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Role of Tryptophanyl Residues in Tobacco Acetolactate Synthase

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Acetolactate synthase (ALS) catalyzes the first common step in the biosynthesis of valine, leucine, and isoleucine. ALS is the target of three classes of herbicides, the sulfonylureas, the imidazolinones, and the triazolopyrimidines. Five mutants (W266F, W439F, W490F, W503F, and W573F) of the ALS gene from *Nicotiana tabacum* were constructed and expressed in *Escherichia coli*, and the enzymes were purified. The W490F mutation abolished the binding affinity for cofactor FAD and inactivated the enzyme. The replacement of Trp573 by Phe yielded a mutant ALS resistant to the three classes of herbicides. The other three mutations, W266F, W439F, and W503F, did not significantly affect the enzymatic properties and the sensitivity to the herbicides. These results indicate that the Trp490 residue is essential for the binding of FAD and that Trp573 is located at the herbicide binding site. The data also suggest that the three classes of herbicides bind ALS competitively. © 1999 Academic Press

Acetolactate synthase (ALS, EC 4.13.18; also referred to as acetohydroxy acid synthase) catalyzes the first common step in the biosynthesis of the branched-chain amino acids, valine, leucine and isoleucine, in plants and microorganisms. ALS can either catalyze the condensation of 2-acetolactate from two molecules of pyruvate in the first step of the valine and leucine synthetic pathway, or the formation of 2-aceto-2-hydroxybutyrate from pyruvate and 2-ketobutyrate in the second step of isoleucine biosynthesis. ALS requires cofactors including thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), and a divalent metal ion, Mg^{2+} or Mn^{2+}, for its catalytic activity. Requirement of FAD is an unusual feature of ALS, since the reaction catalyzed involves no redox step. The enzyme is a key controlling point for the level of branched-chain amino acids in both prokaryotes and eukaryotes. The control involves feedback inhibition of enzyme activity by end-product amino acids or repression of the synthesis of the enzyme or both (1–3). Strong interest has been raised in ALS since several structurally unrelated classes of herbicides, including the sulfonylureas (4, 5), the imidazolinones (6), the triazolopyrimidines (7, 8), and the pyrimidyl-oxybenzoates (9, 10), have been shown to specifically inhibit the enzyme. In bacteria, three ALS isozymes differing in substrate preference and feedback regulation have been identified and well characterized (11, 12). Bacterial ALS is a tetramer composed of two large catalytic subunits (59–60 kDa) and two small regulatory subunits (9–17 kDa) (12). The low abundance and labile nature of plant ALS have severely hampered the purification and biochemical characterization of the enzyme. So far, only the enzymes from barley (13, 14) and wheat (15) have been obtained in apparently pure forms. The ALS genes from *Arabidopsis thaliana* (16) and tobacco (17) have been functionally expressed in *E. coli* as glutathione S-transferase fusion proteins, and the enzymes have been purified. Herbicide-resistant ALS genes from *A. thaliana* have been constructed, expressed in *E. coli*, and the enzymes have been purified (18). In the past, commercial crops resistant to ALS-inhibiting herbicides have been obtained by physical selection of herbicide-insensitive plants. The studies of mutants have shown that several sites can lead to herbicide resistance. The most frequent mutation is at W464F (*E. coli* ALS II numbering) which has been found in yeast (29), oliseedrape (30), tobacco (28) and cocklebur ALS (31), and has been introduced into *E. coli* (32). A second resistant site identified in several organisms is at position 100, having been found in *A. thaliana* (33, 34), yeast (35, 36) and tobacco (29, 37). The mutation of Pro196 to Gln in ALS from *N. tobacum Su*RA-C3 resulted in an insensitivity to the sulfonylurea herbicide (29). In addition, two-point mutation of

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Abbreviations used: ALS, acetolactate synthase; GST, glutathione *S*-transferase; GSH, glutathione; PCR, polymerase chain reaction; wALS, wild-type ALS; mALS, mutant ALS; IPTG, isopropyl-β-D-thiogalactoside; EPPS, *N*-(2-hydroxyethyl) piperazine-*N*′-3-propanesulfonic acid, TPP, thiamin pyrophosphate; FAD, flavin adenine dinucleotide; TP, a triazolopyrimidine sulfonamide.

