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Roles of lysine 219 and 255 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. The ALS is the target of several classes of herbicides, including the sulfonylureas, the imidazolinones, and the triazolopyrimidines. The roles of three well-conserved lysine residues (K219, K255, K299) in tobacco ALS were determined using site-directed mutagenesis. The mutation of K219Q inactivated the enzyme and abolished the binding affinity for cofactor FAD. However, the secondary structure of the enzyme was not changed significantly by the mutation. Both mutants, K255F and K255Q, showed strong resistance to three classes of herbicides Londax (a sulfonylurea), Cadre (an imidazolinone), and TP (a triazolopyrimidine). In addition, there was no difference in the secondary structures of wALS and K255F. On the other hand, the mutation of K299Q did not show any significant effect on the kinetic properties or any sensitivity to the herbicides. These results suggest that Lys219 is located at the active site and is likely involved in the binding of FAD, and that Lys255 is located at a binding site common for the three herbicides in tobacco ALS.

Keywords: Acetolactate synthase; Tobacco; Site-directed mutagenesis; Herbicide; Resistance

The first enzymatic step common to the biosynthesis of branched-chain amino acids is catalyzed by acetolactate synthase (ALS, EC 4.1.3.12; also known as acetohydroxy acid synthase). The enzyme catalyzes two parallel reactions, the condensation of two molecules of pyruvate to give rise to 2-acetolactate in the first step of the valine and leucine synthetic pathway and 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, thiamin pyrophosphate (TPP), flavin adenine dinucleotide (FAD), and divalent metal ions, Mg^{2+} or Mn^{2+}. ALS has attracted much interest 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].

ALS activity is found in bacteria, yeast, and plants resulting from one or more isozymes. In bacteria, three ALS isozymes have been studied extensively in terms of their genetic regulation, kinetic properties, feedback regulation, and sensitivity to herbicidal inhibitors [7–10]. Each of the isozymes exists as a tetramer composed of two large catalytic subunits (59–60 kDa) and two small regulatory subunits (9–17 kDa) [7]. In contrast to the bacterial enzyme, the 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 thalania* [11], *Brassica napus* [12], *Gossypium hirsutum* [13], *Nicotina tabacum* [11], *Zea mays* [14], and *Xanthium* sp. [15] have been cloned and characterized.

☆ *Abbreviations:* ALS, acetolactate synthase; mALS, mutant ALS; wALS, wide-type ALS; CD, circular dichroism; EPPS, *N*-(7-hydroxyethyl) piperazine-*N*′-3-propanesulfonic acid; FAD, flavine adenine dinucleotide; GSH, glutathione; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactoside; PCR, polymerase chain reaction; TP, a triazolopyrimidine sulfonamide; TPP, thiamin pyrophosphate.

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ALS genes from *A. thalania* [16] and tobacco [11] have been functionally expressed in *E. 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 laboratories have revealed that Tryp490 [19], Cys411 [20], and His487 [21] residues are essential for catalytic functions in tobacco ALS and that Tryp573 [19], Ala121 [22], and Ser652 [23] are possibly located at a herbicide-binding site. More recently, crystallization and preliminary X-ray diffraction analysis of the catalytic subunit of *Saccharomyces cerevisiae* ALS have been reported [23].

In this study, we carried out site-directed mutagenesis of the three well-conserved lysine residues (K219, K255, and K299) in tobacco ALS, and analyzed the effects of 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, Sephadex G-25, 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 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).

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 [24]. All manipulations of the DNA were carried out using the technique reported previously [25]. The PCR was also performed as described previously [26]. 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′
K219Q, 5′-GATCGATTACCCAGCATAATTATC-3′
K255F, 5′-TTGATTGATGTACCTTTCGATATTC-3′
K255Q, 5′-TTGATTGA TGTACCTCAGGATATTC-3′
K299Q, 5-TCTGAGTCACAGAAGCCTGTTTTG-3′

The bold bases 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 in 50 mM KCl, 10 mM Tris (pH 7.5), and 1.5 mM $MgCl_2$ in 100 μl. Each reaction was performed for 30 cycles of the following programs; 94 °C, 1 min; 60 °C, 1 min; 72 °C, 2 min. The resulting DNA was subjected to a second PCR with the universal primer NKB1 5′-CATCTCC**GGATCC**ATGTCCACTA CCCAA-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 [25]. Each transformant was sequenced to ensure the correct base mutation in the mutant ALS gene and cultured to obtain the mutant protein.

DNA sequence analysis. DNA sequencing was carried out by the dideoxy chain-termination procedure [27]. Each mutant ALS was sequenced and identified.

