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Chemical Modification of *Serratia marcescens* Catabolic α-Acetolactate Synthase

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The catabolic α-acetolactate synthase purified from *Serratia marcescens* ATCC 25419 was rapidly inactivated by the tryptophane-specific reagent, N-bromosuccinimide, and the arginine-specific reagent, phenylglyoxal. The enzyme was inactivated slowly by the cysteine-specific reagent N-ethylmaleimide. The second-order rate constants for the inactivation by N-bromosuccinimide, phenylglyoxal. and N-ethylmaleimide were 114,749 $M^{-1}min^{-1}$, 304.3 $M^{-1}min^{-1}$, and 5.1 $M^{-1}min^{-1}$, respectively. The reaction order with respect to N-bromosuccinimide, phenylglyoxal, and N-ethylmaleimide were 1.5, 0.71, and 0.86, respectively. The inactivation of the catabolic α-acetolactate synthase by these modifying reagents was protected by pyruvate. These results suggest that essential tryptophane, arginine, and cysteine residues are located at or near the active site of the catabolic α-acetolactate synthase.

Keywords: Active site, Catabolic α-acetolactate synthase, Chemical modification.

Introduction

Acetolactate synthase (ALS) belongs to a family of homologous thiamin pyrophosphate (TPP)-dependent enzymes, and catalyzes processes in which the common first step is decarboxylation of pyruvate (Green, 1989). The enzymes of this family including biosynthetic ALS, catabolic α-ALS, pyruvate oxidase, pyruvate dehydrogenase, and pyruvate decarboxylase differ widely, however, with regard to the fate of the TPP-bound "active aldehyde" intermediate formed by the decarboxylation (Hawkins *et al.*, 1989). ALS catalyzes the condensation of the (hydroxyethyl-TPP) carbanion intermediate, with either pyruvate or α-ketobutyrate, to one of two possible alternative physiologically-significant acetohydroxy acid products, acetolactate (precusor of valine and leucine) and acetohydroxybutyrate (precusor of isoleucine), respectively (Tse and Schloss, 1993). On the other hand, catabolic α-ALS catalyzes the condensation of the (hydroxyethyl-TPP) carbanion intermediate with only pyruvate to acetolactate (Umbarger, 1987).

In a previous study, we had reported that the enzyme activity of an anabolic ALS from *Serratia marcescens* was rapidly inactivated by the thiol-specific reagent p-chloromercuribenzoate, the tryptophane-specific reagent N-bromosuccinimide, and the arginine-specific reagent phenylglyoxal (Choi and Kim, 1995). In a recent study of *E. coli* ALS II, the replacement of Trp464 leads to a decrease in the substrate specificity, by 8~20 folds, even though another aromatic residue (Phe or Tyr) was introduced instead of tryptophane (Ibdah *et al.*, 1996). *E. coli* ALS I was also irreversibly inactivated by the alkylation with 3-bromopyruvate (Silverman and Eoyang, 1987).

Although intensive studies have been carried out on the mechanism of the interaction of the herbicides with anabolic ALS isozymes from enteric bacteria and plants, there is relatively little information available on the amino acid residues involved in the enzyme reaction of catabolic α-ALS, except that the enzyme activity of catabolic α-ALS was decreased by modification of the sulfhydryl group by 5,5′-dithiobis (2-nitrobenzoate) (Tveit and Stormer, 1969). In this present report, we describe the chemical modification of the purified valine-resistant catabolic α-ALS with various chemical modifying reagents. We present evidence that tryptophane, arginine, and cysteine residues are at or near the active site of the catabolic α-ALS from *Serratia marcescens*.

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Materials and Methods

Materials DEAE-Sepharose, Phenyl-Sepharose, and protein molecular weight markers were purchased from Pharmacia Co. (Uppsala, Sweden). Cocarboxylase (TPP), phenylglyoxal (PGO), flavin adenine dinucleotide (FAD), dithiothreitol (DTT), N-ethylmaleimide (NEM), phenylmethylsulfonylfluoride (PMSF), pyridoxal-5-phosphate (PLP), sodium pyruvate, creatine, magnesium chloride, and glyoxylate were purchased from Sigma Chemical Co. (St. Louis, USA). N-bromosuccinimide (NBS) was purchased from Fluka Co. (Buchs, Switzerland). Hydroxylapatite was obtained from Bio-Rad (Richmond, USA); α-Naphthol was purchased from Kanto Chemical Co. (Tokyo, Japan). All other reagents used were of first reagent grade.

