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Roles of conserved methionine residues in tobacco acetolactate synthase☆

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Abstract

Acetolactate synthase (ALS) catalyzes the first common step in the biosynthesis of valine, leucine, and isoleucine. ALS is the target of several classes of herbicides, including the sulfonylureas, the imidazolinones, and the triazolopyrimidines. The conserved methionine residues of ALS from plants were identified by multiple sequence alignment using ClustalW. The alignment of 17 ALS sequences from plants revealed 149 identical residues, seven of which were methionine residues. The roles of three well-conserved methionine residues (M350, M512, and M569) in tobacco ALS were determined using site-directed mutagenesis. The mutation of M350V, M512V, and M569V inactivated the enzyme and abolished the binding affinity for cofactor FAD. Nevertheless, the secondary structure of each of the mutants determined by CD spectrum was not affected significantly by the mutation. Both M350C and M569C mutants were strongly resistant to three classes of herbicides, Londax (a sulfonylurea), Cadre (an imidazolinone), and TP (a triazolopyrimidine), while M512C mutant did not show a significant resistance to the herbicides. The mutant M350C was more sensitive to pH change, while the mutant M569C showed a profile for pH dependence activity similar to that of wild type. These results suggest that M512 residue is likely located at or near the active site, and that M350 and M569 residues are probably located at the overlapping region between the active site and a common herbicide binding site.

Keywords: Acetolactate synthase; Tobacco; Site-directed mutagenesis; Herbicide; Resistance; Conserved methionine; Multiple alignments; ClustalW

Acetolactate synthase (ALS, EC 4.1.3.18; also referred to as acetohydroxy acid synthase) catalyzes the first common step in the biosynthesis of valine, leucine, and isoleucine in microorganisms and plants. ALS catalyzes two parallel reactions, the condensation of two molecules of pyruvate to give rise to 2-acetolactate in the first step of valine and leucine biosynthetic pathway, and the condensation of pyruvate and 2-ketobutyrate to yield 2-aceto-2-hydroxybutyrate in the second step of isoleucine biosynthesis [1].

ALS requires three cofactors for its catalytic activity, thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), and divalent metal ion, Mg^{2+} or Mn^{2+}. Strong interest has been raised in ALS since it was demonstrated to be the target of several classes of modern and potent herbicides, including the sulfonylureas [2,3], the imidazolinones [4], and the triazolopyrimidines [5,6].

In bacteria, three ALS isozymes have been purified and studied extensively in terms of their genetic regulation, kinetic properties, feedback regulation, and sensitivity to herbicidal inhibitors [7–10]. Each of the isozymes is a tetramer of two large catalytic subunits (59–60 kDa) and two small regulatory subunits (9–17 kDa) [1]. In contrast to the bacterial enzyme, the

☆ *Abbreviations:* ALS, acetolactate synthase; mALS, mutant ALS; wALS, wild-type ALS; CD, circular dichroism; FAD, flavine adenine dinucleotide; GSH, glutathione; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactoside; PCR, polymerase chain reaction; TP, triazolopyrimidine sulfonamide; TPP, thiamine pyrophosphate.

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structure and biochemical properties of ALS from eukaryotes have not been well characterized since purification of eukaryotic ALS is severely hampered by its extreme instability and very low abundance.

A number of ALS genes from *Arabidopsis thaliana* [11], *Brassica napus* [12], *Gossypium hirsutum* [13], *Nicotiana tabacum* [11], *Zea mays* [14], and *Xanthium* sp. [15] have been cloned and characterized. The ALS genes from *A. thaliana* [16] and tobacco [17] have been functionally expressed in *Escherichia coli*, and each of the enzymes has been purified. Various herbicide-resistant ALS mutants from several plants have been obtained by spontaneous or induced mutation under field or laboratory conditions, and by site-directed mutagenesis (summarized in [1,18]).

Recently, site-directed mutagenesis studies in our laboratory revealed that K219 [19], W490 [20], C411 [21], and H487 [22] residues are essential for catalytic function of tobacco ALS, and that K255 [19], W573 [20], A121 [23], and S652 [23] are possibly located at herbicide-binding site. More recently, preliminary X-ray diffraction analysis of the catalytic subunit of *Saccharomyces cerevisiae* ALS was reported [24].

