A Dixon plot of inhibition of ALS from PX4 by L-Leu revealed a degree of nonlinearity at all concentrations of inhibitor tested (Fig. 3A), suggesting partial inhibition (27). Hence, the data were replotted as double reciprocal plots of inhibitor concentration *versus* fractional inhibition (Fig. 3B), which indicated a y axis intercept slightly greater than unity. At 10 mM pyruvate, the intercept at y axis was 1.139 ± 0.043. The Dixon plots obtained in the case of ALS from CS-resistant variants were even more nonlinear than in the case of Px4 (Fig. 3C for CSR2). A double reciprocal plot of inhibitor concentration and fractional inhibition indicated that the y axis intercept was clearly greater than unity (Fig. 3D). For example, at 40 mM pyruvate, the y axis intercept of a double reciprocal plot of inhibitor concentration *versus* fractional inhibition was 1.30 ± 0.02 for CSR2.

Based on the graphical analyses of inhibition data discussed above, partial competitive inhibition of ALS by L-Leu was hypothesized to fit the data most closely, and K_i values were calculated as described by Webb (27). The equation used was:

$$i = \frac{(I)\,(\alpha - 1)}{(I)\,(\alpha + [S]/K_s) + K_i[1 + (S)/K_s]}$$

where i is the fractional inhibition, (I) is the inhibitor concentration, (S) is the substrate concentration, K_s is $K_{m_{app}}$, and α was calculated using double reciprocal plots of $[I]$ and i as described by Webb (27). K_i values for L-Leu for CSR2 and CSR6 were about threefold and 10-fold higher than for Px4, respectively (Table II).

An Examination to Find Whether *D. innoxia* Had More Than One ALS

One explanation for the partial inhibition of ALS would be that *D. innoxia* contains more than one ALS (see below), a question that was addressed by using enzyme kinetic analyses. Px4 enzyme extract was mixed with that from CSR2 and the velocity *versus* $v/[s]$ plot (Eadie-Scatchard plot; ref. 21) was analyzed. The data from Px4 (Fig. 4A) and from CSR2 (Fig. 4B) conformed to straight lines, whereas the plot from the mixture of Px4 and CSR2 indicated two distinct linear regions (Fig. 4C). The absence of two distinct linear regions in CSR2 whose ALS $K_{m_{app}}$ was severalfold greater than that of Px4 (*i.e.* note the different scales in Fig. 4, A and B) in this plot suggested the absence of more than one ALS in *D. innoxia.*

Table I. *Kinetic Constants of ALS from Wild-Type (Px4) and Su-Resistant Variants of D. innoxia*

Cell Line	$K_{m_{app}}$ Pyruvate	V_{max}
	mM	μmol acetoin (mg protein)$^{-1}$·(h)$^{-1}$
Px4	0.89 ± 0.1	0.80 ± 0.02
CSR6	5.82 ± 0.2	0.46 ± 0.01
CSR2	14.88 ± 0.9	1.47 ± 0.06
CSR10	4.91 ± 0.3	0.36 ± 0.01
CSR15	3.05 ± 0.2	0.88 ± 0.01
SMR1	3.14 ± 0.2	0.39 ± 0.01

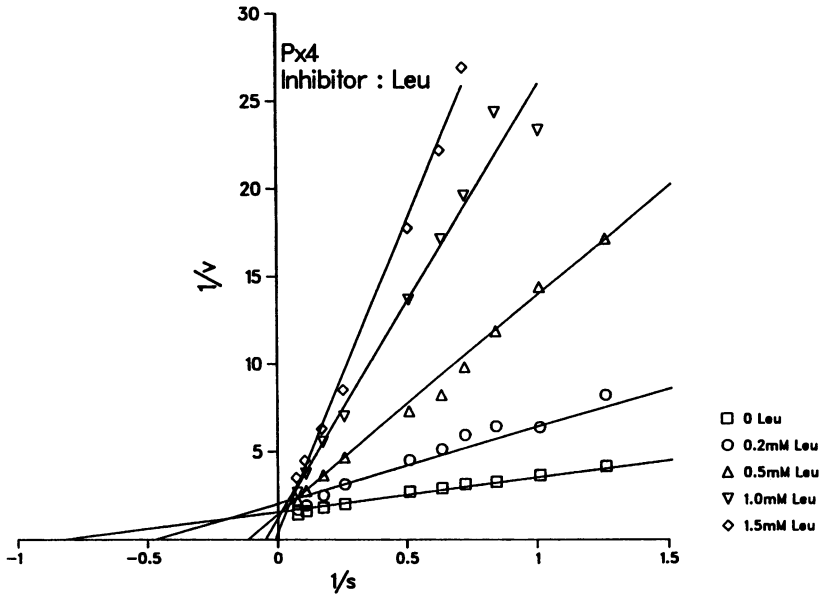

Figure 2. Double reciprocal Lineweaver-Burk plot of velocity (v) data at varying (0–1.5 mM) levels of L-Leu for ALS from wild-type *D. innoxia* cells. The substrate (s) pyruvate and inhibitor L-Leu levels were varied, and other assay conditions were as described in "Materials and Methods."