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Pro196 by Ala and Trp573 by Leu confer resistance to the sulfonylureas and the imidazolinones for ALS from another tobacco mutant *Su*RB-S4 HRA (29). Recently, chemical modification studies have elucidated that one cysteinyl residue and one tryptophanyl residue are essential for catalytic function in the recombinant tobacco ALS (19).

In this report, we described site-directed mutagenesis of tryptophanyl residues in tobacco ALS and biochemical properties of the mutant enzymes.

MATERIALS AND METHODS

Materials. Bacto-tryptone, yeast extract, and Bacto-agar were purchased from Difco Laboratories (Detroit, MI). Restriction and modifying enzymes were from TaKaRa SHUZO Co. (Shiga, Japan) and Boehringer-Mannheim (Mannheim, Germany). Thrombin protease and epoxy-activated Sephrose 6B were obtained from Pharmacia Biotech (Uppsala, Sweden). GSH, Sephadex G-25, TPP, FAD, α-naphthol, and creatine were purchased from Sigma Chemical Co. (St. Louis, MO). Oligonucleotides were obtained from JENOTECH (Taejeon, Korea). The plasmid pSB1 containing the wild-type tobacco ALS *Su*RB gene was provided by Dr. B. J. Mazur (E. I. DuPont de Nemours Co., Wilmington, USA). The expression vector pGEX-2T was obtained from Dr. Sang-Ki Paik (Chungnam National University, Taejon, Korea). Londax, a sulfonylurea herbicide, and Cadre, an imidazolinone herbicide, were provided by Dr. Dae-Whang Kim (Korea Research Institute of Chemical Technology, Taejon, Korea). TP, a triazolopyrimidine sulfonamide, was obtained from Dr. Sung Keon Namgoong (Seoul Women's University, Seoul, Korea).

Site-directed mutagenesis of tobacco ALS. Site-directed mutagenesis was performed directly on the plasmid derived from pGEX-2T containing tobacco ALS cDNA. All manipulations of the DNA were carried out using standard techniques (20). The first polymerase chain reaction (PCR) was carried out with the oligonucleotide primer NKB2 and each mutagenic fragment as internal primers, with the underlined bases changed: NKB2, 5′-CCCGGGGATCCTCAAAGTCAATAGG-3′.

BamHI. W266F, 5′-ACCTGACTTCGATCAGCC-3′; W439F, 5′-TTCTGCTTTCAGGCAGGA-3′; W490F, 5′-CCAGATGTTCGCTGCTCA-3′; W503F, 5′-ACGCCAATTCTTGACATC-3′; W573F, 5′-GGTGGTTCAATTCGAGGATC-3′. PCR was carried out as described previously (21). Each reaction mixture contained 50 ng of template DNA, 90 pmol of mutagenic primer and universal primer NKB2, 200 μM dNTPs in 50 mM KCl, 10 mM Tris (pH 8.3) and 1.5 mM $MgCl_2$ in 100 μl. Each reaction was performed for 30 cycles of the following programs: 94°C, 1 min; 55°C, 1 min; 72°C, 2 min. The resulting DNA was subjected to the second PCR with the universal primer NKB1, 5′-CATCTCCGGATCCATGTCCACTACCCAA-3′.

BamHI. PCR products were double digested with *Nco*I and *Bgl*II, isolated from 1% agarose gel, and cloned into the expression vector which was prepared from the *Nco*I/*Bgl*II-excised pGEX-*w*ALS. The resulting pGEX-*m*ALS was used to transform *E. coli* strain XL1 Blue MRF′ cell using a standard $CaCl_2$ transformation instruction (20). Each transformant was sequenced to ensure the correct base mutation in the *m*ALS gene, and cultured to obtain the mutant protein.

