Identification of the Reactive Sulphydryl Group of 1-Aminocyclopropane-1-carboxylate Deaminase

Mamoru Honma, Jun Kawai, and Masataka Yamada

Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan
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1-Aminocyclopropane-1-carboxylate (ACC) deaminase, a pyridoxal phosphate enzyme that catalyzes cyclopropane ring-opening and deamination of ACC, formed a quinoid intermediate with d-alanine, as shown by the appearance of a 510-nm absorption band. The presence of d-alanine also stimulated the inactivation of ACC deaminase with iodoacetamide. The increase of absorbance at 510 nm and the stimulation of the enzyme inactivation were temperature-dependent with a critical point at around 20°C, indicating a conformational change of the enzyme. To identify a reactive thiol group, this stimulated inactivation and an iodoacetamide derivative, N-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulfonic acid were used. The residue that was modified by the specific reagent was monitored by absorbance at 350 nm through the digestion by lysylendopeptidase and the fractionation of peptides, and it was located at Cys-162 near the midpoint of the whole peptide chain of the ACC deaminase.

A pyridoxal phosphate enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC 4.1.99.4) catalyzes a reaction including the ring-opening and deaminating of ACC, which is an intermediate in the biosynthesis of the fruit-ripening hormone ethylene from l-methionine. The ACC deaminase has been found in microorganisms, but has never been found in higher plants with the exception of transgenic plants. The ACC deaminase from two strains of Pseudomonas sp. was sequenced from protein and DNA to consist of 338 residues. Expression of the ACC deaminase in plants was reported to produce significant results in the reduction of ethylene generation by plant tissues and in the delayed ripening process of fruits, greatly extended shelf lives.

ACC has two enantiomeric β-methylene groups in a cyclopropane ring. A regiospecificity in ring-opening reaction was resolved, but its mechanism remains unresolved. Previous studies reported that 2 mm 5,5'-dithiobis(2-nitrobenzoic acid) inhibited ACC deamination completely. This inhibition was reversible and accompanied with modification of one mole of thiol groups per mole of subunit. But it is not clear whether any thiol group is involved in the ring-opening mechanism. In this paper, we describe the identification of this reactive thiol group among 6 cysteine residues in a subunit of the ACC deaminase of Pseudomonas sp.

Methods

Enzyme. ACC deaminase was prepared from Pseudomonas sp. ACP and its activity was assayed as reported previously. The concentration of ACC deaminase was measured spectrophotometrically using E280 7.45.

Modification of thioles in ACC deaminase. ACC deaminase, 0.5 mg, was incubated in 0.4 ml of 0.05 M potassium phosphate, pH 8.5, containing 0.5 M d-alanine and 0.1 M N-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulfonic acid (1,5-I-AEDANS; Aldrich Chemical Company, Inc.) at 30°C. After 120 min of incubation, 4 μl of 100 mm 2-mercaptoethanol was added to inactivate the remaining reagent and the resulting mixture was dialyzed against 0.05 M potassium phosphate, pH 8.5. Following which, the binding of pyridoxal phosphate to the enzyme was done by reduction with an addition of solid sodium borohydride and further dialyzed as described above. The modified and reduced enzyme was denatured in 6 M guanidine hydrochloride containing 0.5 μl of 2-mercaptoethanol for 15 h under nitrogen at room temperature. Pyridylethylidation of the remaining cysteine residues of the denatured enzyme was done by an addition of 0.75 μl of vinylpyridine and reaction for 1 h. The reaction mixture was acidified with 10 μl of glacial acetic acid and diluted with an equal volume of water. The modified ACC deaminase was purified with an Asahipak C4P-50 column (4.6 x 150 mm) at 30°C with a gradient of 0 to 40% acetonitrile in 0.1% trifluoroacetic acid. Protein fractions were collected and evaporated to dryness.

Lysylendopeptidase digestion and analysis of peptide. The purified fraction of modified ACC deaminase was dissolved in one μl of 8 M urea, pH 9.0, per μg of protein. This was followed by dialysis with an equal volume of 0.01 M Tris, pH 9.0, addition of 1 μg of lysylendopeptidase (Wako Pure Chemicals Co.) per 50 μg of protein, and incubation at 30°C for 23 h. Peptides were separated with an Asahipak C8P-50 column (4.6 x 150 mm) and a gradient of 0 to 40% acetonitrile in 0.1% trifluoroacetic acid. Chromatograms were monitored at 216 nm for peptides, 325 nm for pyridoxyl groups, and 350 nm for the reactive thiol group modified by 1,5-I-AEDANS. Sequences of peptides were analyzed with a protein sequencer (Applied Biosystems Model 477A-120A system).

Proton NMR spectrum of alamine. Alanine and ACC deaminase in 0.2 ml of D2O containing 0.05 M potassium phosphate, pH 8.5, were incubated at 30°C for 4 days in the presence of 1 mM sodium azide. The proton NMR spectra of alamine in the mixtures were checked during the incubation period and their peaks were assigned by the description of Roberts and Jardetzky.

