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Lys296 and Arg299 residues in the C-terminus of MD-ACO1 are essential for a 1-aminocyclopropane-1-carboxylate oxidase enzyme activity

Ahrim Yoo [a,1], Young Sam Seo [b,1], Jin-Won Jung [c], Soon-Kee Sung [d], Woo Taek Kim [b,*], Weontae Lee [c,e,*], Dae Ryook Yang [a,*]

[a] Department of Chemical and Biological Engineering, Korea University, Seoul 136-713, Republic of Korea
[b] Department of Biology, College of Science, Yonsei University, Seoul 120-749, Republic of Korea
[c] Department of Biochemistry and National Research Laboratory of HTSD-NMR and Application, Yonsei University, Seoul 120-749, Republic of Korea
[d] Plant Biotechnology Team, Dongbu Advanced Research Institute, Daejeon 305-708, Republic of Korea
[e] Protein Network Research Center, College of Science, Yonsei University, Seoul 120-749, Republic of Korea

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Abstract

The 1-aminocyclopropane-1-carboxylate (ACC) oxidase catalyzes the last step in the biosynthesis of ethylene from ACC in higher plants. The complex structure of ACC oxidase/Fe^{2+}/H_2O derived from *Petunia hybrida* has recently been established by X-ray crystallography and it provides a vast structural information for ACC oxidase. Our mutagenesis study shows that both Lys296 and Arg299 residues in the C-terminal helix play important roles in enzyme activity. Both K296R and R299K mutant proteins retain only 30–15% of their enzyme activities with respect to that of the wild-type, implying that the positive charges of C-terminal residues are involved in enzymatic reaction. Furthermore, the sequence alignment of ACC oxidases from 24 different species indicates an existence of the exclusively conserved motif (Lys296-Glu301) especially in the C-terminus. The structure model based on our findings suggests that the positive-charged surface in the C-terminal helix of the ACC oxidase could be a major stabilizer in the spatial arrangement of reactants and that the positive-charge network between the active site and C-terminus is critical for ACC oxidase activity.

Keywords: Comparative modeling; Molecular dynamics; Site-directed mutagenesis; 1-Aminocyclopropane-1-carboxylate oxidase; C-terminus; Substrate binding

1. Introduction

The gaseous plant hormone, ethylene, regulates many processes of plant growth, development, and senescence (Abeles et al., 1992; Yang and Hoffman, 1984). 1-Aminocyclopropane-1-carboxylate (ACC) oxidase catalyzes the last step in the biosynthesis of ethylene from ACC in higher plants (Kim and Yang, 1994; Yang and Hoffman, 1984). The first identification of a cDNA clone, pTOM13, encoding ACC oxidase was achieved in tomato fruit through an RNA antisense strategy (Hamilton et al., 1990). The deduced amino-acid sequence of pTOM13 shows homology to that of flavone 3-hydroxylase, which requires both Fe^{2+} and ascorbate for its enzyme activity (Hamilton et al., 1991). Thereafter, it was demonstrated that purified ACC oxidase (MD-ACO1) from ripening apples (*Malus domestica* Borkh.) requires CO_2 as an activator of enzyme activity (Dong et al., 1992).

The ACC oxidase is a member of the non-heme iron enzyme family in which most of their members require Fe^{2+} and 2-oxoglutarate as a co-substrate.

* Corresponding authors. Fax: +82 2 362 9897 (W. Lee).
E-mail addresses: wtkim@yonsei.ac.kr (W.T. Kim), wlee@spin.yonsei.ac.kr (W. Lee), dryang@korea.ac.kr (D.R. Yang).
[1] These authors contributed equally.

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However, ACC oxidase and isopenicillin N synthase (IPNS, another member of this family) do not require 2-oxoglutarate (Schofield and Zhang, 1999). The crystal structures of both apo- and metal-complexed ACC oxidase derived from *Petunia hybrida* have recently been reported (Zhang et al., 2004). Since the ACC oxidase of *P. hybrida* has a high sequence identity to the MD-ACO1, the crystal structure (PDB ID: 1WA6) provides an insight for the structure–function studies of the MD-ACO1. The common Fe^{2+}-binding motif (His-X-Asp-X(54)-His) and the putative co-substrate hydrogen-binding residues (Arg-X-Ser) are well conserved as other members in the non-heme iron enzyme family. Before the structure is reported by X-ray crystallography, we previously predicted a three-dimensional model of the MD-ACO1 using site-directed mutagenesis and comparative modeling methods (Seo et al., 2004). Our structure model was used to establish the binding mode with the Fe^{2+}, the substrate (ACC) and the co-substrate (ascorbate). Although the crystal and predicted structures show some differences in overall topologies, the active sites in our model were consistent with those of the crystal structure. These findings support the results of our previous study: the ascorbate is located in cofactor Fe^{2+}-binding pocket, which consists of His177, Asp179, and His234; the ascorbate interacts with the side chains of Arg244 and Ser246, composing hydrogen-bonding network in the hydrophobic wide cleft of the active site near the C-terminus.