Analysis of Bartlett et al. [25] showed that, among 178 enzymes, whose structures were determined by X-ray analysis, more than 1% of catalytic residues were methionine. Duggleby and Pang [1] identified that six methionine residues are conserved in acetolactate synthase. The study of the conserved methionine residues in *Arabidopsis* ALS suggested that both M124 and M351 residues are located at or near herbicide-binding site [1].

In this study, we identified the conserved residues in plant ALSs by multiple sequence alignment and carried out site-directed mutagenesis of three well-conserved residues (M350, M512, and M569) in tobacco ALS, and then analyzed the effects of the mutation on the kinetic parameters, the structure of the enzyme, and the inhibition by herbicides.

Materials and methods

Materials. Bacto-tryptone, yeast extract, and Bacto-agar were purchased from Difco Laboratories (Detroit, USA). Restriction enzymes were purchased from Takara Shuzo (Shiga, Japan) and Boehringer–Mannheim (Mannheim, Germany). GSH, TPP, FAD, α-naphthol, and creatine were obtained from Sigma Chemical (St. Louis, USA). Thrombin protease and epoxy-activated Sepharose 6B were obtained from Pharmacia Biotech (Uppsala, Sweden). *E. coli* XL1-blue cells containing expression vector pGEX-ALS were provided by Dr. Soo-Ik Chang (Chungbuk National University, Cheongju, Korea). Oligonucleotides were obtained from Jenotech (Taejon, Korea). Londax (a sulfonylurea herbicide) and Cadre (an imidazolinone herbicide) were kindly provided by Dr. Dae-Whang Kim (Korea Research Institute of Chemical Technology, Taejon, Korea). TP, a triazolopyrimidine derivative, was obtained from Dr. Sung-Keon Namgoong (Seoul Women's University, Seoul, Korea).

Multiple sequence alignment of ALS sequences from plants. We aligned 17 ALS sequences of plants using ClustalW program [26], which was integrated in BIOEDIT Software [27] provided by North Carolina State University.

Database set consists of ALS sequences from following species: *Amaranthus powellii*, *Amaranthus retroflexus*, *Amaranthus* spp., *A. thaliana*, *B. napus* (ALS I, ALS II, and ALS III), *Bassia scoparia*, *Cyanidium caldarium*, *Guillardia theta*, *Nicotiana tabacum* (SuRA and SuRB), *Porphyra purpurea*, *Raphanus raphanistrum*, *Spirulina platensis*, *Solanum ptychanthum*, and *Volvox carteri.* All sequences were obtained from GenBank database and the ALS sequence of *B. scoparia* was from a biotype that is resistant to herbicides [28].

Site-directed mutagenesis. Site-directed mutagenesis of tobacco ALS was performed directly on the plasmid derived from pGEX-2T containing tobacco ALS cDNA, using the PCR megaprimer method [29]. All manipulations of the DNA were carried out using the technique reported previously [30]. The PCR was also performed as described by Saiki et al. [31]. The first PCR was carried out with oligonucleotide primer NKB2 and each mutagenic fragment as internal primers with the underlined bases changed:

NKB2, 5′-CCCG**GGATCC**TCAAAGTCAATA-3′
M350C, 5′-GTTGGGTTGTCATGGTACTGTTTA-3′
M350V, 5′-GTTGGGTGTGCATGGTACTGTTTA-3′
M512C, 5′-GGAGCATGCGGATTTGGTTTG-3′
M512V, 5′-GGAGCAGTTGGATTTGGTTTG-3′
M569A, 5′-GGGAGCGGTGGTTCAATGGGA-3′
M569C, 5′-GGGATGCGTGGTTCAATGGG-3′
M569V, 5′-GGGAGTAGTGGTTCAATGGGA-3′