Results

Reaction of ACC deaminase to d-alanine

Inhibition of ACC deaminase by d-alanine is much lower than that by l-alanine (competitive inhibition constant 5.3 mm), as shown in a previous paper. Competitive inhibition constants for d-alanine were calculated to be 660 mm at 30°C and 610 mm at 20°C at pH 8.5. In the other hand, an addition of a high concentration of d-alanine caused the appearance of a new band at 510 nm in the ACC deaminase spectrum (Fig. 1), indicating x-proton

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate, 1,5-I-AEDANS, N-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulfonic acid.
elimination of d-alanine. In fact, the proton NMR spectrum of 1 M d-alanine in D₂O had the signal of an a-proton that disappeared after incubation with ACC deaminase (26.7 units/ml) at pH 8.5 at 30°C for 4 days, while that of l-alanine did not have any alteration during the 4 days of incubation. The absorbance at 510 nm of ACC deaminase depended on d-alanine concentration and temperature. Concentrations of d-alanine required for half of the maximum change in the absorbance at 510 nm were 310 mM at 30°C and 450 mM at 20°C. Figure 2 shows that the absorbance at 510 nm of ACC deaminase (0.97 mg/ml) with 0.5 M d-alanine rose with increasing temperature, with the critical point being at around 20°C.

Effects of d-alanine on ACC deaminase inactivation with iodoacetamide

To investigate the effects of several amino acids on the ACC deaminase inactivation, the ACC deaminase was incubated with 10 mM iodoacetamide and 100 mM or 200 mM amino acid under the same conditions as those for the enzyme assay (Table). The remaining activities showed that L-amino acids protected the enzyme more effectively than the d-forms, and the substrate ACC was much more effective than its analog a-aminoisobutyrate. However, d-alanine was stimulative rather than protective for the inactivation. This stimulation depended on the d-alanine concentration (Fig. 3) and concentrations for a half of maximum stimulation were 320 mM at 30°C and 540 mM at

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Fig. 1. Effects of d-Alanine on Absorption Spectra of ACC Deaminase. ACC deaminase (1.65 mg/ml) was held at pH 8.5 and 25°C in 0, 0.2, 0.4, and 0.8 mM d-alanine (top to bottom at 420 nm and bottom to top at 330 nm and 510 nm).

Fig. 2. Effects of Temperature on Absorbance at 510 nm in the Presence of 500 mM d-Alanine.

Fig. 3. Effects of d-Alanine on the ACC Deaminase Inactivation by Iodoacetamide. ACC deaminase (0.17 mg/ml) was incubated with 1 mM iodoacetamide at pH 8.5 and 30°C in the presence of 0, 75, or 300 mM d-alanine (1, 2, or 3, respectively). The resulting mixture (30 μl) was used for assay of the remaining activity.

Fig. 4. Effects of Temperature on the ACC deaminase Inactivation by 1 mM Iodoacetamide in the Presence of 500 mM d-Alanine. Conditions are the same as Fig. 3 except for concentration of d-alanine.
20°C with 1 mM iodoacetamide. These values are similar to those for the absorbance at 510 nm. The similarity between the stimulation of the inactivation and the absorbance at 510 nm was also shown in temperature dependence (Fig. 4), indicating a critical point at around 20°C.

Modification of ACC deaminase with N-(iodoacetamideth- yl)-1-aminonaphthalene-5-sulfonic acid (1,5-I-AEDANS)

As shown in Fig. 3, ACC deaminase was slightly inactivated by incubation with 1 mM iodoacetamide for 120 min in the absence of d-alanine, but it was significantly inactivated in the presence of 300 mM d-alanine. To locate the modification site in the inactivated ACC deaminase, 1,5-I-AEDANS was used. This naphththalene derivative showed an absorption band at 337 nm, ε: 6800, with 88% of the absorption maximum at 350 nm. The modification site was monitored with absorbance at 350 nm where absorbance of the pyridoxyl group was significantly lower. The reagent, 1,5-I-AEDANS inactivated the ACC deaminase more effectively than iodoacetamide, that is, the ACC deaminase, at 30 µg/100 µl, was inactivated after 60 min of incubation with 1 mM reagent by 31% and 82.9% in the absence and in the presence of 0.5 mM d-alanine, respectively, while the inactivation with 0.1 mM reagent was 10% and 68.6% in the absence and in the presence of the d-alanine, respectively under the same conditions. When the ACC deaminase, at 500 µg/400 µl, was incubated with 0.1 mM 1,5-I-AEDANS and 0.5 mM d-alanine as described in Methods, the degree of the inactivation was 70.5%. Fixation of pyridoxal phosphate, pyridylethylated free thiol groups, and purification of the modified ACC deaminase followed.

