Substitutions of Alanine for Cysteine at a Reactive Thiol Site and for Lysine at a Pyridoxal Phosphate Binding Site of 1-Aminocyclopropane-1-carboxylate Deaminase

Toyotaka Murakami, Miwa Kiuchi, Hiroyuki Ito, Hirokazu Matsui, and Mamoru Honma

Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan
Received September 10, 1996

1-Aminocyclopropane-1-carboxylate (ACC) deaminase catalyzes the cyclopropane ring fragmentation and deamination of ACC. Replacement of cysteine with alanine at a reactive thiol site, Cys-162, of ACC deaminase did not affect the enzyme activity, in spite of the previous result that modification of Cys-162 caused complete loss of the enzyme activity. Substitution of glycine or valine for the cysteine residue gave a higher $K_m$ for ACC without a significant change of the $k_\text{cat}$, indicating that changes of the amino acid side chain had structural effects on substrate binding.

Replacement of lysine with alanine at the pyridoxal phosphate (PLP) binding site of the ACC deaminase caused a lower content of PLP and loss of detectable activity of ACC deamination. This mutant enzyme, K51A, showed absorption peaks at 330 nm and 405 nm. The peak at 405 nm was shifted to about 425 nm by the addition of ACC, $\delta$-l-alanine, and $\delta$-l-serine. The formation of aldime complexes indicated by the spectral shift was reversible. It is suggested that lysine 51 affects the formation of holoenzyme and is important in catalysis.

Key words: 1-aminocyclopropane-1-carboxylate deaminase; site directed mutagenesis; reactive thiol group; pyridoxal phosphate enzyme

A pyridoxal phosphate (PLP) enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC 4.1.99.4) catalyzes a cleavage of ACC (Fig. 1), which is an intermediate in the biosynthesis of the plant hormone ethylene from l-methionine. This cleavage reaction includes cyclopropane ring fragmentation and deamination of the ACC to form $\alpha$-ketobutyrate and ammonia. The following findings have been reported with regard to the cyclopropane ring fragmentation of ACC; 1) the cyclopropane ring was opened at a bond between $\alpha$ carbon and pro-S methylene carbon as shown in Fig. 1, 2) one of the C-3 methylene hydrogens of the product, $\alpha$-ketobutyrate, was exchanged with solvent hydrogen during the enzyme reaction. Two alternative mechanisms consistent with these findings were discussed in a previous paper (Fig. 2). In the first one (1), the ring fragmentation is done by attack of an active-site nucleophile at a methylene carbon, and in the second one (2), it is initiated concerted by a proton abstraction from pro-S $\beta$-methylene. As noted previously, the ACC deaminase reaction can be regarded as an intramolecular $\gamma$ elimination of the PLP enzyme. Therefore, is conceivable, but unproven, that ACC deaminase catalysis proceeds by a nucleophilic attack. It was previously reported that ACC deaminase processed the $\delta$-isomers but not l-isomers of serine and $\beta$-chloroalanine to pyruvate. Thus there is one active-site base for proton removal from the $\alpha$ carbon of the $\delta$-amino acid. Since the ACC places a pro-S methylene carbon in line with the $\alpha$ proton of the $\delta$-amino acid in the substrate-PLP aldime complex (Fig. 3), it is also conceivable that the active-site base acts as a nucleophile in ACC deaminase catalysis.3 We previously described identification of the 162nd cysteine residue as a site of a reactive

![Fig. 1. Reaction of ACC Deaminase.](image)

**Abbreviations:** ACC, 1-aminocyclopropane-1-carboxylate; PLP, pyridoxal phosphate; SDS, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

![Fig. 2. Suggested Mechanisms of Cyclopropane Ring Opening Reaction in ACC Deaminase Catalysis.](image)
Materials and Methods

**Bacterial strains, plasmids, plagues, and restriction enzymes.** The *Escherichia coli* strains used were JM109 as a host of plasmids and CJ236, MV1184, and BMH71-18 for Kunkel’s method. These strains were purchased from Takara Shuzo Co., Ltd., Japan. The construction of a ACC deaminase gene containing plasmid pCG1472 was described previously.  