DNA sequence analysis. Sequencing was carried out by the dideoxy chain-termination procedure (21). Each mutant ALS gene was sequenced and identified.

Expression and purification of wALS and mALS. Bacterial strains of *E. coli* XL1-Blue cells were transformed by expression vector encoding mutant ALS gene and GST. Cells were then grown aerobically at 37°C in Luria-Bertani (LB) medium to an OD_{600} of 0.7–0.8. Cells were induced with 0.3 mM isopropyl-β-D-thiogalactoside (IPTG), and were cultured additionally for 4 h at 30°C. Cells were harvested by centrifugation at 5000*g* for 15 min. Purification of wALS and mALS was performed as described by Chang *et al.* (19). The harvested cells were resuspended with the standard buffer (50 mM EPPS, pH 7.5, 10% (v/v) ethylene glycol, 1 mM pyruvate, 10 mM $MgCl_2$) containing 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 on a GSH-coupled Sepharose 6B column preequilibrated with the standard buffer. After removing unbound proteins by washing with sufficient volume of the standard buffer, the GST-ALS fusion protein was recovered from the column with the elution buffer (50 mM Tris–HCl, pH 7.5, 15 mM GSH, 10% ethylene glycol). To obtain the pure ALS, the fusion protein desalted by Sephadex G-25 was digested with thrombin protease (10 U/mg protein) overnight at 4°C. The ALS was purified by another GSH-affinity chromatography step. The isolated proteins were identified by SDS–PAGE analysis (22), with protein concentration determined by the method of Bradford (23).

Enzyme assay. The ALS activity was measured according to the method of Westerfeld (24) with a modification as previously reported (14). The assay solution contained 20 mM potassium phosphate (pH 7.0), 0.5 mM TPP, 10 μM FAD, 20 mM pyruvate, 50 mM $MgCl_2$, and the enzyme in the presence or absence of various concentrations of inhibitors in a final volume of 200 μl. After incubating at 37°C for 1 h, the enzyme reaction was stopped by the addition of 20 μl of 6 N H_2SO_4, and the reaction product acetolactate was allowed to decarboxylate at 60°C for 15 min. The acetoin formed by acidification was incubated with 200 μl of 0.5% creatine and 200 μl of 5% α-naphthol at 60°C for 15 min. Then, the absorbance of the colorized mixture was measured at 525 nm. One unit (U) of activity was defined as the amount required to form 1 μmol of acetolactate per minute under the assay conditions as described above. Specific activities of *w*ALS and *m*ALS were expressed as units (U) per mg protein. The values of K_m and V_{max} were calculated by the least-squares fitting of the data to the equation using the nonlinear regression method.

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

Spectroscopic measurements. Absorption spectra were recorded on a Beckman DU650 UV/VIS spectrophotometer. Protein solutions were dispensed into a 1-ml black-walled quartz cuvette, and the absorbances of each sample were measured over the range 250–550 nm. Fluorescence emission spectra were recorded with a HITACHI F-3000 fluorescence spectrophotometer. The fluorescence of FAD in *w*ALS and W490F in 25 mM Tris–HCl buffer was measured by excitation at 450 nm. Emission spectra were scanned over the range of 400 to 700 nm under the same conditions.