The bold bases in the NKB2 primer are *Bam*HI restriction sites. Each reaction mixture contained 50 ng of template DNA, 25 pmol of mutagenic primer, and universal primer NKB2, 200 μM dNTPs, and *Taq* polymerase in 50 mM KCl, 10 mM Tris (pH 7.5), and 1.5 mM $MgCl_2$ in 100 μl. The resulting DNA was subjected to a second PCR with the universal primer NKB1 5′-CATCTCC**GGATCC**ATGTCCACTACC CAA-3′. The PCR products were double digested with *Nco*I and *Bgl*II, and cloned into the expression vector, which was prepared from the *Nco*I/*Bgl*II-excised pGEX-wALS. The resulting pGEX-mALS was used to transform the *E. coli* strain XL1-Blue cells using standard $CaCl_2$ transformation instructions [30]. Each transformant was identified by digestion of plasmid with *Bam*HI, transformants giving correct restriction map were sequenced to ensure the correct base mutation in the mutant ALS gene. *E. coli* BL21-DE3 cells carrying correct mutant ALS plasmid were cultured to obtain the mutant protein.

DNA sequence analysis. DNA sequencing was carried out by the dideoxy chain-termination procedure [32] (sequencing service provided by Macrogen, Seoul, Korea). Each mutant ALS was sequenced and identified.

Expression and purification of tobacco wALS and mALS. Bacterial strains of *E. coli* BL21-DE3 cells containing the expression vector pGEX-ALS were grown at 37 °C in Luria–Bertani (LB) medium containing 50 μg/ml ampicillin to an OD_{600} of 0.7–0.8. Expression of the pGEX-ALS gene was induced by adding 0.1–0.3 mM isopropyl-D-thiogalactoside (IPTG). Cells were grown for an additional 3 h at 30 °C and harvested by centrifugation at 5000*g* for 30 min. Purification of wALS and mALS was carried out as described previously by Chang et al. [17]. The cell pellets were suspended with the standard buffer (50 mM Tris–HCl, pH 7.5, 1 mM pyruvate, 10% (v/v) ethylene glycol, and 10 mM $MgCl_2$) containing protease inhibitors (2 μg/ml leupeptin, 4 μg/ml aprotinin, and 2 μg/ml pepstatin A). The cell suspension was then lysed by sonication at 4 °C. The homogenate was centrifuged at 20,000*g* for 20 min and the supernatant was re-centrifuged. The supernatant was applied to the GSH–coupled Sepharose 6B column and unbound proteins were removed by washing with the standard buffer. Then, the GST-ALS fusion protein was recovered from the column with an elution buffer (50 mM Tris–HCl, pH 7.5, 15 mM GSH, and 10% (v/v) ethylene glycol). To obtain the cleaved ALS, the purified

GST-ALS was incubated overnight at 4 °C with thrombin (10 U/mg protein). The ALS was purified by an additional step of GSH-affinity chromatography. The isolated protein was identified by SDS–PAGE analysis [33] and the protein concentration was determined by the method of Bradford [34].

Enzyme assay. Enzyme activities of the purified wALS and mALS were measured according to the method of Westerfeld [35] with a modification as reported previously [36]. The reaction mixture contained a 50 mM potassium phosphate buffer (pH 7.5), 1 mM TPP, 10 mM $MgCl_2$, 20 μM FAD, 100 mM pyruvate, and the enzyme in the absence or presence of various concentrations of inhibitors. Assay was terminated by adding 6 N H_2SO_4 and then the reaction product acetolactate was allowed to decarboxylate. The acetoin formed by acidification was colorized with 0.5% creatine and 5% α-naphthol. The absorbance of the reaction mixture was determined at 525 nm. The continuous enzyme inhibition assay was carried out at 20 °C. The consumption of pyruvate was measured by monitoring the change in absorbance at 333 nm and data were collected at 1-min interval [37].

Spectroscopic measurement. Absorption spectra were recorded on Beckman DU-600 Spectrophotometer. The protein solution was dispensed in 1 ml black-walled quartz cuvettes and the spectrum of each sample was scanned over the range of 250–550 nm. Fluorescence emission spectra were recorded with a Perkin–Elmer Luminescence Spectrophotometer LS50B. The fluorescence spectra of FAD bound to wALS and mALS were scanned over the range of 450–650 nm by exciting at 450 nm. The CD spectra were recorded over the range of 200–250 nm on a Jasco J-710 Spectropolarimeter set at 20–50 mdeg sensitivity, 1 mm resolution, 3 U accumulation, 5 s response, and at a scanning speed of 200 nm/min. A protein solution of 0.3–0.8 mg/ml was assayed in a 1-mm path length cylindrical quartz cell.

