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Functional Amino Acid Residues of Recombinant Tobacco Acetolactate Synthase

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Acetolactate synthase (ALS) is the common enzyme in the biosynthetic pathways leading to leucine, valine, and isoleucine. Tobacco ALS was expressed in *E. coli* and purified to homogeneity. The recombinant tobacco ALS was inactivated by thiol-specific reagents, N-ethylmaleimide (NEM) and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB). Inactivation of the ALS by NEM followed pseudo-first order kinetics and was first order with respect to the modifier. The substrate pyruvate protected the enzyme against the inactivation by NEM and DTNB. Extrapolation to complete inactivation of the enzyme by DTNB showed modification of approximately 2 out of 4 total cysteinyl residues (or 2 cysteinyl and 1 cysteinyl residues), with approximately 1 residue protected by pyruvate. The tobacco ALS was also inactivated by the tryptophan-specific reagent, N-bromosuccinimide (NBS), and was similarly protected by pyruvate. The kinetics of the inactivation was first-order with respect to NBS. The present data suggest that cysteinyl and tryptophanyl residues play a key role in the catalytic function of the enzyme.

Keywords: Acetolactate synthase, 5,5′-Dithio-bis-(2-nitrobenzoic acid), N-bromosuccinimide, N-ethylmaleimide, Tobacco.

Introduction

Acetolactate synthase (ALS, EC 4.1.3.18) is an enzyme catalyzing the first common step in the biosynthesis of valine, leucine, and isoleucine in bacteria, yeast, and higher plants. ALS catalyzes the condensation of 2-acetolactate from two molecules of pyruvate in the first step of the valine and leucine synthetic pathway, and the formation of 2-aceto-2-hydroxybutyrate from pyruvate and 2-ketobutyrate as the second step of isoleucine biosynthesis. ALS is the target site of several classes of structurally diverse herbicides, including the sulfonylureases (LaRossa and Schloss, 1984; Ray, 1984), the imidazolinones (Shaner *et al.*, 1984), the triazolopyrimidines (Kleschick, 1984), the pyrimidyl-oxy-benzoates (Babczinski and Zelinski, 1991; Choi *et al.*, 1992), the pyrimidyl-thio-benzenes (Choi *et al.*, 1992), and the 4,6-dimethoxypyrimidines (Shim *et al.*, 1995). ALS is a key controlling point for the levels of the branched chain amino acids. This involves feedback inhibition of the enzyme activity by the end products of the pathway (valine, leucine, and isoleucine) in both microorganisms and plants (Miflin and Cave, 1972; Gollop *et al.*, 1983).

In bacteria, ALS has been purified to homogeneity and well characterized with respect to its molecular weight, subunit composition, and isozyme pattern (Eoyang *et al.*, 1984; Schloss *et al.*, 1985). The low abundance and labile nature of plant ALS have severely hampered the purification and biochemical characteristics of this enzyme. Although recently ALS was purified to homogeneity from barley, only limited studies of its characterization were carried out since the yield of the purification was too low (Chong *et al.*, 1997). The ALS genes from several sources have been cloned (Mazur and Falco, 1989), and the ALS genes from *Arabidopsis* and *Brassica napus* have been expressed in *E. coli* and *Salmonella typhmurium*, respectively, but none of these plant ALSs have been purified to homogeneity (Wiersma *et al.*, 1990). Recently, the tobacco ALS gene was cloned, expressed in *E. coli*, and purified to homogeneity (Chang *et al.*, 1997). In this study, we carried out a chemical modification of the purified recombinant tobacco ALS with cysteine-specific and tryptophan-specific reagents. We present here evidence that a cysteinyl and a tryptophanyl residue are essential for the catalytic function of the tobacco ALS.

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Materials and Methods

Reagents Bacto-tryptone, yeast extract and Bacto-agar were purchased from Difco Laboratories (Detroit, USA). EPPS, glutathione, N-bromosuccinimide, 5,5′-dithio-bis-(2-nitrobenzoic acid) and N-ethylmaleimide were obtained from Sigma Chemical Co. (St. Louis, USA). Microbio-spin 6 columns were from Bio-Rad Lab. (Hercules, USA). Thrombin protease and epoxy-activated Sepharose 6B were purchased from Pharmacia Biotech. (Uppsala, Sweden). All other chemicals were of the highest commercial grade available.