Expression and purification of tobacco wALS and mALS. Bacterial strains of *E. coli* XL1-Blue cells containing the expression vector pGEX-ALS were grown at 37 °C in Luria–Bertani (LB) medium containing 50 μg/ml ampicilin 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 6–7 h at 20 or 30 °C and harvested by centrifugation at 5000 rpm for 30 min. Purification of wALS and mALS was carried out as described previously by Chang et al. [17]. The cell pellets were suspended in a standard buffer (50 mM Tris–HCl, pH 7.5, 1 mM pyruvate, 10% (v/v) ethylene glycol, 10 mM $MgCl_2$) containing protease inhibitors (2 μg/ml leupeptin, 4 μg/ml aprotinin, 2 μg/ml pepstatin A). The cell suspension was then lysed by sonication at 4 °C. The homogenate was centrifuged at 20,000 rpm 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, 10% (v/v) ethylene glycol). To cleave the ALS from GST, the purified GST–ALS was subjected to overnight digestion 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 [28] and the protein concentration was determined by the method of Bradford [29].

Enzyme assay. Enzyme activities of the purified wALS and mALS were measured according to the method of Westerfeld [30] with a modification as reported previously [31]. 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 6N H_2SO_4, 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.

Spectroscopic measurement. Absorption spectra were recorded on a Beckman DU-600 Spectrophotometer. The protein solution was dispensed in 1 ml back-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 190–750 nm on a Jasco J-710 Spectropolarimeter set at 20–50 mdeg sensitivity, 1 mm resolution, 2 units of accumulation, 5 s response, and at a scanning speed of 200 nm/min. A protein solution of 0.6–0.7 mg/ml was assayed in a 400 μl cylindrical quartz cell of 1 mm path length.

Results

Expression and purification of tobacco ALS

Four mutants (K219Q, K255Q, K255F, and K299Q) of the tobacco ALS gene were cloned including part of the chloroplast transit peptide into the bacterial expression plasmid pGEX-2T. Each of the mutant ALSs was expressed in *E. coli* as a GST–ALS fusion protein. Expression for mutants K255Q, K255F, and K299Q

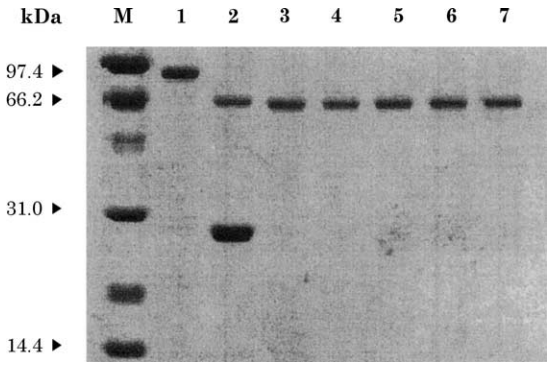

Fig. 1. SDS–PAGE analysis of the purified wALS and the mutant ALSs. Each sample was electrophoresed on 11% polyacrylamide gel containing SDS, then the gel was stained with Coomassie Blue. M, molecular marker, 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa; Lane 1, GST–wALS fusion protein; Lane 2, thrombin-digested wALS and GST; Lane 3, purifed wALS, Lane 4, K219Q; Lane 5, K255F; Lane 6, K255Q; Lane 7, K299Q.

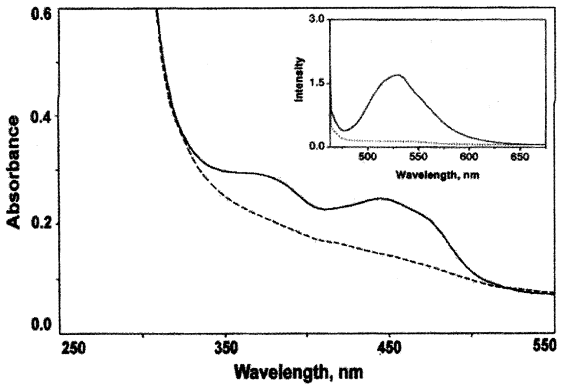

Fig. 2. Absorption spectra of wALS and K219Q. The concentration of each enzyme was 14 μM in 50 mM Tris–Cl buffer (pH 7.5). wALS, solid line; K219Q, dashed line; inset is the fluorescence spectra of wALS and K219Q. The concentration of each enzyme was 0.5 μM in 50 mM Tris–Cl buffer (pH 7.5).

was induced by the addition of 0.3 mM IPTG, but the optimum level of IPTG for expression of K219Q ALS was 0.1 mM. Each of the mutants was successfully expressed as a soluble form, as judged by the SDS–PAGE (data not shown). The resulting GST–ALS was purified to homogeneity in a single step by GSH-Sepharose 6B affinity chromatography. Lane 1 in Fig. 1 is a sample of the purified GST–wALS. The purified GST–ALS protein was subjected to digestion with thrombin protease. The cleaved ALS enzyme was purified to homogeneity by an additional GSH-affinity chromatography step. A single band of wALS, K219Q, K255Q, K255F, and K299Q at 65 kDa, corresponding to M_r of the ALS subunit, was apparent on the SDS–PAGE (Fig. 1).