Results

Identification of conserved methionines

The alignment of 17 ALS sequences from plants (see Materials and methods for details) had revealed 149 identical residues (data not shown). Of these, there were seven identical methionine residues, namely M332, M347, M350, M489, M512, M542, and M569 (numbering according to tobacco sequence). These residues were found in high consensus regions (data not shown). In this paper we report the site-directed mutagenesis study on three of the identical residues: M350, M512, and M569.

Expression and purification of tobacco ALS

All mutants were expressed and purified successfully to homogeneity by two steps of affinity chromatography, which employed a GSH-coupled 6B Sepharose column (see Materials and methods for details). SDS–PAGE data showed that all mutants had the same molecular mass as that of wild-type enzyme (Fig. 1).

Kinetic properties of wALS and mALS

The mutant and wild-type enzymes were characterized in term of kinetic parameters, including V_{max}, K_m, K_c for FAD, TPP, and K_i^{app} of three herbicides, Londax, Cadre,

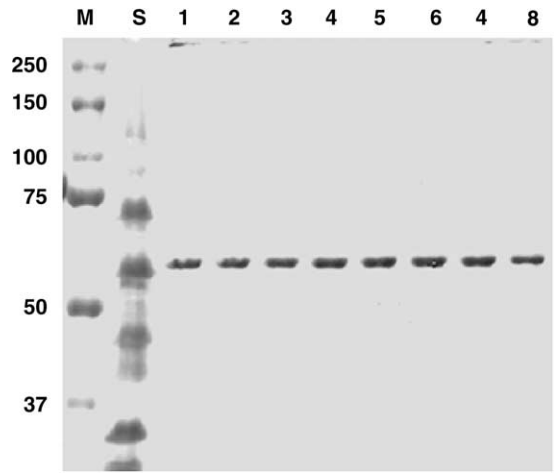

Fig. 1. SDS–PAGE of wild-type and mutant enzymes. Each sample was electrophoresed on a 10% polyacrylamide gel containing SDS and the gel was stained with Coomassie blue. M, molecular markers, 250, 150, 100, 75, 50, and 37 kDa; S, cell lysate; 1, wALS; 2, M350C; 3, M350V; 4, M512C; 5, M512V; 6, M569A; 7, M569C; and 8, M569V.

and TP. The substrate and cofactor saturation curves for wALS and mutants, M350C, M512C, and M569C were hyperbolic (data not shown) as reported previously for wALS [20]. The values of V_{max}, and K_m for the substrate were determined by fitting the data to Eq. (1), while the values of activation constant (K_c) were obtained by fitting the data to Eq. (2), by the non-linear least-squares and Simplex methods for error minimization [38]:

$$v = V_{max}/(1 + K_m/[S]), \quad (1)$$

$$v = V_0 + V_{max}/(1 + K_c/[C]). \quad (2)$$

In these equations, v is the reaction velocity, V_{max} is the maximum velocity, V_0 is the activity without adding cofactors, K_m is Michaelis–Menten constant, K_c is activation constant, $[S]$ is substrate concentration, and $[C]$ is added cofactor concentration. Table 1 shows the values of V_{max}, K_m, and K_c for the cofactors, and K_i^{app} for the inhibition by herbicides. Since all valine-substituted and M569A mutants were inactive at various assay conditions, it was not possible to measure the kinetic parameters for substrate and cofactors as well as herbicides. The K_m values for pyruvate of M350C, M512C, and M569C were 56.69, 75.27, and 361.48 mM, respectively. The K_c values of these mutants for FAD were greatly different, while those values for TPP were not significantly different.

The pH-dependent activities of M350C and M569C mutants were also determined along with that of wild type. As shown in Fig. 2, the pH-dependent activity profile of M569C mutant overlapped with that of the wild-type enzyme, while M350C mutant had a sharper profile. These data suggest that substitution of M350 by cysteine results in a mutant whose activity is more sensitive to pH change.