Expression and purification of GST-ALS The bacterial strain of *E. coli* XL1-Blue cell transformed by pGEX-ALS2 (co-encoded tobacco ALS gene and glutathione S-transferase (GST) gene) was prepared and cells were grown aerobically for 9 h at 30°C as described by Chang *et al.* (1997). The cells were induced with 0.3 mM isopropyl-β-D-thiogalactoside (IPTG) and grown for an additional 3 h at 30°C. Cells were harvested by centrifugation at 5000 × *g* for 15 min. The purification of GST-ALS was essentially performed as described by Chang *et al.* (1997). The harvested cells were resuspended with the resuspension buffer (50 mM EPPS, 10% ethylene glycol, 1 mM pyruvate, 10 mM $MgCl_2$, 2 μg/ml Leupeptin, 4 μg/ml Aprotinin, 2 μg/ml Pepstatin A) and lysed by sonication at 4°C (14 micron, 5 s × 5 times). The homogenate was centrifuged at 20,000 × *g* for 20 min and the supernant was then applied to a GSH-coupled Sepharose 6B column pre-equilibrated with the resuspension buffer. After removing unbound proteins by elution with 10 vol of the buffer, the GST-ALS fusion protein was recovered from the column with the elution buffer (50 mM Tris-HCl, pH 9.6, 15 mM GSH). The active fractions were pooled and stored in aliquots in liquid nitrogen. To obtain the pure recombinant tobacco ALS from the fusion protein, the GST-ALS was digested with thrombin overnight at 4°C. The ALS was separated from GST by applying the mixture solution to the GSH-coupled Sepharose 6B column.

Enzyme assay and protein determination The enzyme activities of purified GST-ALS and ALS were measured according to the method of Westerfeld (1943) with a modification. The assay mixture contained 20 mM potassium phosphate (pH 7.0), 0.5 mM TPP, 10 μM FAD, 20 mM pyruvate, 50 mM $MgCl_2$, plus enzyme in the final volume of 200 μl. After incubation at 37°C for 1 h, the reaction was stopped by adding 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 mixture was measured at 525 nm. The concentration of protein was determined by the method of Bradford (1976).

Chemical modification of the recombinant tobacco ALS The modification reaction of tobacco ALS with various concentrations of NEM was carried out in 50 mM phosphate buffer, pH 7.0, at 25°C. Modification reactions were initiated by adding the reagent to the enzyme solution which had been preincubated for 10 min at 25°C. Aliquots of reaction mixture were removed at time intervals and assayed for activity.

To remove unreacted excess reagents, pyruvate, and reaction products, the reaction mixture was applied to the Micro Bio-spin 6 column. Activity was expressed as the ratio of the activity of modified enzyme, V, to that of the control subjected to the same conditions but in the absence of modifying reagent, V_c, multiplied by 100. Modification with DTNB was performed as described in the procedure for modification with NEM, but in 25 mM HEPES buffer (pH 7.6) containing 20 mM EDTA, 50 mM $MgCl_2$. The concentration of 2-nitro-5-mercaptobenzoate (TNB) released from the reaction of DTNB with cysteinyl residues was determined using $\varepsilon_{412} = 1.415 \times 10^4\ M^{-1}cm^{-1}$ (Ellman, 1959).

The modification of tryptophanyl residues of the ALS using NBS was carried out as described in the procedure for modification with NEM.

Results

Expression and purification of GST-ALS Recombinant tobacco ALS was expressed in *E. coli* as a protein fused with GST. The fusion protein GST-ALS was purified in a single step of a glutathione-Sepharose. The recombinant ALS was obtained by digestion of the GST-ALS fusion protein with thrombin. The purified ALS was functionally active and showed a single band on SDS-PAGE (data not shown).