RESULTS

Expression and purification of tobacco ALS. We cloned mutants (W266F, W439F, W490F, W503F, and W573F) of the tobacco ALS gene containing part of the chloroplast transit peptide into the bacterial expression plasmid pGEX-2T. Each of the ALS mutants was expressed in *E. coli* as a GST-ALS fusion protein. Expression was induced by the addition of 0.3 mM IPTG. Each of the mutants was successfully expressed, and all the proteins were soluble, as judged by SDS–PAGE (data not shown). Each of the resulting GST-ALS fusion proteins was purified to near homogeneity in a single step by GSH-Sepharose 6B affinity chromatography. The purified GST-ALS was subjected to diges-

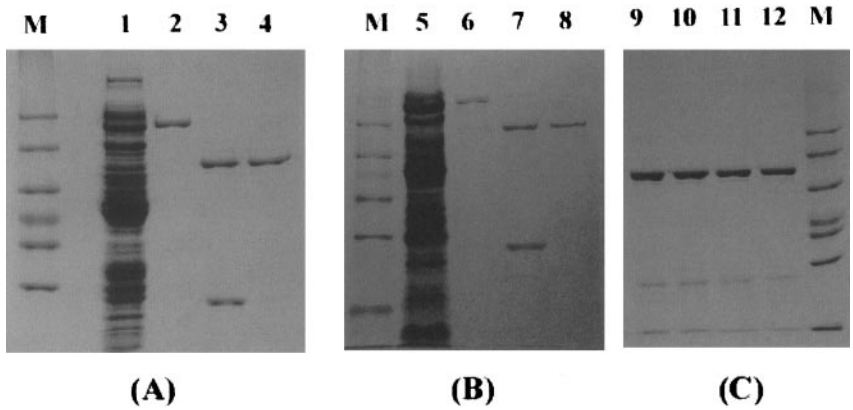

FIG. 1. Purification of *w*ALS and each *m*ALS from *E. coli* transformed with pGEX-ALS2 after induction of the *tac* promotor by 0.3 mM IPTG, as described under Materials and Methods. Samples were electrophoresed on 10% polyacrylamide gels containing SDS, then gels were stained with Coomassie blue. Molecular markers indicate 97.4, 66.2, 55.0, 42.7, 31.0, and 21.5 kDa molecular masses from the top, respectively. (A) Purification of *w*ALS. M, molecular marker; lane 1, soluble cell lysate; lane 2, GST-*w*ALS fusion protein; lane 3, thrombin-digested fusion protein; lane 4, purified *w*ALS. (B) Purification of W490F. M, molecular marker; lane 5, soluble cell lysate; lane 6, GST-W490F; lane 7, thrombin-digested fusion protein; lane 8, purified W490F. (C) Purification of *m*ALS. Lane 9, purified W266F; lane 10, purified W439F; lane 11, purified W503F; lane 12, purified W573F; M, molecular marker.

tion with thrombin protease for 16 h at 4°C. The cleaved ALS enzyme was purified to homogeneity by an additional GSH-affinity chromatography step. A single band at 65 kDa, corresponding to ALS, was apparent on SDS–PAGE (Fig. 1).

Kinetics properties of tobacco ALS. The kinetic properties of tobacco ALS were determined using purified ALS which was not fused with GST. Substrate saturation curves for wild-type and three mutant enzymes, W266F, W439F, and W503F, were hyperbolic as reported previously for wild-type ALS (25), but the saturation curve for W573F somewhat deviated from a hyperbolic curve (data not shown). The mutant W490F showed no detectable enzymatic activity when it was assayed under the standard assay conditions described under Material and Methods or under other conditions of longer incubation time and higher substrate concentration. Table I shows the kinetic data of K_m and V_{max} for *w*ALS and *m*ALS obtained by fitting data to Eq. [1] by nonlinear least-squares and the Simplex method for error minimization (26). Among the three mutants, W266F, W439F, and W503F, the values of K_m are somewhat different (0.5- to 3-fold) from that of the wild type. The differences in K_m are not large enough, indicating that the substrate-binding site is not substantially perturbed by the mutations. However, the K_m value for W573F is approximately 20-fold higher than that of wild-type. This mutation seriously affected substrate-binding. The values of V_{max} (U/mg protein) for the four mutants except for W490F showed some differences from the wild-type value, but it is not clear whether the differences were due to the intrinsic properties of the mutants or due to varying stabilities of the mutants.