Table 1
Kinetic properties of wild-type and mutant enzymes

	K_m (mM)	V_{max} (U/mg protein)	K_{FAD} (μM)	K_{TPP} (mM)	IC_{50}		
					Cardre (μM)	Londax (nM)	TP (μM)
wALS	3.317	0.427	1.26	0.47	1.96	26.72	36.34
M350C	56.695	0.031	51.54	0.83	ND	907.44	ND
M350V	No enzymatic activity						
M512C	75.27	0.29	4.06	0.17	20.59	28.59	4.52
M512V	No enzymatic activity						
M569A	No enzymatic activity						
M569C	361.48	0.017	26.28	0.81	113.73	ND	ND
M569V	No enzymatic activity						

TP, a newly synthesized triazolopyrimidine; ND, not detectable within the range of 0–128 μM.

Spectral properties of wALS and mALSs

Although expressed as intact proteins (Fig. 1), the valine- and alanine-substituted mutants did not give any detectable activity under various assay conditions. To understand further the mechanism of inactivation, we measured the absorption and fluorescence spectra of wild-type and mutant enzymes. The data obtained showed that none of the alanine- and valine-substituted mutants contained any trace of FAD (Fig. 3 and data not shown). In contrast, all cysteine-substituted mutants showed a trace of FAD. The presence of FAD in M350C and M569C mutants was not clearly visible by absorption spectra (Fig. 3); however, it was detected on fluorescence spectra (Fig. 3, inset).

To determine the changes in secondary structure of the mutants, the CD spectra of wild-type and mutant enzymes were measured. Fig. 4 shows that the CD spectra of M350V, M512V, and M569V mutants almost completely overlapped with that of wild type. However, the CD spectrum of M569A mutant substantially differed from that of wild type. Hence, it seems that the substitution of M569 by alanine perturbed the correct conformation of the enzyme.

Inhibition of ALS by herbicides

The sensitivities of three mutants, M350C, M512C, and M569C to herbicides, were determined for three classes of herbicides, Londax (a sulfonylurea), Cadre (an imidazolinone), and TP (a triazolopyrimidine). The K_i^{app} were determined by fitting the data to the following equation:

$$v_i = v_0/(1 + [I]/K_i^{app}). \quad (3)$$

In this equation, v_i and v_0 represent the rates in the presence or absence of the inhibitor, respectively, and $[I]$ is the concentration of the inhibitor. The K_i^{app} is the apparent K_i that is the concentration of the inhibitor giving 50% inhibition under a standard assay condition, which is also known as IC_{50}. M350C and M569C mutants were highly resistant to the three tested classes of herbicides. However, the M512C mutant was weakly resistant only to Cardre, not resistant to Londax, and

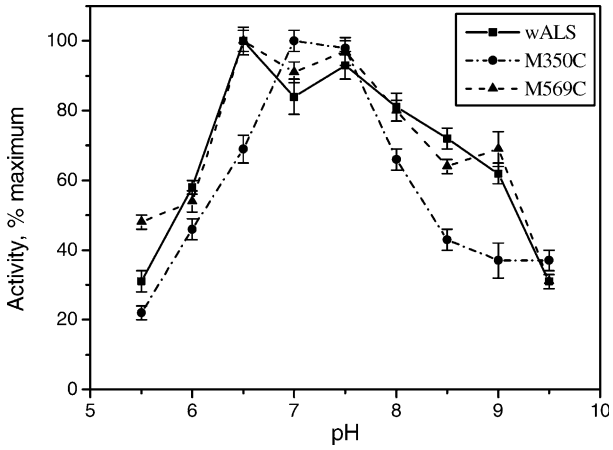

Fig. 2. Activity of wild-type and mutant enzymes as a function of pH (arbitrary unit). The enzyme assay was carried out as described in Materials and methods with buffer at following pH values: 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5.