Inactivation of tobacco ALS by N-ethylmaleimide Recombinant tobacco ALS was inactivated by incubation with NEM in 0.1 M phosphate buffer (pH 7.0). Complete inactivation could be achieved by prolonged incubation with the reagent. Figure 1A shows the semilogarithmic plot of the inactivation by various concentrations of NEM *versus* incubation time. The inactivation reaction followed pseudo-first order kinetics, the rate of which was a function of the concentration of the reagent. The second-order rate constant for the inactivation of the enzyme with NEM was $9.42\ M^{-1}min^{-1}$ in 0.1 M phosphate buffer at 25°C. The reaction order with respect to NEM for the inactivation was about 1.04, which was determined from the slope of the double logarithmic plot as described previously (Choi and McCormick, 1981) (Fig. 1B, inset). The resulting data suggest that the enzyme was inactivated by the modification of one essential cysteinyl residue. In the presence of the substrate pyruvate, inactivation of the enzyme by NEM was drastically retarded (Fig. 2). At saturation level, pyruvate protected the enzyme almost completely against inactivation by NEM, while the enzyme lost its activity by more than 60% after incubation of the enzyme with 3 mM for 40 min without pyruvate. These data suggest that a cysteinyl residue plays a key role in the activity of the tobacco ALS.

Stoichiometric analyses of inactivation and Cys modification of tobacco ALS by DTNB Treatment of the tobacco ALS with 1 mM DTNB in HEPES buffer (pH 7.6) at 25°C caused a rapid appearance of TNB, and simultaneously the enzyme was rapidly inactivated. The number of cysteinyl residues modified by DTNB was

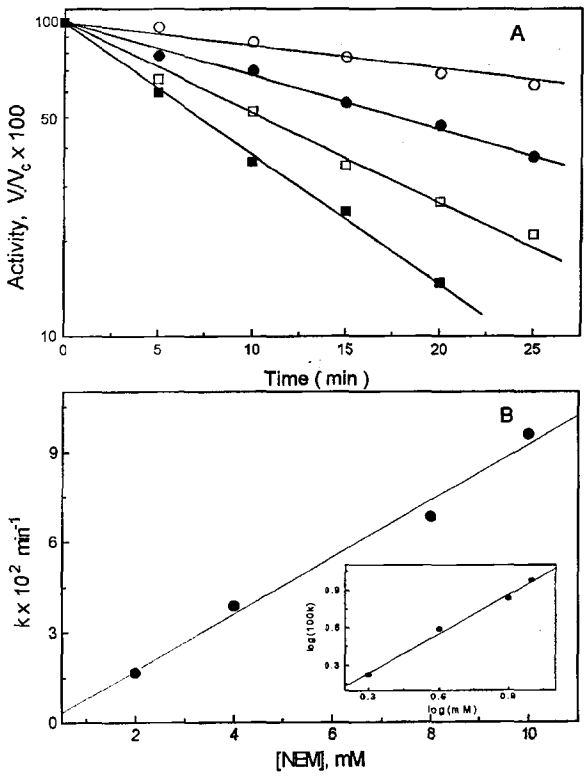

Fig. 1. A. Inactivation of recombinant tobacco ALS by N-ethylmaleimide. The enzyme (0.08 mg/ml) was incubated with 2 mM (○), 4 mM (●), 8 mM (□), 10 mM (■) N-ethylmaleimide in 0.1 M photassium phosphate buffer, pH 7.0 at 25°C. B. Plots of the pseudo-first order rate constant for inactivation (k) determined from the slopes of semilogarithmic plots of Fig. 1A against concentrations of the reagent (R = 0.993). The inset is the plots of log(pseudo-first order rate constant) *vs.* log(N-ethylmaleimide) (R = 0.996).

calculated using $\varepsilon_{412} = 1.415 \times 10^4\ M^{-1}\,cm^{-1}$ for the molar absorption coefficient of TNB (Ellman, 1959). Figure 3 shows the correlation of inactivation of the enzyme with the number of cysteinyl residues modified. From extrapolation of the linear portion, approximately 2.25 moles of DTNB were reacted with one mole of ALS for complete inactivation of the enzyme. It suggests that about two cysteinyl residues per ALS monomer are modified with DTNB for complete inactivation of the enzyme.