DISCUSSION

SU herbicides are reversible, slow, tight binding inhibitors of ALS (9). Hence, kinetic analyses of inhibition by CS of ALS would require a continuous type assay to measure both initial and the final steady state K_i values. However, the data on CS inhibition of ALS obtained in fixed-time assays for the enzyme from Px4, CSR2, and CSR6 clearly indicate that ALS from CSR2 and CSR6 variants exhibits manyfold greater resistance to CS than the wild-type enzyme.

Our results here and those reported previously (15) showed that the Km_{app} values for pyruvate in the case of ALS isolated from SU-resistant variants (CSR2, CSR6, and CSR10) were manyfold greater than that for the wild type. This suggested that the herbicide resistance mutation somehow altered pyruvate binding to the ALS molecule. This alteration could also have physiological implications, for example, by resulting in less efficient synthesis of branched-chain amino acids *in vivo*. In support of this hypothesis, our studies on the free amino acid contents of wild type and CSR6 showed that the CSR6 variant had significantly smaller pools of free branched-chain amino acids than did wild-type cells (B. Rathinasabapathi, J. King, manuscript in preparation). More recently, others using an ALS from a TP-resistant cotton cell variant have shown a higher Km_{app} for pyruvate than in the case of the wild-type enzyme (25). Contrary to this observation (25)

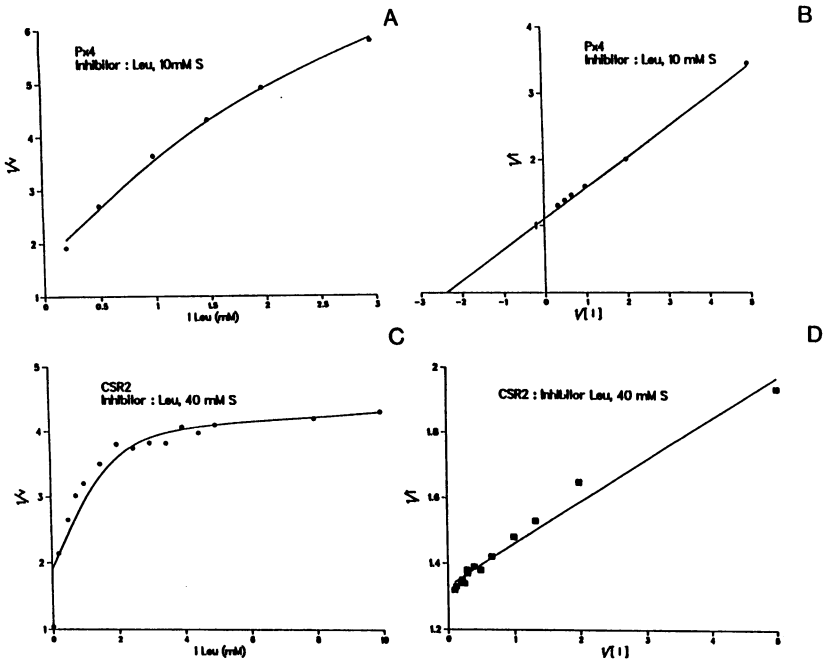

Figure 3. A, Dixon plot (1/*v versus* [*I*]) for inhibition of ALS from wild-type Px4 by L-Leu. Pyruvate concentration used was 10 mM. B, Double reciprocal plot of fractional inhibition (*i*) *versus* inhibitor concentration ([*I*]) for inhibition of ALS from wild-type Px4 by L-Leu (data used for the Dixon plot in A). C, Dixon plot (1/*v versus* [*I*]) for inhibition of ALS from CSR2 by L-Leu. Pyruvate concentration used was 40 mM. D, Double reciprocal plot of fractional inhibition (*i*) *versus* inhibitor concentration ([*I*]) for inhibition of ALS from CSR2 by L-Leu (data used for the Dixon plot in C).