It is known that ACC oxidase requires CO_2 as an activator to increase the yield of ethylene unlike other members of non-heme enzyme family, but the activation mechanism by CO_2 is less understood. Rocklin et al. (1999) proposed the single turnover reaction mechanism based on experimental data from the electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) studies (Rocklin et al., 2004, 1999). The single turnover reaction mechanism begins with ACC and O_2 binding to Fe^{2+} simultaneously, thereby the oxidized iron promotes one-electron reduction to form a peroxy intermediate. They suggested that the CO_2/bicarbonate would mediate the proton transfer from the peroxy intermediate to high-valent iron species, which are thought to be the actual oxidizing reagent for the ACC to produce ethylene (Solomon et al., 2000). They thought that the bicarbonate could be stabilized by an electrostatic interaction with Arg299 (Rocklin et al., 1999) or Arg244 (Solomon et al., 2000). In this report, the structure–functional approach which combines the site-directed mutagenesis and the comparative modeling methods (Seo et al., 2004) was applied to find the exact role of the charged residues in the C-terminus for ACC oxidase activity. The previous results already showed that the Arg244 participates in the ascorbate binding. The Arg299 of the MD-ACO1 located on the last helix forming the C-terminus. Since the C-terminal helix locates far away from the active sites in the crystal structure of the *P. hybrida* (PDB ID: 1WA6), the C-terminus of the protein is not likely involved in the enzyme activity. However, the sequences in the C-terminal helix are exclusively conserved for all known ACC oxidases. And the Lys296 or Arg299 of MD-ACO1 has possibility to be aligned to Gln330 of IPNS which has the on–off binding property. In case of absence of substrate, the metal is coordinated by Fe^{2+}-binding motif (His-X-Asp-X(54)-His), Gln330 and two water molecules. On the contrary, in case of existence of substrate (L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV)), the thiolate group of ACV replaces the metal binding of Gln330 (Roach et al., 1997). These local conformational changes of the IPNS are known as an important mechanism to protect the metal site from chelation as well as from autooxidation leading to damaging oxygen and radical adducts (Ivano et al., 2001). It could be inferred from the reactive properties of the IPNS in the same family that the Arg299 and Lys296 have something to do with enzyme activity. The site-directed mutagenesis was used in order to investigate whether the residues in the C-terminal helix could participate in the oxidation reaction. The nine residues (Leu-X(7)-Ala) near to C-terminal helix form a long loop in the crystal structure. If the loop is flexible in aqueous solution, its flexibility may lead to move the C-terminal helix toward the active sites. The molecular dynamic simulation was performed to examine whether the local conformational changes occur in the ACC oxidase similar to that of the IPNS.

2. Materials and methods

2.1. Cloning and expression of wild-type and mutant MD-ACO1 proteins

The MD-ACO cDNA from *M. domestica* Borkh. cv. Golden Delicious apple was introduced into pET20b+ (Novagen Inc., Madison, WI, USA) overexpression plasmid, containing an isopropyl β-D-thiogalactoside-inducible promoter, and used for expression of wild-type recombinant MD-ACO1. The mutant MD-ACO1 clones were produced by site-directed mutagenesis using the Quik Change™ site-directed mutagenesis system (Stratagene, La Jolla, CA, USA). Both sense and antisense mutagenic oligonucleotide primers for site-directed mutagenesis are presented in Table 1. The coding region of each mutant MD-ACO1 clone was sequenced for confirming point mutation.

Both wild-type and mutant plasmids were transformed into *Escherichia coli* BL21 (DE3) p*Lys*S competent cells using conventional methods. The transformed *E. coli* cells were grown in Luria–Bertani broth with 100 μg/mL ampicillin, initially at 37 °C until the optical density reached 0.4–0.5 at a wavelength of 600 nm. Thereafter, the cells were cooled to 27 °C and 0.4 mM isopropyl β-D-thiogalactoside was added. After induction of recombinant protein, the culture was continued for 4 h at 27 °C. The grown cells were harvested by centrifugation and resuspended in an extraction buffer, containing 50 mM MOPS (3-(*N*-morpholino)propanesulphonic acid; pH 7.4), 1 mM dithiothreitol