The extent of inactivation and the number of cysteinyl residues modified by DTNB in the absence and presence of the pyruvate are listed in Table 1. The number of cysteinyl residues which are essential for activity and protected by pyruvate was determined by comparison, at the same reaction time, of the total number of cysteinyl residues modified and the extent of inactivation for samples incubated in the absence and presence of the substrate. If the difference in modified cysteinyl residues is linearly related to the difference in activation, pyruvate protects approximately 1.22 cysteinyl residues, and modification of these cyteinyl residues causes inactivation of the enzyme.

Inactivation of recombinant tobacco ALS by N-bromosuccinimide As shown in Figs. 4A and 4B, inactivation of ALS by the modification with NBS followed pseudo-first order kinetics, and the inactivation rate was a function of the NBS concentration. From these data, the second-order rate constant was calculated to be $10.3\ M^{-1}\,min^{-1}$ in 0.1 M phosphate buffer at 25°C. The plot of log(pseudo-first order rate constant) against log(NBS) gave the reaction order of 0.93 with respect to NBS (Fig. 4B, inset). It means that the ALS is inactivated by modification of one essential tryptophan residue of

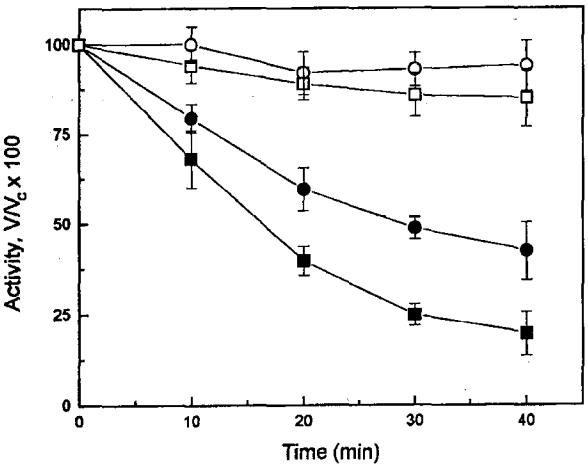

Fig. 2. Protective effect of pyruvate on the inactivation of the recombinant tobacco ALS by N-ethylmaleimide. ALS (0.08 mg/ml) was incubated with 3 mM (○) and 5 mM (□) N-ethylmaleimide in the presence of 50 mM pyruvate and with 3 mM (●) and 5 mM (■) reagent in the absence of pyruvate.

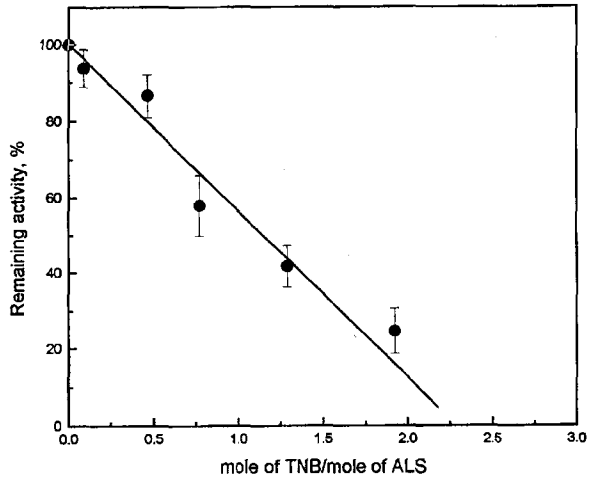

Fig. 3. Correlation of inactivation of the recombinant tobacco ALS with the number of cysteinyl residues modified (R = −0.981). ALS (1 μM) was incubated with 1 mM DTNB in 25 mM HEPES (pH 7.6) containing 25 mM EDTA and 50 mM $MgCl_2$ at 25°C.

Table 1. Effects of substrate pyruvate on the inactivation of ALS and the modification of cysteinyl residues by DTNB.