Table II. *Inhibitor Constants of ALS from Wild-Type (Px4) and CS-Resistant Variants of D. innoxia for Inhibition by L-Leu*

Cell Line	L-Leu K_i
	mM
Px4	0.07 ± 0.01
CSR2	0.19 ± 0.04
CSR6	0.74 ± 0.50

and our results, Saari *et al.* (17) found that the $K_{m_{app}}$ values of ALS from SU-sensitive and -resistant *Kochia scoparia* biotypes did not differ significantly. However, their (17) studies were carried out with a SU-resistant biotype of *K. scoparia* occurring naturally under field conditions, whereas the *D. innoxia* variants used in our study and the cotton variant (25) were isolated from cell cultures. Cell culture conditions might allow the isolation of a broader spectrum of SU-resistant mutations including ones that might be significantly harmful or even lethal at the whole plant level. Further enzyme kinetic evaluations of ALS from both cell culture-generated SU-resistant mutants and mutants selected by screening seedlings could elucidate this point. Our results presented here, thus, suggest the need for critical evaluations of SU-resistant mutants at the whole plant level to assess whether some of the mutants have decreased yield or competitive fitness compared with their herbicide susceptible counterparts. It has often been observed that atrazine-resistant biotypes exhibit lower yield and ecological fitness than the susceptible biotypes (7, 23). Hence, in the context of genetic engineering of SU resistance into crop plants (10), the screening of SU-resistant mutants for competitive fitness and yield will be an important aspect of evaluation.

Previous studies in the literature had shown that branched-chain amino acids inhibited barley ALS in a competitive fashion (12). The inhibition of the pea enzyme by L-Val was markedly nonlinear and described as approximately competitive (6). None of the kinetic studies on ALS available in the literature, however, describes partial inhibition of ALS by branched-chain amino acids. The reasons for this could be because the detection of partial inhibition would need diagnostic analyses other than the often-used Lineweaver-Burk plot. Even in Dixon plots the nonlinearity observed might have been overlooked in other cases since the wild-type enzyme might exhibit only a slight nonlinearity. In our studies, since the nonlinearity of Dixon plots was pronounced in the case of CS-resistant variants, we were able to identify the phenomenon and check our data carefully. This caused us to calculate K_i values more accurately than had been done in other reported cases.

The K_i value for L-Leu reported here and also previously (15) for ALS from CSR6 was an order of magnitude higher than that of the wild-type ALS. This was in accordance with our previous studies where ALS from CSR6 and three other CS-resistant variants was shown to be less sensitive to inhibition by branched-chain amino acids (14). Feedback insensitivity of ALS from a TP-resistant cotton variant has also been reported more recently (25).