Identification of the modified reactive residue

The ACC deaminase has 11 lysine residues including a pyridoxal phosphate binding site. The reduced and pyridylethylated ACC deaminase, 68 µg, was digested with lysylendopeptidase and the digest was separated by reversed phase chromatography into 10 main peaks (Fig. 5B). With the ACC deaminase, which was modified by 1,5-I-AEDANS in advance, the reversed phase chromatogram had 2 new peaks; 11 and 12 in Fig. 5A, and a drop in 2 peaks; 2 and 8. The new peak 12 was the only one that had a higher absorbance at 350 nm, and the sequence of the peptide of this peak was the same as that of peak 8 except for a cysteine residue, that is, PYAIPAGXSDDHPLGG. This sequencing shows that the residue modified with 1,5-I-AEDANS was Cys-162 (Fig. 7). A peak involving pyrdoxyllysine was lower in the 1,5-I-AEDANS modified ACC deaminase than in the control, indicating that this modification caused a decrease of the aldime bond of pyridoxal phosphate with the lysine residue 51. Consequently a new peptide, peak 11, appeared.

Discussion

Four findings were reported in preceding studies on the mechanism of the ACC deaminase reaction: 1) the ACC deaminase catalyzed deamination of (1S, 2S) 2-ethyl-ACC to α-ketocaproic acid, deamination of β-chloro-d-alanine and d-serine to pyruvate, and deamination of ACC through the cleavage of a bond between pro-S methylene and α-carbon atom of pro-R methylene of the ACC was replaced stereoselectively with one of the solvent; 3) L-alanine was a stronger inhibitor of ACC deamination than d-alanine; 4) ACC deaminase was inactivated by modification of a thiol group per its subunit.

The proton NMR spectrum of alanine in D₂O showed an α-proton exchange of d-alanine with a deuterium of ACC deaminase, but it did not show that of l-alanine under the same conditions. This explains the deamination of β-chloro-d-alanine and d-serine by ACC deaminase, and this is consistent with the stereospecific cleavage of the cyclopropane ring in ACC deamination. The direction of bond cleavage around the α-carbon of d-serine by the ACC deaminase is the same as that of a ring-opening bond cleavage in the case of ACC. Therefore, l-serine is not a substrate for ACC deaminase but instead a good inhibitor. Proton elimination from the α-carbon of d-alanine was also indicated by the appearance of a new band at 510 nm in the optical absorption spectrum that showed the occurrence of the quinoidal form of pyridoxal phosphate (Fig. 1). Another observation concerning d-alanine was the stimulation of ACC deaminase inactivation by a thiol reagent, iodoacetamide (Fig. 3). Concentration of d-alanine for the half value of the maximum absorbance at 510 nm and that of the stimulated inactivation were close to each other, as shown in “Results”, indicating that these two phenomena were due to the same change of the enzyme by d-alanine.

In the case of aspartate aminotransferase, it was reported that the substrates or their analogs stimulated the reactivity of a certain thiol group of the enzyme with thiol reagents. This was associated with the hypothesis that substrate binding caused a conformational change of the enzyme, which was indicated by crystallographic studies. Reactivity of a thiol group in the ACC deaminase was depressed by the addition of ACC or L-alanine, the structure of which was like a part of the substrate ACC. Therefore, the stimulation of this reactivity by d-alanine

Fig. 5. Chromatograms of Lysylendopeptidase Digests of the ACC Deaminase.

Lysylendopeptidase digests of 79 µg of ACC deaminase modified with 1,5-I-AEDANS (A) and 68 µg of control ACC deaminase (B) were fractionated by reversed phase chromatography, being monitored by absorbance at 216, 325, and 350 nm.

Fig. 6. Structure of ACC and D-Serine.

Dotted lines drawn across the bonds indicate cleavage sites by ACC deaminase.
is thought to be due to an intermediate of the enzyme with the quinoidal pyridoxal phosphate conjugate, not to the substrate binding itself. The temperature dependence of this stimulated inactivation showed a critical point at around 20°C (Fig. 4). The same critical temperature was shown with absorbance at 510 nm of the ACC deaminase in the presence of D-alanine (Fig. 2) and by $k_{cat}$ values of the ACC deamination. These results suggested that these reactions were affected by a certain conformational change of the enzyme.

An amino acid sequence between 34 and 52, including a pyridoxal phosphate binding site of the ACC deaminase, was homologous with a sequence from 73 to 88 of tryptophan synthase $\beta$-subunit, the three-dimensional structure of which has been reported (Fig. 7). The ACC deaminase is shorter by 59 residues than the tryptophan synthase $\beta$-subunit, especially in an N-terminal side, when two sequences are aligned by matching at pyridoxal phosphate binding sites. The tryptophan synthase $\beta$-subunit is composed of two domains, the N- and C-domains, divided at Phe-204, and has three tryptic cleavage sites, Lys-272, Arg-275, and Lys-283, which were identified in a long hinge region. If the structures are assumed to be similar in these two enzymes, a tryptic cleavage site of the ACC deaminase at Arg-250 would correspond to the hinge region of the $\beta$-subunit, and a reactive thiol group of the ACC deaminase, Cys-162 would be near a position corresponding to the dividing point between the N- and C-domains.

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References