Time (min)	Addition to incubation mixture: 1. None		2. 50 mM Pyruvate		Difference (1–2)		No. of Cys modified to 100% Inact.
	Inact. (%)	No. of Cys modified	Inact. (%)	No. of Cys modified	Inact. (%)	No. of Cys modified	
5	38.6	0.82	5.26	0.48	33.34	0.34	1.02
10	69.8	1.63	8.29	0.81	60.88	0.82	1.35
15	76.0	1.79	5.82	0.88	70.20	0.91	1.30

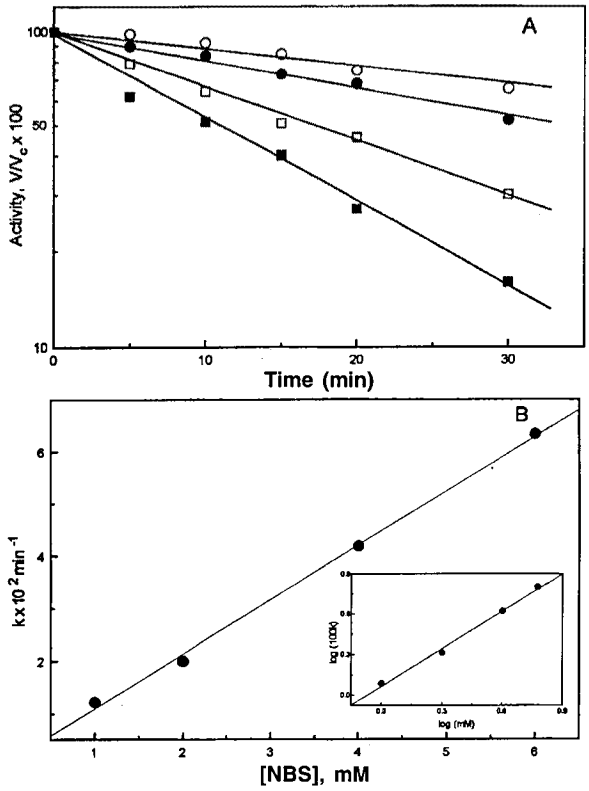

Fig. 4. A. Inactivation of recombinant tobacco ALS by N-bromosuccinimide. The enzyme (0.08 mg/ml) was incubated with 1 mM (○), 2 mM (●), 4 mM (□), 6 mM (■) N-bromosuccinimide in 0.1 M photassium phosphate buffer, pH 7.0 at 25°C. B. Plots of the pseudo-first order rate constant for inactivation (k) determined from the slopes of semilogarithmic plots of Fig. 4A against concentrations of N-bromosuccinimide (R = 0.999). The inset is the plots of log(pseudo-first order rate constant) *vs.* log(N-ethylmaleimide) (R = 0.997).

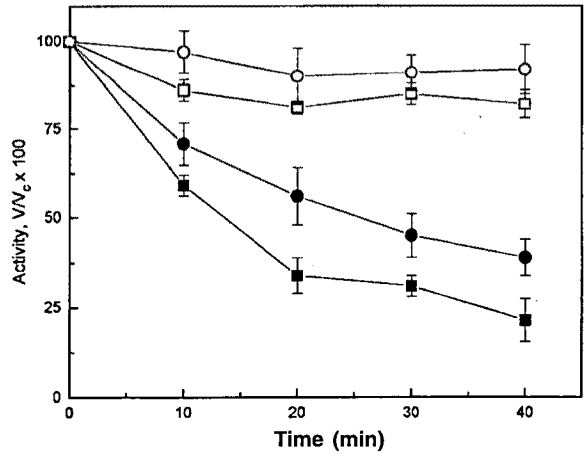

Fig. 5. Protective effect of substrate on the inactivation of the recombinant tobacco ALS by N-bromosuccinimide. ALS (0.08 mg/ml) was incubated with 3 mM (○) and 5 mM (□) N-bromosuccinimide in the presence of 50 mM pyruvate and with 3 mM (●) and 5 mM (■) reagent in the absence of pyruvate.