Bacterial strain and culture condition The bacterial strain used in this study was *Serratia marcescens* ATCC 25419. Cells (3 L) were grown aerobically for 24 h in 5 L culture flasks containing minimal medium at 37°C with shaking at 60 rpm on a reciprocal shaker. The cells were harvested by centrifugation (15,000 × *g*, 30 min). The minimal medium contained 22 mM potassium phosphate monobasic, 51 mM potassium phosphate dibasic, 8 mM ammonium sulfate, 0.4 mM magnesium sulfate, and 0.5% glucose.

Preparation of crude extracts About 15 g of wet cells were resuspended in column buffer (20 mM potassium phosphate buffer, pH 7.0, 0.1 mM TPP, 0.5 mM DTT, 10 μM FAD, 2 mM $MgCl_2$, and 10% glycerol). The cell suspension was then subjected to ultrasonic treatment for 30 s at 5 min intervals on ice 15 times, and centrifugated at 15,000 × *g* for 40 min. The supernatants were pooled and this crude extract was used for the purification of the catabolic α-ALS.

Enzyme assay and protein determination The activity of catabolic α-ALS was assayed using the method of McEwen and Silverman (1980). One millilitre reaction mixture containing 0.1 M sodium phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 20 mM sodium pyruvate, 80 μg TPP, 20 μg FAD, and 10% glycerol was incubated with enzyme for 30 min at 37°C. The enzyme reaction was stopped by adding 5 μl of 50% (v/v) sulfuric acid. After the addition of 200 μl of 0.5% creatine and 200 μl of 5% α-naphthol in 2.5 N NaOH solution, the reaction mixture was further incubated for 30 min at 37°C. After centrifugation at 15,000 × *g* for 5 min, the absorbance of the supernatant of the reaction mixture was measured at 540 nm. One unit of enzyme activity represents the formation of 1 μmole acetolactate/min, and the specific activity is expressed as U/mg of protein at standard conditions. The amount of protein was determined by the Lowry method (1951).

Enzyme purification The valine-resistant catabolic α-ALS from *Serratia marcescens* ATCC 25419 was purified to homogeneity by 50~80% ammonium sulfate fractionation, DEAE-Sepharose ion exchange chromatography, phenyl-Sepharose hydrophobic chromatography, and hydroxylapatite chromatography, as described by Joo and Kim (1997).

Chemical modification Modifications of the cysteine, arginine, and tryptophane residues of the purified catabolic α-ALS were carried out as described by Lundblad and Noyes (1984). For the modification of the cysteine residue, the enzyme solution (0.13 μM) was reacted with various concentrations of N-ethylmaleimide (NEM) in 50 mM potassium phosphate buffer (pH 7.0) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$, and 10% glycerol at 25°C. The arginine residue of the enzyme was modified by reacting the enzyme (0.13 μM) with various concentrations of phenylglyoxal (PGO) in 50 mM sodium bicarbonate buffer (pH 8.4) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$, and 10% glycerol at 25°C.

Modification of the tryptophane residue of the enzyme (0.13 μM) was carried out by reacting the enzyme with various concentrations of N-bromosuccinimide (NBS) in 50 mM sodium acetate buffer (pH 4.5) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$ and 10% glycerol at 25°C. At regular time intervals, aliquots were removed for the measurement of residual enzyme activity.

Alkylation by bromopyruvate Alkylation of the cysteine residue of the enzyme was carried out by reacting the enzyme solution (0.13 μM) with various concentrations of bromopyruvate in 50 mM sodium phosphate buffer (pH 7.0) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$, and 10% glycerol at 28°C (Silverman and Eoyang, 1987). At regular time intervals, aliquots were removed for the measurement of residual enzyme activity.