K219Q mutant

Although the K219Q mutant of tobacco ALS was expressed and purified as a soluble protein (Fig. 1), it showed no detectable activity under various assay conditions, including much higher concentrations of substrate and cofactors and a longer incubation time. To understand the inactivation mechanism, the binding of cofactor FAD to K219Q was determined by spectral measurements. Although the absorption spectrum of FAD bound to wALS was not well resolved, two peaks around 370–390 and 450–480 were evident (Fig. 2), similar to those reported previously [19]. In contrast, the spectrum of K219Q showed no peak in the region of 350–500 nm, superimposed on a background that rises progressively at a lower wavelength (Fig. 2).

The fluorescence emission spectrum of K219Q showed no peak around 530 nm by exciting at 450 nm, which is the emission peak of FAD bound to wALS (Fig. 2 inset) [25]. Both absorption and fluorescence spectra of the K219Q indicated that the mutant does not bind the cofactor FAD. The CD spectrum of K219Q mutant almost overlapped with that of the wild-type ALS. This result suggests that the mutation of K219Q did not cause any significant change in the secondary structure, which could abolish binding affinity for FAD and catalytic activity.

Kinetic properties of wALS and mALS

The mutants and wild-type enzyme were characterized with respect to the kinetic properties, including V_{max}, K_m, K_c for FAD, TPP, and K_i^{app} of three herbicides, Londax, Cadre, and TP (Table 1). The substrate and cofactor saturation curves for wALS and mutants, K255F, K255Q, and K299Q were hyperbolic as reported previously for wALS [19] (data not shown). The values of V_{max}, K_m for the substrate and activation constant (K_c) for each cofactor were determined by fitting the data to Eq. (1) by the non-linear least-squares and Simplex methods for error minimization [32]

$$v = V_{max}/(1 + K/[X]). \quad (1)$$

In this equation, v is the reaction velocity, V_{max} is the maximum velocity, and K is K_m or K_c depending on whether the varied component $[X]$ is a substrate or cofactor. Table 1 shows the values of V_{max}, K_m, K_c for the cofactors, and K_i^{app} for inhibition by herbicides.

The K219Q mutant was totally inactive under various assay conditions. Thus, it was not possible to measure the affinity for the substrate, cofactors and herbicides, as was done for the active enzymes. The K_m values of K255F and K255Q were 109.4 and 115.5 mM, respectively, and the values were approximately 6- to 7-fold higher than those of wALS. However, the K_c values of both mutants K255F and K255Q for FAD and TPP were marginally different (less than 2-fold) from those of wild-type ALS. Therefore, the mutation did not occur at

Table 1
Kinetic parameters and K_i^{app} values of wALS and mALSs

	K_m	V_{max}	K_c		K_i^{app} values for inhibitors		
	Pyruvate (mM)	U mg^{-1}	FAD (μM)	TPP (mM)	Londax (nM)	Cadre (μM)	TP[a] (nM)
wALS	17.06	1.87	2.47	0.04	8.23	1.22	5.27
K219Q	No enzymatic activity						
K255F	109.38	1.02	1.40	0.03	ND[b]	ND	ND
K255Q	115.53	2.17	2.07	0.02	ND	ND	ND
K299Q	26.23	4.15	7.42	0.37	9.02	1.09	3.01

For each enzyme, the values shown are the best fitting estimations of the parameter obtained from regression analysis.
[a] TP, a newly synthesized derivative of triazolopyrimidine.
[b] ND – not detected ($\leqslant$2 μM of Londax, $\leqslant$1 mM of Cadre, $\leqslant$1 mM of TP).

the active site of the enzyme. The mutation of K299Q had little effect on the K_m value and K_c values for FAD and TPP.