Plasmid pUC18 was purchased from Takara Shuzo Co., pTrc99A from Pharmacia Biotech, and pBluescript, M13mp18, and M13mp19 from Toyobo Co., Ltd., Japan. All the restriction enzymes used were from Takara Shuzo Co.

**Expression vectors.** The vector used for Cys-162 replacement was pUC19, which resulted in the same structure as pCG1472. For Lys-51 replacement, a new expression vector, pACD2, was prepared from pCG1472 and plasmid pTrc99A to establish overexpression. The NdeI (blunt end)-SacI fragment of pCG1472 was inserted into BamHI (blunt end)-SacI site of pTrc99A. The sequence of pACD2 around the N-terminal is ATGGAGATTATG, corresponding to a sequence with an excess of 3 residues, Met-Glu-Phe. Strain JM109 containing pACD2 produced a much higher level of ACC deaminase than the original bacterium.

**Site-directed mutagenesis.** Mutagenesis was done by the method of Kunkel.  

Templates for this method were prepared from pU11 fragments of pCG1472 and M13mp19 or from BamHI-EcoRI fragments of pCG1472 and M13mp18. The following oligonucleotides were synthesized by an Applied Biosystems DNA Synthesizer, 3800B:

- TGGTATG TAC CGA GGC GCC TGC CGG-3’ (C162A)
- TGGTATG GTA GCC GCC TGC CAT CAC CGG-3’ (C162G)
- TGGTATG GTA GCC GCC TGC CAT CAC CGG-3’ (C162S)
- TGGTATG GTA GCC GCC TGC CAT CAC CGG-3’ (C162V)
- TGGTATG GTA GCC GCC TGC CAT CAC CGG-3’ (C162Q)
- TGGTATG GTA GCC GCC TGC CAT CAC CGG-3’ (K51A)
- TGGTATG GTA GCC GCC TGC CAT CAC CGG-3’ (K51A)

To identify the obtained Cys-162 mutant plasmids, Tkb111 II fragments of 240 base pairs containing Cys-162 site were sequenced with an Applied Biosystems DNA Sequencer 370A. The sequence around the mutagenic site of the K51A mutant plasmid was confirmed and a lysylendopeptidase fragment containing residue 51 was sequenced using Applied Biosystems Protein Sequencer 477A.

**Growth of bacteria and purification of enzymes.** The bacterial host strain E. coli JM109 containing pCG1472 or its mutant plasmids was grown in 1% Trypton, 0.5% yeast extract, and 0.5% NaCl at 37°C. The same strain containing pACD2 or its mutant plasmids were incubated in 1.6% Trypton, 1% yeast extract, 0.5% NaCl, 0.005% ampicillin, and 0.01% pyrimidine (pH 7.0) at 30°C. In this case, isopropyl-β-D-thiogalactoside was added in a final concentration of 2 mM when bacterial growth proceeded to the degree of absorbance unit 1.0 at 600 nm, and further incubation for 15h followed. Bacterial cells containing pACD2 from 50 ml of culture were suspended in 4 ml of 0.05 M potassium phosphate, pH 7.5, and treated with a French press. The cell extract obtained by centrifugation of a homogenate was fractionated by 25 to 60% saturation of ammonium sulfate. A precipitate was dissolved in 0.05 M potassium phosphate, pH 7.5, and dialyzed against the same buffer. From the resulting solution, ACC deaminase was purified using a DEAE-cellulose column (1×9 cm) and 0 to 0.3 M potassium chloride (40 ml), a butyl-Toyopearl column (1×7 cm) and 25 to 0% saturated ammonium sulfate (40 ml), and gel filtration with Sephadex G-150 (1.5×60 cm). The purification procedure is summarized in Table I. The purity of the ACC deaminase was shown by a single band on an SDS-polyacrylamide gel electrophoresis and analysis of a single N terminal sequence. Buffer solution used in the purification process was 30% glycogen when mutagenic ACC deaminase was unstable. For pCG1472 and its mutants, a DEAE-Toyopearl column (1×5 cm) and 0 to 0.25 M potassium chloride (100 ml) were used instead of the gel filtration.