Protection of the catabolic α-acetolactate synthase from inactivation by pyruvate Protection against the inactivation of the enzyme by NBS, PGO, and NEM was carried out by preincubation of the enzyme (0.13 μM) with 10 mM pyruvate for 15 min at 37°C before the addition of each modifying reagent. After reaction with each modifying reagent at 25°C, aliquots of the reaction mixture were removed at regular time intervals for the measurement of the enzyme activity.

Reaction of the catabolic α-acetolactate synthase with other chemical modifiers The purified enzyme solution (0.13 μM) was incubated with given concentrations (1 mM and 2 mM) of several modifying reagents such as pyridoxal-5'-phosphate (PLP) and phenylmethylsulfonylfluoride (PMSF) in 50 mM potassium phosphate buffer (pH 7.0) containing 10 μM FAD, 0.1 mM TPP, 2 mM $MgCl_2$, and 10% glycerol. After incubation with the modifying reagents for 20 min at 25°C (Lundblad and Noyes, 1984), aliquots were removed and the remaining enzyme activity was measured as described above. Stock solutions of PMSF were prepared by dissolving it in 99% ethanol just before use. The ethanol concentration of the reaction mixture was kept at less than 5% so that it would not affect the enzyme activity.

Effect of divalent metal ions on the catabolic α-ALS Purified enzyme solution was dialysed overnight against column buffer without $MgCl_2$. The dialyzed overnight solution was incubated with various concentrations of divalent metal ions such as Fe^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Hg^{2+}, and Zn^{2+}, in column buffer without $MgCl_2$ at 37°C. Aliquots were removed at various incubation times, and the remaining enzyme activity was measured as described above.

Results and Discussion

Inactivation of catabolic α-acetolactate synthase

Inactivation by N-bromosuccinimide (NBS): The effect of NBS, which is specific to the tryptophane residue at acidic pH, on the catabolic α-ALS was examined in 50 mM sodium acetate buffer (pH 4.5) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$, and 10% glycerol. A semilog plot of remaining activity against incubation time showed a biphasic mode, i.e., initially rapid and then slow, indicating that two types of tryptophane residues affecting the enzyme activity are present in the catabolic α-ALS (Fig. 1). From the initial, fast inactivation reaction with NBS in Fig. 1, the second-order rate constant of 114,749 M^{-1} min^{-1} at 25°C was determined from the slope of the linear relationships between the K_{obs} values and NBS concentrations (Fig. 1B). The reaction order with respect to NBS was determined from a plot of $\log(1/t_{1/2})$ against log(100 × [NBS]). This plot showed a straight line with a slope equal to n, where n is the number of inhibitor molecules reacting with each active site of the enzyme to produce an inactive enzyme–inhibitor complex (Lundblad and Noyes, 1984). A double logarithmic plot of the reciprocal of the half-time of inactivation against reagent concentration yielded a reaction order of 1.5 with respect to the inhibitor (Fig. 1C), suggesting that there may be one essential tryptophane residue which has a significant effect on the enzyme activity, and a less active tryptophane residue that affects the enzyme activity less. In contrast with the valine-sensitive biosynthetic ALS isozyme from *Serratia marcescens*, the reactivity of NBS with the catabolic α-ALS was much higher than that with the anabolic ALS isozyme. The second-order rate constant was 15,000 M^{-1} min^{-1} with NBS for the valine-sensitive anabolic ALS from *Serratia marcescens* (Choi and Kim, 1995).

Inactivation by phenylglyoxal (PGO): The purified catabolic α-ALS was inactivated by incubation with PGO in 0.05 M sodium bicarbonate buffer (pH 8.4) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$, and 10% glycerol. The results of inactivation by various PGO concentrations are shown in Fig. 2 as a semi-logarithmic plot. The second-order rate constant (K) for the inactivation of the catabolic α-ALS by PGO was 304.3 M^{-1} min^{-1} at 25°C (Fig. 2B). The reaction order with respect to PGO was determined from a plot of $\log(1/t_{1/2})$ against log(100 × [PGO]). The reaction order with respect to PGO was 0.71 from a double logarithmic plot of the reciprocal of the half-time of inactivation against reagent concentration (Fig. 2C). The result suggests that the inactivation is the result of the reaction of one arginine residue per active catabolic α-ALS. It was reported that the reaction order of the inactivation by PGO was 0.75 for the valine-sensitive biosynthetic ALS from *Serratia marcescens* (Choi and Kim, 1995).