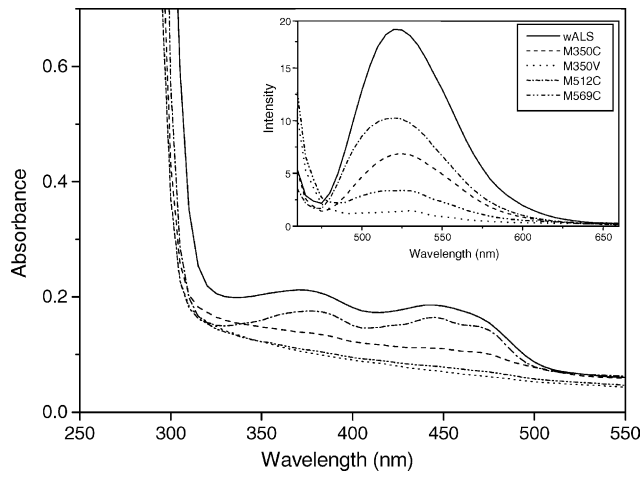

Fig. 3. Absorption and fluorescence spectra of wild-type and mutant enzymes. The concentration of each enzyme was 1.2 mg protein/ml in 50 mM Tris–Cl buffer (pH 7.5).

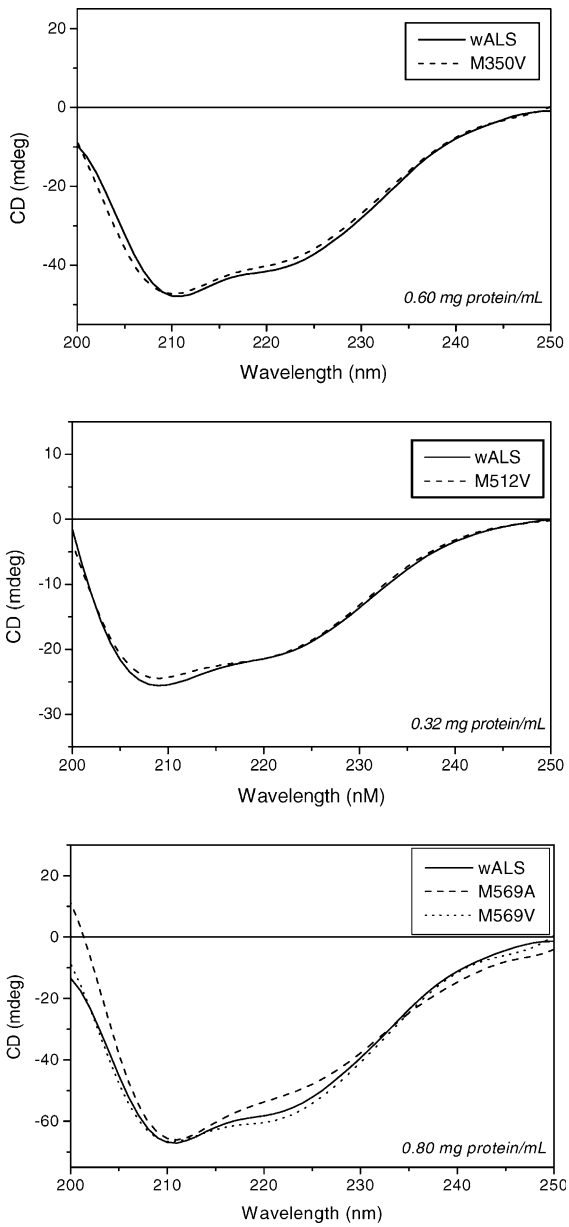

Fig. 4. CD spectra of wild-type and mutant enzymes. Each protein was present at a concentration ranging from 0.32 to 0.80 mg/ml in 10 mM potassium phosphate buffer (pH 7.5).