**Assays.** Deamination activity toward ACC was measured as described previously. The concentration of ACC deaminase was dosed spectrophotometrically using E$_{1%}$550 = 7.45. PLP was measured by the method of Wada and Snell.

**Electrophoresis.** Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was done by method of Laemmli. Gels were used for protein staining and immunoblot with antibody produced against the purified ACC deaminase.

**Results**

**Activity of C162A mutant enzyme**

As shown in our previous paper, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reacted effectively with a thiol group of the ACC deaminase subunit and inhibited the enzyme activity. The reaction of the thiol group with iodoacetamide was stimulated by the presence of d-alanine, and use of this reaction revealed a reactive thiol group located in Cys-162. Replacement of Cys-162 by alanine, however, did not affect the ACC deaminase activity. The kinetic parameters of C162A were almost the same as those of the wild-type enzyme (Table II). This activity was not inhibited by DTNB under the conditions in which the wild-type enzyme activity was done (Table III), and inhibition by iodoacetamide was not stimulated by d-alanine in the C162A enzyme, which was more labile rather in the presence of d-alanine.

**Substitution of various amino acids for cysteine 162**

To consider the difference between inactivation by thiol reagents and the activity of C162A, we tested substitution of various amino acids at Cys-162. Table II shows that $K_m$ for ACC depends on the length of the side chain of the amino acid at residue 162, and there is no significant difference in $k_0$, except for a little lower value of C162S, the side chain of which has a polar group.

We failed in isolation of the C162Q mutant enzyme although the DNA sequence was correct. A cell extract with 0.05 M potassium phosphate, pH 7.5, showed no activity.

**Table I. Purification of Wild-type ACC Deaminase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>144</td>
<td>48.4</td>
<td>0.34</td>
<td>100</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>20.4</td>
<td>21.9</td>
<td>1.07</td>
<td>45</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>6.0</td>
<td>15.4</td>
<td>2.57</td>
<td>32</td>
</tr>
<tr>
<td>Butyl-Toyopearl</td>
<td>4.3</td>
<td>14.3</td>
<td>3.33</td>
<td>30</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>1.7</td>
<td>8.9</td>
<td>5.24</td>
<td>18</td>
</tr>
</tbody>
</table>

phosphate, pH 7.5, and treated with a French press. The cell extract obtained by centrifugation of a homogenate was fractionated by 25 to 60% saturation of ammonium sulfate. A precipitate was dissolved in 0.05 M potassium phosphate, pH 7.5, and dialyzed against the same buffer. From the resulting solution, ACC deaminase was purified using a DEAE-cellulose column (1×9 cm) and 0 to 0.3 M potassium chloride (40 ml), a butyl-Toyopearl column (1×7 cm) and 25 to 0% saturated ammonium sulfate (40 ml), and gel filtration with Sephadex G-150 (1.5×60 cm). The purification procedure is summarized in Table I. The purity of the ACC deaminase was shown by a single band on an SDS-polyacrylamide gel electrophoresis and analysis of a single N terminal sequence. Buffer solution used in the purification process was 30% glycogen when mutagenic ACC deaminase was unstable. For pCG1472 and its mutants, a DEAE-Toyopearl column (1×5 cm) and 0 to 0.25 M potassium chloride (100 ml) were used instead of the gel filtration.

**Assays.** Deamination activity toward ACC was measured as described previously. The concentration of ACC deaminase was dosed spectrophotometrically using $E_{1%}$550 = 7.45. PLP was measured by the method of Wada and Snell.

**Electrophoresis.** Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was done by method of Laemmli. Gels were used for protein staining and immunoblot with antibody produced against the purified ACC deaminase.

**Results**

**Activity of C162A mutant enzyme**

As shown in our previous paper, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reacted effectively with a thiol group of the ACC deaminase subunit and inhibited the enzyme activity. The reaction of the thiol group with iodoacetamide was stimulated by the presence of d-alanine, and use of this reaction revealed a reactive thiol group located in Cys-162. Replacement of Cys-162 by alanine, however, did not affect the ACC deaminase activity. The kinetic parameters of C162A were almost the same as those of the wild-type enzyme (Table II). This activity was not inhibited by DTNB under the conditions in which the wild-type enzyme activity was done (Table III), and inhibition by iodoacetamide was not stimulated by d-alanine in the C162A enzyme, which was more labile rather in the presence of d-alanine.