Inactivation by N-ethylmaleimide (NEM): Purified enzyme was inactivated by incubation with NEM in 0.05 M

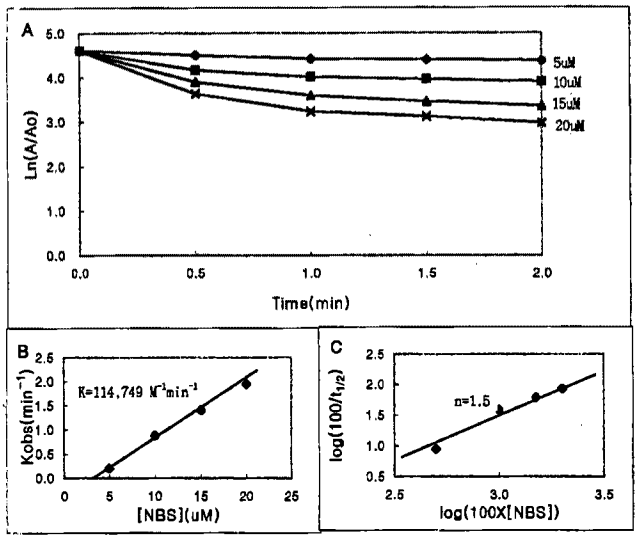

Fig. 1. (A) Inactivation of the catabolic α-ALS by NBS. The enzyme (0.13 μM) was incubated with various concentrations of NBS in 50 mM sodium acetate buffer (pH 4.5) at 25°C. (B) Plot of the pseudo first-order rate constant for the inactivation of the catabolic α-ALS (Kobs) obtained at various concentrations of NBS. (C) Double-logarithmic plot of the half-times of inactivation of the catabolic α-ALS at various NBS concentrations.

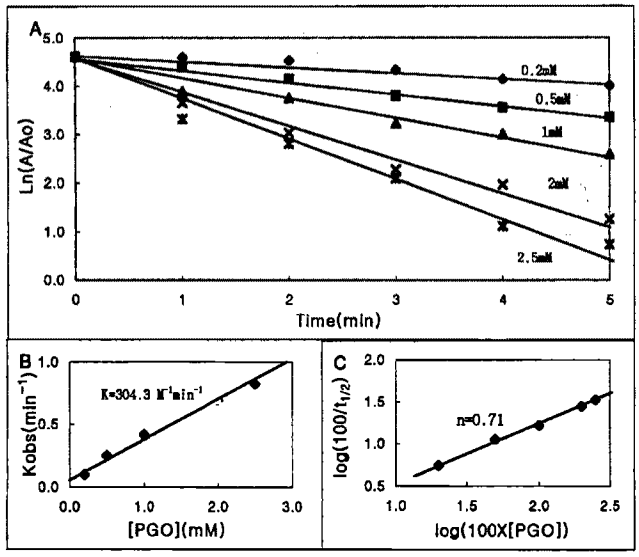

Fig. 2. (A) Inactivation of the catabolic α-ALS by PGO. The enzyme (0.13 μM) was incubated with various concentrations of PGO in 50 mM potassium phosphate buffer (pH 7.0) at 25°C. (B) Plot of the pseudo first-order rate constant for the inactivation of the catabolic α-ALS (K_{obs}) obtained at various concentrations of PGO. (C) Double-logarithmic plot of the half-times of inactivation of the catabolic α-ALS at various PGO concentrations.