W490F mutant. Although the W490F mutant appeared to be expressed and purified normally as the other ALS enzymes (Fig. 1B), it showed no detectable enzymatic activity under various assay conditions. To

TABLE I

Kinetic Parameters and IC_{50} Values of Wild-Type ALS and Mutant ALS

	K_m for pyruvate (mM)	V_{max} (U/mg protein)	IC_{50} value for inhibitors		
			Londax (nM)	Cadre (μM)	TP[a] (nM)
Wild-type	20.8	2.07	5.67 ± 0.33	2.24 ± 0.11	2.77 ± 0.27
W266F	62.6	3.82	6.93 ± 0.81	2.54 ± 0.12	4.86 ± 0.48
W439F	12.3	0.90	6.07 ± 0.05	2.72 ± 0.20	3.06 ± 1.48
W490F			No enzymatic activity		
W503F	12.7	1.48	27.25 ± 4.43	3.91 ± 0.37	18.8 ± 2.33
W573F	407.0	0.79	N.D.[b]	N.D.	N.D.

[a] TP, a newly synthesized derivative of triazolopyrimidine sulfonamide.
[b] N.D., not detected (≤2 μM Londax, ≤1 mM Cadre, ≤2 μM TP).

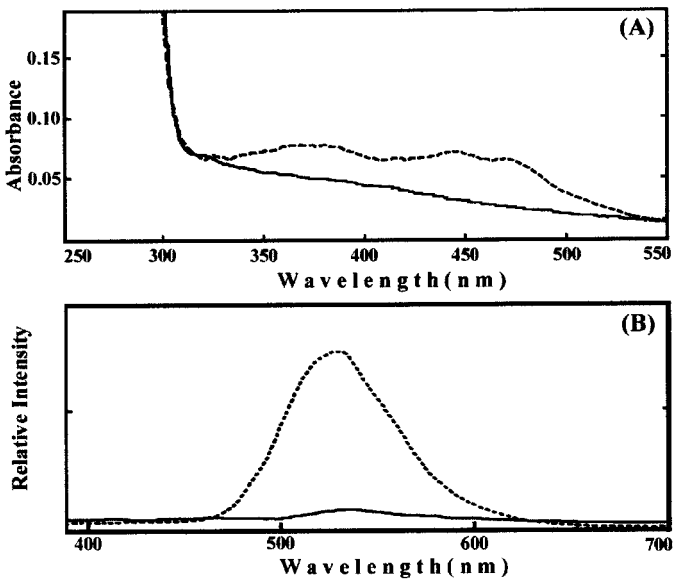

FIG. 2. Absorption (A) and fluorescence (B) spectra of wALS and W490F. Each protein was present at a concentration of 7 μM in 25 mM Tris–HCl buffer (pH 7.5). Wild-type protein is indicated by the broken line whereas the W490F mutant is indicated by the solid line.

confirm an essential role of Trp490 in catalytic function, the reverse mutation from W490F to F490W was carried out. Interestingly, the enzymatic activity was fully regenerated in the re-mutagenized enzyme (data not shown). The results indicate that Trp490 is located at the active site of the enzyme and is critical for enzymatic activity. Since Trp has a nonpolar aromatic side chain, Trp490 is probably involved in the binding of cofactor FAD or TPP. To determine the binding of FAD to the mutant W490F, spectral measurements for wild-type and mutant ALS were carried out. Although the absorption spectrum of FAD bound to wild-type ALS was not well resolved, two peaks around 370–390 and 450–480 nm were evident, consistent with the spectrum reported previously for bacterial ALS II (Fig. 2A) (27). In contrast, the spectrum of W490F showed no peak in the region of 350–500 nm, superimposed on a background that rises progressively at lower wavelengths (Fig. 2A). This background may be attributed to light-scattering by protein aggregates. The fluorescence emission spectrum of wild-type ALS showed a peak around 530 nm with excitation at 450 nm, corresponding to the emission of FAD bound to ALS (Fig. 2B). However, the W490F mutant did not show any emission peak with excitation at 450 nm (Fig. 2B). Evidently, the W490F mutation caused a change in the conformation of ALS that impaired FAD binding, thus destroying the catalytic activity of the enzyme.