ALS. As with the case of inactivation of the ALS by cysteine-specific reagents, the substrate pyruvate protected the enzyme effectively against inactivation by NBS (Fig. 5). These results suggest that the trytophanyl residue is probably located at or near the active site of the ALS.

Discussion

ALS has been purified to homogeneity from several bacteria and well characterized with respect to its isozyme pattern, subunit structure, and some enzymatic properties (De Felice *et al.*, 1978; Eoyang and Silverman 1984; Schloss *et al.*, 1985; Barak *et al.*, 1988), but low abundance and extreme lability have hampered purification and biochemical studies of the enzyme from plant sources. Recently, we have purified ALS to homogeneity from etiolated barley shoots, but only limited structural and biochemical studies have been made with the pure ALS since the overall yield of the purification was exceptionally low (Chong *et al.*, 1997).

In this work, we have expressed tobacco ALS as a fusion protein of GST-ALS in *E. coli* and purified it to homogeneity in a single step of glutathione-Sepharose column chromotography according to the method of Chang *et al.* (1997). The pure recombinant tobacco ALS was obtained from GST-ALS fusion protein by digestion with thrombin and then by passing through the glutathione-

Sepharose column (Chang *et al.*, 1997). The purified ALS have been subjected to chemical modification in order to identify amino acid residues at the active site of the enzyme. The recombinant tobacco ALS was inactivated by treatment of the sulfhydryl reagents, NEM and DTNB. With NEM, complete inactivation could be achieved, and the inactivation reaction followed pseudo-first order kinetics and was first-order with respect to the reagent, indicating that the inactivation proceeds in an all-or-none fashion. The substrate of ALS, pyruvate, was very effective in protecting the enzyme against inactivation by NEM, and almost complete protection could be achieved at the saturation level. Previously, maleimides were shown to react selectively with sulfhydryl groups of a number of enzymes (Parniak and Kaufman, 1981; Takata *et al.*, 1991; Bradshaw and Dunlap, 1993; Zapata *et al.*, 1993). Together, the results strongly suggest that the modification of an essential cysteinyl residue is fully responsible for the inactivation of ALS by NEM. One could presume that the conformational change induced by modification of the cysteinyl residues might cause inactivation of the enzyme. To correlate the inactivation of the enzyme with the number of cysteinyl residues modified, the enzyme was treated with another sulfhydryl-specific reagent, DTNB. The moles of modified cysteine could be determined by measuring absorbance at 412 nm which is the absorbance maximum in the visible region for TNB, the product of the reaction of DTNB with cysteine (Ellman, 1959). Extrapolation to complete inactivation yielded about 2 cysteinyl residues modified per monomer of the ALS. In parallel experiments, the enzyme was treated with DTNB in the presence of pyruvate. Pyruvate prevented the inactivation of the enzyme and simultaneously protected the cysteinyl residues from modification. The analyses of those data demonstrated that pyruvate protects about 1 cysteinyl residue, and modification of this cysteinyl residue causes inactivation of the enzyme. DNA sequence analyses showed that the tobacco ALS gene encodes 4 cysteinyl residues (Mazur *et al.*, 1987). Among them, 2 residues seem to be able to react with DTNB, and another 2 residues may be embedded in the interior of the protein or crosslinked as a disulfide bridge.

The tryptophan-specific reagent, N-bromosuccinimide, also inactivated the tobacco ALS. The treatment of the enzyme with NBS caused a time-dependent loss of activity, and the inactivation was a function of the NBS concentration. The substrate pyruvate also effectively protected the enzyme against inactivation by NBS. These results implicated that inactivation of the enzyme by NBS most likely occurs due to modification of a tryptophanyl residue at the active site of the ALS. In *E. coli* ALS II, Trp464 was proposed to contribute the substrate specificity by site-directed mutagenesis (Ibdah *et al.*, 1996). Trp552 of cocklebur ALS was reported to play a key role in the enzyme function (Bernasconi *et al.*, 1995).

To determine the positions of the essential cysteinyl and tryptophanyl residues in the primary structure of the ALS, site-directed mutagenesis of the tobacco ALS gene is ongoing in our laboratory.

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