potassium phosphate buffer (pH 7.0) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$, and 10% glycerol. The results of inactivation with various NEM concentrations are shown in Fig. 3 as a semi-logarithmic plot. The second-order rate constant (K) for the inactivation of the catabolic α-ALS by NEM was 5.08 M^{-1} min^{-1} at 25°C (Fig. 3B). The reaction order with respect to NEM was 0.86 from a double logarithmic plot of the reciprocal of the half-time of inactivation against reagent concentration (Fig. 3C) suggesting that inactivation is the result of the reaction of one cysteine residue per active catabolic α-ALS. It has been reported that the reaction order of the inactivation by DTNB was 0.94 for the valine-sensitive biosynthetic ALS from *Serratia marcescens* (Choi and Kim, 1995). From these results it can be seen that the amino acid residues involved in the enzyme reaction of the catabolic α-ALS are similar to those of the valine-sensitive biosynthetic ALS isozyme from *Serratia marcescens,* although the reaction kinetics with each modifying reagent was somewhat different (Choi and Kim, 1995). Purified catabolic α-ALS was inactivated by bromopyruvate, an alkylating reagent, in a time- and dose-dependent fashion (Table 1). This result was consistent with the data of chemical modification with NEM. It has been reported that the cysteinyl residues 44 and 67 in *E. coli* ALS II reacted with bromopyruvate, and the cysteinyl residue 47 is prevented from reacting with bromopyruvate by TPP (Schloss and Aulabaugh, 1990). From the result, we suppose that the cysteinyl residue reacting with

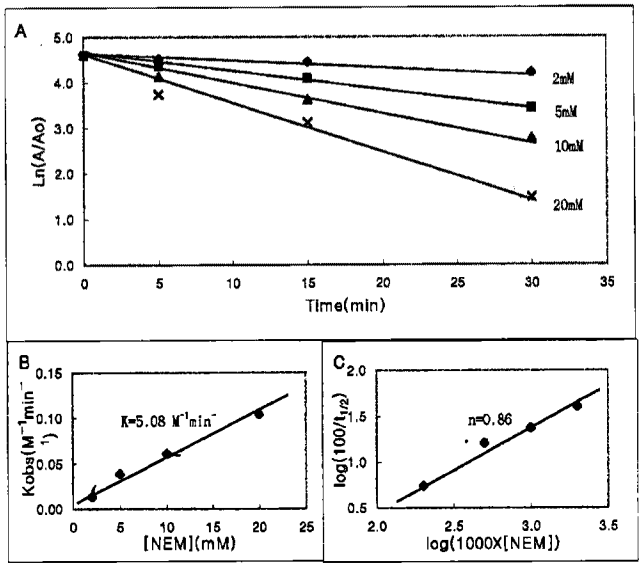

Fig. 3. (A) Inactivation of the catabolic α-ALS by NEM. The enzyme (0.13 μM) was incubated with various concentrations of NEM in 50 mM potassium phosphate buffer (pH 7.0) at 25°C. (B) Plot of the pseudo first-order rate constant for the inactivation of the catabolic α-ALS (K_{obs}) obtained at various concentrations of NEM. (C) Double-logarithmic plot of the half-times of inactivation of the catabolic α-ALS at various NEM concentrations.

Table 1. Effect of bromopyruvate on the catabolic α-ALS.

	Remaining activity (%)		
	0.5 mM	1.0 mM	2.0 mM
0	100.0	100.0	100.0
at 10 min	58.3	51.1	30.4
at 20 min	50.4	44.2	27.6

bromopyruvate in catabolic α-ALS corresponds to the cysteinyl residue 67 in *E. coli* ALS II.