Interaction of ALS with herbicides. The activities of *w*ALS and *m*ALS were assayed in the presence of three representative kinds of herbicides, a sulfonylurea, Londax, an imidazolinone, Cadre, and a triazolopyrimidine, TP. The IC_{50} value for inhibition is defined as the concentration of inhibitor [In] that inhibits enzyme activity by 50% under the standard assay condition described:

$$\% \text{ Activity} = 100/(1 + [\text{In}]/IC_{50}), \qquad [2]$$

where % activity equals the activity in the presence of various inhibitor concentrations as a percent of the untreated control activity. IC_{50} values were calculated by non-linear least squares and Simplex methods for error minimization (26). As shown in Table I, the IC_{50} values for W266F and W439F are similar to those of wild-type ALS, but the values of W503F for the inhibition by Londax and TP are 5- and 7-fold higher than those of wild-type, respectively. The inhibition of W573F was not observed even in the presence of higher concentrations of herbicides, such as 2 μM Londax, 1 mM Cadre, and 2 μM TP. These results indicate that the W573F mutation confers a high level of resistance to the three classes of herbicides. Thus, Trp573 is critical for ALS in the binding of the herbicides.

DISCUSSION

Our recent chemical modification studies of tobacco ALS suggest that a Trp residue is probably located at or near the active site of the enzyme (21). Tobacco ALS contains only five Trp residues, as the DNA sequence of the ALS gene has already been determined (29). Thus, we performed point mutations of the Trp residues in tobacco ALS.

Each of the five mutants was characterized with respect to kinetic properties, including K_m, V_{max}, and IC_{50} for the three herbicides, Londax, Cadre, and TP (Table I). The K_m values for the three mutants, W266F, W439F, and W503F, are not substantially different from those for wild-type, suggesting that the substrate-binding site is not seriously perturbed by the mutations. On the other hand, the K_m value for W573F is much higher (~20-fold) than that of wild-type, suggesting that the mutation is near the substrate-binding site or its effect is transmitted to the site through a conformational change in the protein. Although the values of V_{max} for the four mutants except W490F are somewhat different from that of wild-type, the changes are not of such a magnitude to indicate that any of these residues is involved in catalytic activity. However, the mutant W490F did not show any detectable enzymatic activity. We propose that Trp490 is essential for the catalytic activity of tobacco ALS. This conclusion was confirmed by the observation that the enzymatic activity was fully regenerated by the reverse mutation from F490 to W490. The mutant W490F showed no absorption and fluorescence emission peaks corresponding to cofac-

tor FAD, indicating that the mutation of W490F prevents this cofactor from binding to ALS, with a consequent loss of enzymatic activity. Recently, this is supported by the report that the G249A mutant of *E. coli* ALS II obtained by random mutation became totally inactive due to the loss of its affinity for cofactor FAD (27).

We determined the inhibition of five mutant ALS by three classes of herbicides, a sulfonylurea, Londax, an imidazolinone, Cadre, and a triazolopyrimidine, TP. Each mutation of W266F and W439F showed a small effect on the sensitivity to the three herbicides. The mutation of W503F increased resistance to Londax and TP by 5-fold and 7-fold, respectively, but not to Cadre. The mutant W573F exhibited resistance to the three herbicides, Londax, Cadre, and TP. Taken together, these results strongly suggest a broad region for the binding sites of the three classes of herbicides. However, these sites do not substantially overlap; Trp573 is apparently located at the common binding site of the three herbicides.

We conclude that the Trp490 residue in tobacco ALS is essential for catalytic activity as a binding site for cofactor FAD, and that a single mutation of Trp573 by Phe can confer a high level of tolerance for the enzyme against the three chemical classes of herbicides.

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