Inhibition of ALS of herbicides

The sensitivities of three mutants, K255F, K255Q, and K299Q 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 Eq. (2)

$$v_i = v_0/(1 + [I]/K_i^{app}). \quad (2)$$

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 and which is also known as IC_{50}. Three different classes of herbicides, Londax, Cadre, and TP are very potent inhibitors of wALS with K_i^{app} values of 2.23 nM, 1.22 μM, and 5.27 nM, respectively (Table 1). The inhibition of K299Q by each of the herbicides is similar to that of wALS (Table 1, Fig. 4), indicating that the mutant has little effect on the binding of the herbicides. However, two mutants, K255F and K255Q, are strongly resistant to both Londax and Cadre (Figs. 4A and B). The mutation of K255F also conferred a high level of resistance against TP, however, the K255Q mutant is only moderately resistant to TP (Fig. 4C). To determine a change in the secondary structure by the mutations, the CD spectrum of the K255F mutant was compared with that of wALS. As shown in Fig. 3B, the CD spectrum of K255F nearly completely overlapped with that of wALS, indicating that no substantial change in the secondary structure occurred due to the mutation. Taken together, these results suggest that the binding sites of the three classes of herbicides partially overlap and that K255 is apparently located at the common binding site of the three herbicides.

Discussion

Since the first two plants ALS genes were isolated from *Arabidopsis thalania* and *Nicotina tabacum* using the yeast gene ilv_2 [11], a number of plant genes have been cloned and characterized [12–15]. Recently, it became possible to express the plant genes at a high level using a proper expression system and to purify the enzyme to homogeneity [17,33]. From site-directed mutagenesis of tobacco ALS in our laboratory, it was established that Trp490 [19], Cys411 [20], and His427

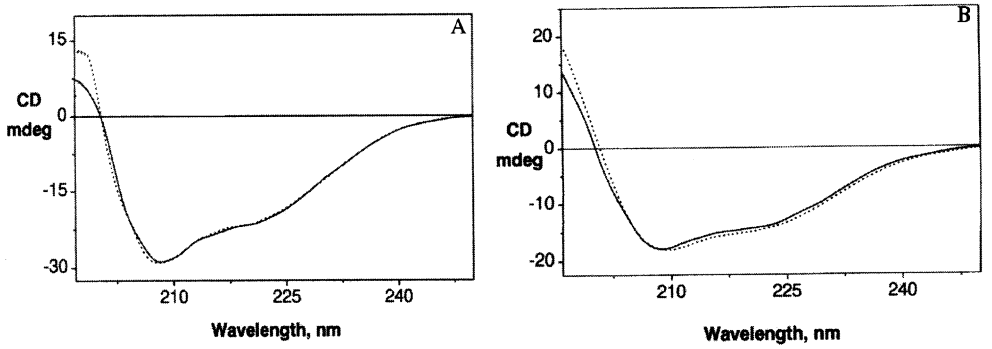

Fig. 3. CD spectra of wALS, K219Q, and K255F. (A) Each protein was present at a concentration of 0.8 mg/ml in 50 mM Tris–SO_4 buffer (pH 7.5). wALS, solid line; K219Q, dotted line. (B) Each protein was present at a concentration of 0.6 mg/ml in 50 mM Tris–SO_4 buffer (pH 7.5). wALS, solid line; K255F, dotted line.

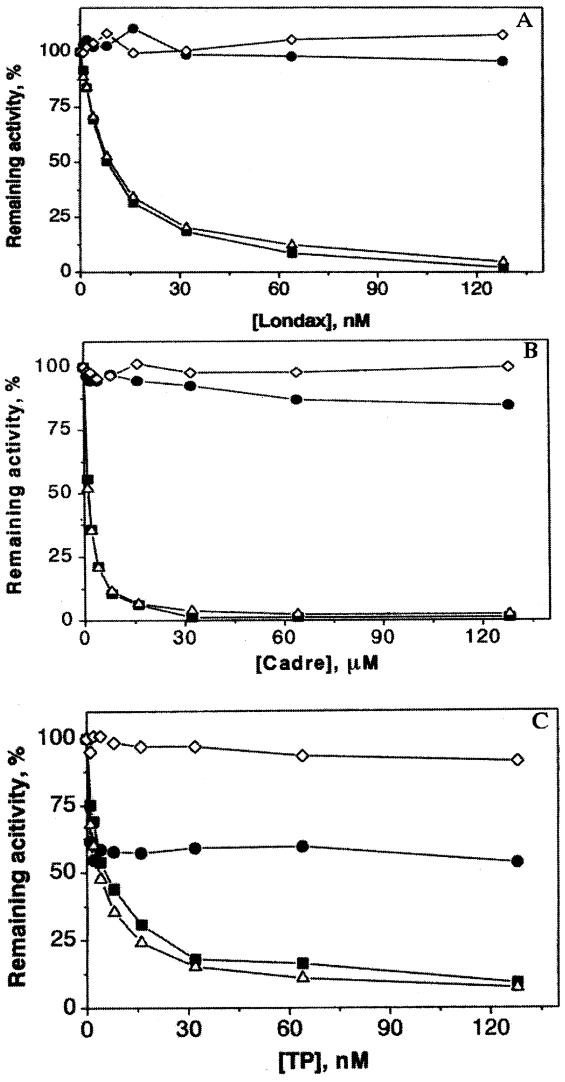

Fig. 4. Inhibition of the purified ALS, wALS, K255F, K255Q, and K299Q by three classes of herbicides, the sulfonylurea Londax (A), the imidazolinone Cadre (B) and the triazolopyrimidine TP (C). wALS (■), K255F (◇), K255Q (●), and K299Q (△).