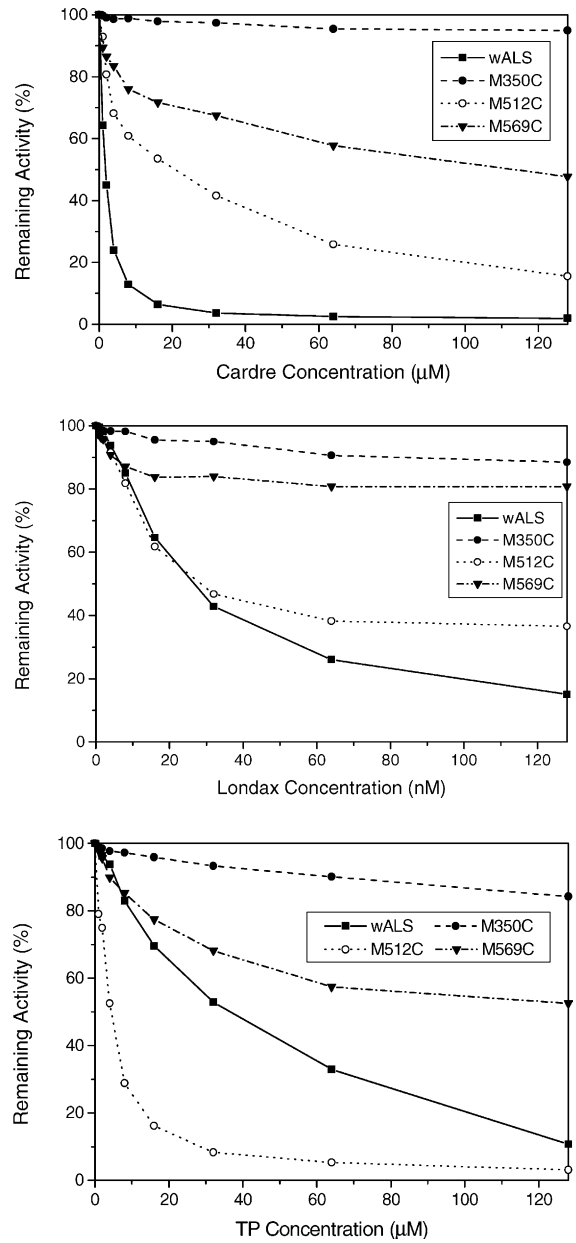

Fig. 5. Effects of three classes of herbicides (the sulfonylurea Londax, the imidazolinone Cardre, and the triazolopyrimidine TP) on wild-type and mutant enzymes.

7-fold more sensitive to TP than that of wild-type enzyme (Table 1, Fig. 5). The continuous inhibition assay of M512C mutant yielded results consistent with those obtained from discontinuous assay (Fig. 6). Furthermore, the activity inhibition of M512 mutant by TP also follows slow-acting mechanism, similar to that reported for wild-type plant ALS [39,40].

Discussion

Bartlett et al. [25] reported that among 178 structurally known enzymes, more than 2% of the total residues are methionines and 1% of the catalytic residues are methionines. To determine the methionine residues in ALS that are involved in catalytic reaction or herbicide binding, we identified all the conserved methionine residues by multiple sequence alignment using ClustalW (see Materials and methods for details). We found 149 identical residues, in which seven residues were methionine (M332, M347, M350, M489, M512, M542, and M569; tobacco numbering), these methionine residues were found to be located in high consensus regions. The results also agreed with those reported by Duggleby and Pang [1] with their dataset which consisted of ALS sequences from plants, yeast, and bacteria. However, in

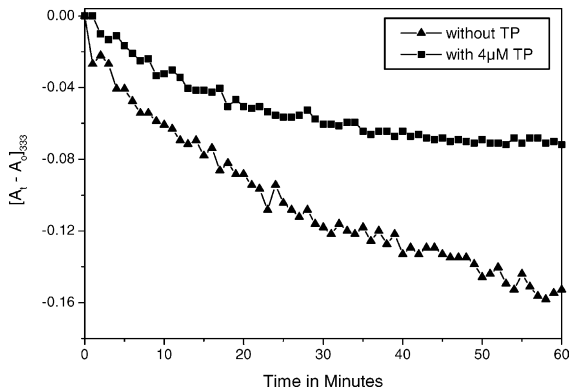

Fig. 6. Continuous assay showed that TP at concentration of 4 μM inhibits over 50% of M512C activity. The assay mixture was same as colorimetric discontinuous method; the mixture was incubated at 20 °C and the change in A_{333} was recorded at 1-min interval.

their report, only six of the above-mentioned residues were identified as highly conserved residues.