Protection against inactivation by pyruvate The inactivation of the enzyme activity by the chemical modifying reagents does not always directly imply that such a residue is present at the active site (Nakanish *et al.*, 1989). However, protection of the enzyme by the substrates or substrate analogues against inactivation would suggest that the amino acid residues that are protected from inactivation by the chemical modifications are located in or near the active sites of the enzyme. As shown in Fig. 4, when the catabolic α-ALS was

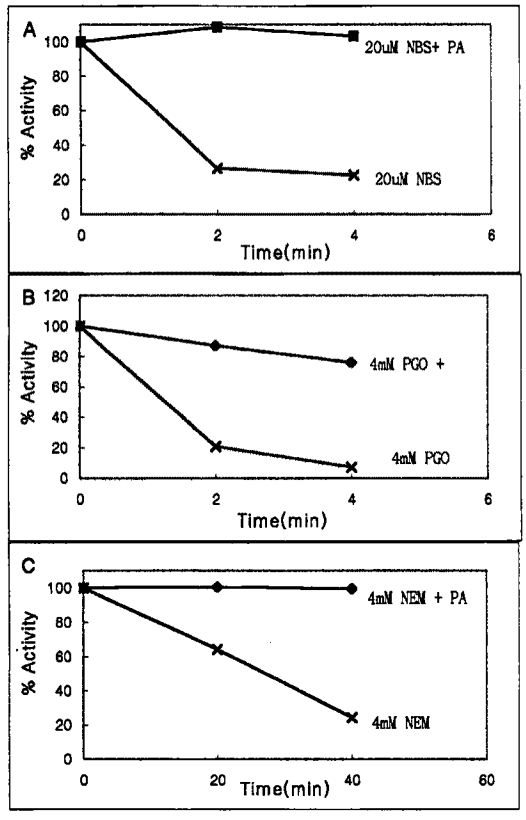

Fig. 4. Protection from chemical modification by pyruvate. The enzyme (0.13 μM) was preincubated with the substrates prior to the addition of each modification reagent. (A) Protection from modification by NBS. (B) Protection from modification by PGO. (C) Protection from modification by NEM.

preincubated with 10 mM pyruvate, the enzyme activity was protected from inactivation by NBS (Fig. 4A), PGO (Fig. 4B), and NEM (Fig. 4C). These results suggest that the tryptophane, arginine, and cysteine residue which are modified by NBS, PGO, and NEM, respectively, are located at or near the active site of the catabolic α-ALS.

Effect of other chemical reagents on the catabolic α-acetolactate synthase The purified enzyme solution was incubated with given concentrations (1.0 and 2.0 mM) of PLP and PMSF in 50 mM potassium phosphate buffer (pH 7.0) for 15 min at 25°C. As shown in Table 2, the catabolic α-ALS was not inactivated by PMSF, a serine-modifying reagent, and PLP, a lysine-modifying reagent. From these results, it can be concluded that serine and lysine are not involved in the reaction of the enzyme.

Effect of divalent metal ions on enzyme activity The purified enzyme that was dialysed for 18 h against column buffer (20 mM potassium phosphate buffer, pH 7.0, 0.1 mM TPP, 0.5 mM DTT, 10 μM FAD, and 10% glycerol) without $MgCl_2$ retained 75% of its original activity. However, the enzyme activity could be fully recovered by addition of divalent metal ions such as Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+}. Thus, the purified catabolic α-ALS is not a metalloenzyme but a metal-activating enzyme (Table 3). In order to determine the effect of divalent metal ions on the acetolactate synthase activity, assays were carried out in the presence of divalent metal ions such as Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, and Ca^{2+} and heavy metals such as Hg^{2+} and Co^{2+}. It was found that enzyme activity was increased approximately 10~100% with 1 mM Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, and Ca^{2+}. On the other hand, the enzyme activity was inhibited by 1 mM Hg^{2+}(Table 3). Complete inactivation of the enzyme by Hg^{2+} suggests that the reduced thiol groups are required for the enzyme activity.

Table 2. Effect of PMSF and PLP on the catabolic α-ALS.

Concentration of the reagent (mM)	remaining activity (%)	
	PMSF	PLP
1.0	99.3	98.4
2.0	96.6	94.5

Table 3. Effect of divalent metal ions on the catabolic α-ALS.

Component[a]	remaining activity (%)
None	100.0
Mg^{2+}	131.0
Mn^{2+}	169.4
Fe^{2+}	56.1
Ca^{2+}	120.1
Zn^{2+}	108.6
Co^{2+}	71.6
Hg^{2+}	26.3

[a] The concentration of metal ion is 1 mM.

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