[21] are essential for catalytic function of the ALS and that the intrachain disulfide bond between Cys163 and Cys309 has an important role in maintaining the correct conformation of the ALS [34].

Recently, our chemical modification study of tobacco ALS suggested that a lysine residue is probably located at the active site of the enzyme (unpublished data). It is also expected that the positively charged side-chain of a lysine residue would interact with the negatively charged substrate or a cofactor. It was noticed by a homology search for ALSs of bacteria, yeast, and plants that three lysine residues, Lys219, Lys255, Lys299, are well conserved. Accordingly, the roles of the lysine residues were investigated by using site-directed mutagenesis. Four mutants of tobacco ALS (K219Q, K255F, K255Q, and K299Q) were successfully expressed as soluble forms and the enzymes were purified to homogeneity (Fig. 1). Each of the mutant enzymes was characterized with respect to the kinetic properties, including V_{max}, K_m, K_c for FAD and TPP, and K_i^{app} of the three herbicides, Londax, Cadre, and TP (Table 1). The replacement of K219 by Gln having an uncharged polar side-chain, K219Q mutation, caused the enzyme to be totally inactive under various assay conditions. To understand the mechanism of inactivation, the spectroscopic properties of K219Q were compared with those of wALS. The purified wALS showed evident absorption and fluorescence emission peaks corresponding to FAD bound to wALS as reported previously [20]. In contrast, the mutant K219Q gave rise to no such absorption nor emission peaks (Fig. 2), indicating that the mutation abolished the binding affinity for FAD with a consequential loss of catalytic function. This result is supported by the recent reports in which the mutants W490F [19], C411A, [20], and H477F [21] of tobacco ALS were inactivated due to the loss of their binding affinity for FAD. The CD spectrum of K219Q was measured to determine the change in the secondary structure, which could hinder the binding of FAD to ALS. Since the CD spectrum of K219Q almost overlapped with that of wALS, the mutation did not cause any change in the secondary structure. Accordingly, it is obvious that the inactivation by the mutation of K219Q is not due to the change in the secondary structure. Taken together, these results suggest that Lys219 residue of tobacco ALS is located at the active site and a positively charged side-chain of the Lys residue likely interacts with a negatively charged FAD cofactor.

The mutations of K255F and K255Q substantially increased their K_m values as much as by 6- to 7-fold. But both mutations showed only a marginal effect on the K_c values for FAD and TPP. Thus, K255 residue is not likely located at the active site of the ALS, but it might be located near the substrate-binding site. And, the possibility that the effects of mutation were transmitted through the protein to the substrate-binding site could not be excluded.

Lately, ALS has been intensively studied, since ALS has been known to be a potential target for various classes of herbicides. 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. Each mutation of K255F and K255Q exhibited a large effect on the sensitivities to the three herbicides. The two mutants K255F and K255Q were strongly resistant to both herbicides, Londax and Cadre. The mutant K255F was also strongly resistant to TP, but K255Q showed less tolerance to TP. To determine whether the secondary structural change of the enzyme by the mutation of K255F is responsible for the resistance to the three

herbicides, the CD spectra of wALS and the K255F mutant were compared. The CD spectra of wALS and K255F showed no significant difference, and accordingly, the resistance of the mutant ALS is not due to a change in the secondary structure. Thus, Lys255 residue is likely located at the binding site common for the three herbicides. These results support the molecular model for the herbicide-binding site of *Arabidopsis thalania* ALS, in which Lys256 residue (corresponding Lys255 of tobacco ALS) is located at imidazolinone binding site [16]. Until recently, it has been reported that in tobacco ALS, Pro191 [35], Trp573 [19], and His351 [21] are involved in the binding of ALS-inhibiting herbicides.

The results of this study suggest that Lys219 is essential for catalytic activity and is likely involved in the binding of cofactor FAD in tobacco ALS and that Lys255 is located at a binding site common for three classes of herbicides, sulfonylureas, imidazolinones, and triazolopyrimidines.

Acknowledgments

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