To explore the functional roles of M350, M512, and M569 residues on recombinant tobacco acetolactate synthase, site-directed mutagenesis was carried out. Seven mutants of tobacco ALS were successfully generated and expressed as soluble forms, and purified to homogeneity.

Though expressed as intact proteins, all the ALS mutants substituted by valine or alanine appeared to be inactive under various assay conditions. Thus, it was not possible to measure kinetic parameters. To understand the mechanism of inactivation of valine- and alanine-substituted mutants, the absorption and fluorescence spectra of wild-type and mutant enzymes were determined. The results (Fig. 3 and data not shown) showed that all mutants substituted by valine or alanine did not give any absorption and fluorescence emission peaks corresponding to FAD bound to mutant ALS, in contrast to wild-type ALS [20–22]. On the other hand, all the mutants substituted by cysteine showed fluorescence and absorption peaks attributable to bound FAD. It appears that the substitutions by valine or alanine abolish the binding affinity of the enzyme for FAD with a loss of catalytic function. Moreover, the CD spectra of all valine-substitution mutants completely overlapped with that of the wild-type enzyme (Fig. 4). Thus, the inactivation by the substitution of methionine by valine is not due to the change of secondary structure of the ALS. The CD spectrum of M569A mutant was substantially different from that of wild type, indicating that substitution by alanine disturbs the native conformation of secondary structure of the enzyme.

Interestingly, all the mutants substituted by cysteine partly retained the enzyme activity toward pyruvate substrate. Each of the mutant enzymes was characterized with V_{max}, K_m, K_c for FAD and TPP, and K_i^{app} for the three herbicides, Londax, Cadre, and TP (Table 1). Since only those substitutions by cysteine retained the enzyme activity, the possibility that the sulfur atom in the side chain plays a role in the catalytic function of the enzyme is not excluded.

Since the non-ionizable side chain of methionine was replaced by the ionizable group of cysteine, we were interested in examining how it would affect the pH-dependent activity. The activities of M350C, M569C mutants, and wild-type enzyme were measured at various pH. Fig. 2 shows that the pH optima for catalytic activity of tobacco ALS are in the broad range from pH 6.5–7.5. The pH-activity profile of M569C mutant nearly completely overlapped with that of the wild-type ALS. On the other hand, the M350C mutant showed a narrower range of pH optima (pH 7.0–7.5) and its activity fell rapidly when pH was out of its optimum range (Fig. 2). This indicated that the M350C mutant was more sensitive to pH. Recently, Huang et al. [37] reported that substitution of ionizable groups in the active site of pyruvate carboxylase from *Zymomonas mobilis* resulted in a change in pH dependence activity profile.

Recently, ALS was identified as a potential target for various classes of herbicides [2–6]. Therefore, the inhibition of three mutant ALSs by the three classes of herbicides, Londax (a sulfonylurea), Cadre (an imidazolinone), and TP (a triazolopyrimidine) was determined. The two mutants M350C and M569C were strongly resistant to the three tested herbicides (Fig. 5). Since the CD spectra of these mutants were almost identical to that of wild type (data not shown), the herbicide resistance was conferred by the substituted amino acid rather than a conformational change. In contrast to these two mutants, M512C mutant did not show a significant resistance to the three tested herbicides. In fact, it was 7-fold more sensitive to TP than wild type (Table 1 and Fig. 5).

Previously, the molecular model for *Arabidopsis* ALS proposed by Ott et al. [16] based on sequence homology to pyruvate oxidase and mutation study suggested that the binding site(s) for herbicides is near to the entry site of substrate and in close proximity to the bound cofactors TPP and FAD. The results from this study that the mutations of M350V and M569V inactivated the enzyme and two mutants M350C and M569C were resistant to the three herbicides support this proposed model. Accordingly, we could propose that the binding sites for the three classes of herbicides are partially overlapping, and the active site and herbicide-binding sites of the ALS also partially overlap.

The present results suggest that M512 residue is likely located at or near the active site of tobacco ALS and that M350 and M569 residues are probably located at the overlapping region between the active site and a common binding site for the three classes of herbicides.

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