Partial inhibition of ALS by L-Leu could be for any one of

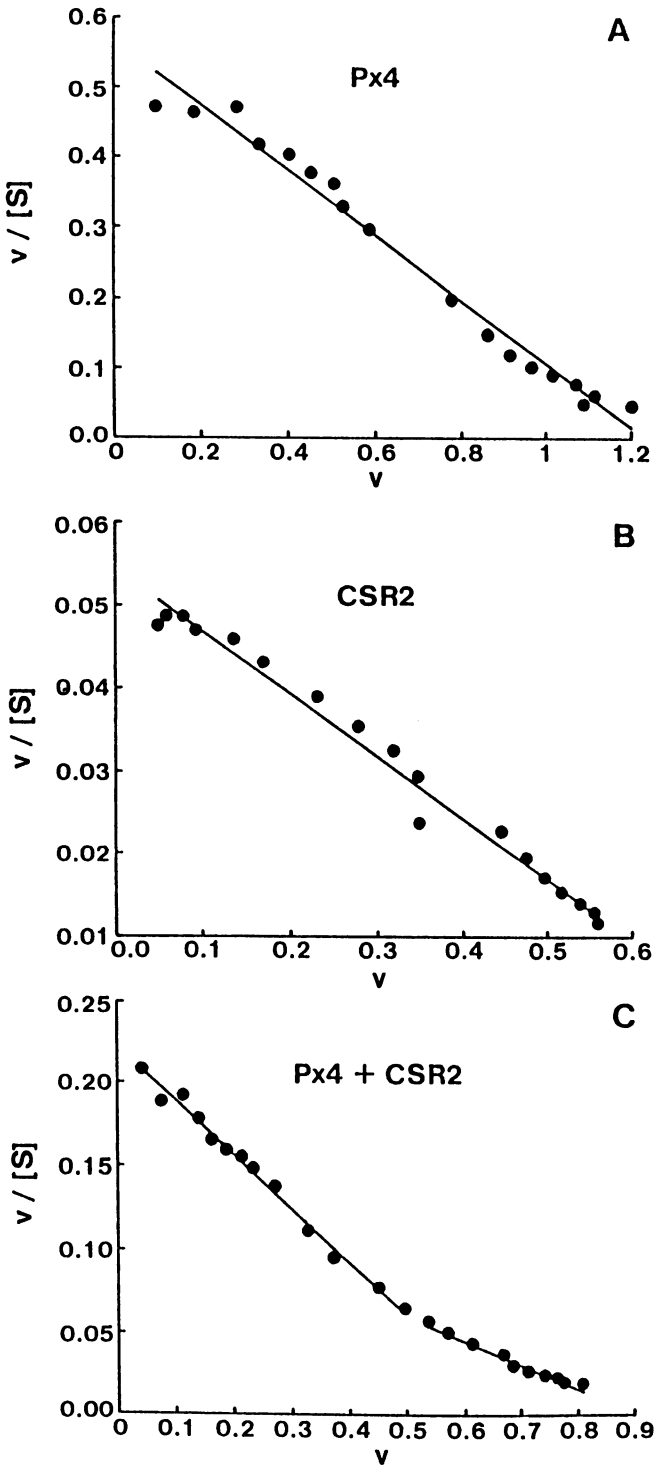

Figure 4. Eadie-Scatchard plot ($v/[S]$ *versus* v) of ALS from wild-type Px4 (A), CS-resistant variant CSR2 (B), and a mixture of extracts (1:1 in terms of protein concentration) from Px4 and CSR2 (C). The substrate (S) pyruvate level was varied, and other assay conditions were as described in "Materials and Methods."

several reasons. In partially competitive inhibition the substrate and the inhibitor bind to the enzyme at different sites, but the substrate binds to the free enzyme with a greater affinity than to the enzyme-inhibitor complex and the enzyme-substrate and enzyme-substrate-inhibitor complexes both yield product with equal facility (21). Based on our results, we propose the hypothesis that ALS from *D. innoxia* has distinct sites for pyruvate, and for branched-chain amino acids such as L-Leu. Furthermore, the mutation for herbicide resistance can lead to an accentuation of the partial mode of inhibition displayed by the wild-type enzyme.

Partial inhibition could also be explained by a model whereby the enzyme has two distinct and mutually exclusive inhibitor sites (21). Studies on ALS from barley (12) suggested that the enzyme had two inhibitor sites, one for Leu and one for Val, although mutual exclusiveness of the sites was not evident. The presence of multiple forms of the same enzyme in a preparation could also give rise to apparent partial inhibition patterns (21). However, several lines of evidence support the hypothesis that *D. innoxia* has a single ALS. Two enzymes, one resistant and another sensitive to the inhibitor, would be present in diploid cells if a mutation for herbicide resistance occurred in a diploid cell and the mutant is heterozygous. But the variants analyzed here were isolated from suspension cultures that were predominantly (>90%) haploid. Also, *D. innoxia* is a true diploid by evolution (4). Hence, the probability of the existence of isozymes is low. If two isozymes of ALS are present in *D. innoxia* and only one is mutated for herbicide resistance and an alteration of K_m results, in a plot of *v versus* $v/[s]$, two distinct linear regions should be evident. This was not the case when such a plot for CSR2 was examined (Fig. 4B) although two distinct linear regions could be shown if extracts of wild type (Px4) and variant (CSR2) were mixed (Fig. 4C). Thus, even if there are several possible explanations for the partial inhibition of ALS by L-Leu, the most plausible proposition would be that the herbicide resistance mutation could give rise to enhanced partial inhibition characteristics in the enzyme.

Schloss *et al.* (20) proposed that the herbicide-specific site of ALS is an evolutionary vestige of the quinone binding site of pyruvate oxidase. They also indicated the possibility that the quinone/herbicide binding site of ALS has some, as yet, undiscovered regulatory or mechanistic role (20). The altered kinetic parameters of CS-resistant ALS with reference to pyruvate and L-Leu reported here suggest that the herbicide binding domain can have either a direct or indirect influence on substrate and amino acid binding sites. Such a hypothesis further suggests a reason for the evolutionary conservation of the herbicide binding domain in ALS from a wide range of prokaryotes and eukaryotes (if not its origin), *i.e.* evolutionary pressure to maintain the substrate and amino acid binding sites could lead, secondarily, to the maintenance of a domain for herbicide binding close by. Further kinetic studies on the relationship between ALS and its structurally diverse inhibitors as well as substrates and cofactors may prove useful in understanding the nature of the herbicide binding domain. Some of the variants having mutations conferring high levels of SU resistance combined with least adverse physiological perturbations could be ideal candidates for somatic hybridization and gene transfer into plants of economic interest.

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