**Substitution of various amino acids for cysteine 162**

To consider the difference between inactivation by thiol reagents and the activity of C162A, we tested substitution of various amino acids at Cys-162. Table II shows that $K_m$ for ACC depends on the length of the side chain of the amino acid at residue 162, and there is no significant difference in $k_0$, except for a little lower value of C162S, the side chain of which has a polar group.

We failed in isolation of the C162Q mutant enzyme although the DNA sequence was correct. A cell extract with 0.05 M potassium phosphate, pH 7.5, showed no activity.
of ACC deaminase and a very weak band corresponding to ACC deaminase on SDS polyacrylamide gel electrophoresis by protein staining and immunoblot, while a cell extract prepared with 1% SDS showed an obvious band.

**Activity of the K51A mutant enzyme**

The ACC deaminase activity of the purified K51A mutant enzyme, which was homogeneous on SDS electrophoresis and showed a single N terminal sequence, was not detected, while the specific activity of wild-type enzyme was 5.2 units/mg, close to that of *Pseudomonas* enzyme. The effects of methyamine and ethylamine were tested, but the presence of 1 to 100 mM each amine did not make the ACC deaminase activity of the mutant enzyme detectable; that was less than 0.04% of the activity of the wild type enzyme.

**The absorption spectrum of the K51A mutant enzyme**

The absorption spectrum of the wild-type enzyme had absorbance bands at 330 nm and 416 nm in the region above 300 nm, and its profile was similar to the published one of the *Pseudomonas* enzyme (Fig. 4). The K51A mutant enzyme showed an absorbance band at 405 nm instead of the 416-nm band. The 405-nm band, which was attributed to the aldehyde form of PLP, was lower than the 416-nm band, in fact, PLP contents were assayed to be 0.21 and 0.70 mol/mol subunit of the mutant and the wild-type enzymes, respectively. The reason for a lower value in the mutant enzyme and identity of the 330-nm band are not known.

**Effects of substrates on the spectrum of the K51A mutant enzyme**

An absorbance band of the K51A mutant enzyme shifted from 405 nm to 425 nm in the presence of ACC (Fig. 5). This spectral shift, which was ascribed to formation of a Schiff base between free PLP and ACC was complete within 15 min after an addition of 10 mM ACC even at 10°C. From the increased absorbance in 10 min of incubation at 30°C, dissociation constants were calculated for ACC, alanine, and serine (Table IV). The constants of D isomers were higher than that of L isomers, indicating a similar relationship to competitive inhibition constants in ACC deamination. Formation of these Schiff base complexes was so reversible that the absorbance band around 425 nm shifted back to around 405 nm by dialysis against 0.05 M potassium phosphate containing 30% glycerol. Measurement of PLP did not show a loss of PLP from the K51A mutant enzyme during the reaction with amino acids and the dialysis of

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_a$ (mM)</th>
<th>$k_o$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>1.7</td>
<td>3.5</td>
</tr>
<tr>
<td>C162A</td>
<td>1.6</td>
<td>3.3</td>
</tr>
<tr>
<td>C162G</td>
<td>4.6</td>
<td>3.2</td>
</tr>
<tr>
<td>C162S</td>
<td>1.9</td>
<td>2.2</td>
</tr>
<tr>
<td>C162V</td>
<td>38</td>
<td>3.4</td>
</tr>
</tbody>
</table>

**Table III. Inactivation with Thiol Reagents**

After incubation of ACC deaminase with reagents for 20 min at 0°C (1) or for 120 min at 30°C (2), residual enzyme activities were measured.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>1 mM DTNB</td>
<td>14.6</td>
</tr>
<tr>
<td>1 mM Iodoacetamide</td>
<td>95.4</td>
</tr>
<tr>
<td>200 mM D-Alanine</td>
<td>85.0</td>
</tr>
<tr>
<td>1 mM Iodoacetamide + 200 mM D-Alanine</td>
<td>32.5</td>
</tr>
</tbody>
</table>

![Fig. 4. Absorption Spectra of Wild-type (broken) and K51A (solid) Enzymes.](image)

![Fig. 5. Effects of ACC on Absorption Spectrum of K51A Enzyme. Mutant enzyme was incubated in 0.05 M potassium phosphate, pH 7.5, containing 0 (C), 0.01 (●), 0.05 (■); or 10 (○) mM ACC at pH 7.5.](image)
enzyme-amino acid complexes.

Discussion
The ring fragmentation of cyclopropane is a key reaction of ACC deaminase catalysis and makes an intermediate of γ elimination of an amino acid which bears no abstractable proton at α carbon (Fig. 1). Nucleophilic attack is conceivable for this ring fragmentation as described in a previous paper.4) The ACC deaminase subunit was found to have a reactive thiol group, the modification of which led to complete loss of the deaminase activity.10) Especially, inhibition by iodoacetamide was stimulated in the presence of D-alanine, not of the L isomer.8) Since D-alanine made an ACC deaminase complex having a 510-nm band in its absorption spectrum and the L isomer did not, it was suggested that a quinoidal form of the enzyme-bound PLP-substrate complex was more reactive to the iodoacetamide, and this reactive thiol group was identified as Cys-162. Results in this paper indicated that the substitution of alanine for Cys-162 caused no loss of the deaminase activity. As the obtained mutant enzyme C162A was not inhibited by thiol reagents under the conditions that inhibited the wild-type enzyme, a reactive thiol group was evidently located at Cys-162. It is clear that the Cys-162 thiol group is not involved in ACC deaminase catalysis as a nucleophilic group. The results suggest that the modification of Cys-162 by thiol reagents made the structural effects against the enzyme activity, and this suggestion was supported by findings of higher $K_m$ for ACC with longer or shorter side chains, C162V or C162G, than alanine at a position of Cys-162 (Table II). The C162V mutant enzyme had a 22-fold higher $K_m$ than the wild-type and C162A mutant enzymes did. Furthermore, the active form of C162Q mutant enzyme was not detected.

The roles of the lysine residues that form internal aldimine with PLP have been investigated in L-aspartate aminotransferase,13 1-α-amino acid aminotransferase,13,14 1-α-histidine decarboxylase,15) and tryptophan synthase16 using mutant enzymes. These studies indicate that these lysine residues are important in other ways besides the internal aldimine formation. The results of this paper show that lysine 51 of ACC deaminase serves specifically in formation of holoenzyme and catalysis, as well as the internal aldimine formation. The PLP contents of the purified K51A mutant enzyme were 0.21 mol/mol subunit, while that of the purified wild-type enzyme was 0.7 mol/mol, indicating that about 80% of the mutant enzyme molecules were in apo-form. This seems to be a reason why the K51A mutant enzyme was so unstable that the presence of 30% glycerol was essential during all the experiments. The low PLP contents was not improved by dialysis against PLP solution, and bound PLP in the mutant enzyme was not removed by the reaction with amino acids and the dialysis against the buffer.

The bound PLP of the K51A mutant enzyme formed external aldimine with substrate ACC as shown by a spectral shift from 405 nm to 425 nm. This spectral shift was saturated by a much lower concentration of ACC than the $K_m$ of original ACC deaminase. The apparent dissociation constants of several amino acids (Table IV) were also much lower than their $K_i$ toward ACC deamination of the original enzyme. Regardless of these lower values, K51A mutant enzyme showed higher affinity to i isomer than to D isomer of alanine and serine. This tendency of stereoselectivity is the same with the original enzyme. So far, there is no evidence to imply that the K51A enzyme catalyzes the cyclopropane ring fragmentation in ACC both from the measurement of a product, z-ketobutyrate, and from analysis of the spectral change. These findings suggest that the lysine 51 residue of ACC deaminase is important in catalysis in a different way from the other PLP enzymes.

References