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Inhibition of apple 1-aminocyclopropane-1-carboxylic acid oxidase, by cyclopropane-1,1-dicarboxylic acid and *trans*-2-phenylcyclopropane-1-carboxylic acid

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Abstract

Cyclopropane-1,1-dicarboxylic acid (CDA) and *trans*-2-phenylcyclopropane-1-carboxylic acid (PCCA) are the main representatives of a group of compounds that are structural analogues of 1-aminocyclopropane-1-carboxylic acid (ACC) and have been proved to have an inhibitory effect on the wound ethylene produced by *Lycopersicum esculentum* fruit discs. During the experiments, that were carried out in this work the inhibition pattern of PCCA and CDA were studied when tested on partially purified apple ACO and their K_i values were determined. A mechanistic proposal was given, in order to explain the kinetic behaviour of the inhibitors. The common feature of these molecules is their cyclopropane ring, with different substitutes mainly at the positions C1 and C2. Two other compounds with similar structure where also tested as inhibitors, in order to clarify the relationship between structure and activity. These compounds are: 2-methyl cyclopropanecarboxylic acid (MCA), and cyclopropanecarboxylic acid (CCA).

Keywords: Apple fruit; Ethylene; 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO) inhibitors

1. Introduction

The generic term "ethylene inhibitors" is applied to a large group of organic and inorganic chemical compounds possessing a common property: the disturbance of ethylene mediated plant regulation. They can be classified in three categories according to their action (Abeles et al., 1992): the first one includes those acting on ACC synthase and therefore inhibit the conversion of *S*-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) according to the pathway proposed by Yang. The second category includes the compounds acting on ACC-oxidase and could be called ACO inhibitors (Adams and Yang, 1979; Yang and Hoffman, 1984; John et al., 1997; Gibson et al., 1998). Finally, the last category includes all the compounds interfering between ethylene and its receptor (Yang and Hoffman, 1984; Sisler et al., 1996).

In this paper we report the inhibition effect of four compounds on partially purified apple ACO, aiming at the development of a new class of non gaseous, environmental friendly "ethylene inhibitors" that can be used for cut flowers and for post harvest treatments of fruits and vegetables. All the compounds tested for their effect on apple ACO extract, are ACC analogues and are proved to inhibit wound ethylene, produced by *Lycopersicum esculentum* fruit discs (Dourtoglou et al., 1999, 2000). Some of them also inhibit tomato fruit maturation (Dourtoglou, unpublished results).

2. Results and discussion

Various compounds have been tested as inhibitors of ACO in order to understand the relationship of ACO active site and ACC degradation. Most of these compounds are L and D amino acids or L and D amino acid derivatives such as hydroxamates and *N*-acetyl amino acids. However, due to low inhibition and high K_i values, most of these compounds were not suitable for

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agricultural use, although they provide a useful tool for the study of ACC oxidation mechanism.

ACO from many fruits (Dilley et al., 1993; Dupille et al., 1993), pear (Vioque and Castellano, 1998) and avocado (Mc Garvey and Christoffersen, 1992) has been isolated and purified. Due to low stability of the native enzyme, a recombinant apple ACO, expressed in *Escherichia coli*, was used to understand the mechanism of ACC oxidation (Kadyrzhanova et al., 1997). α-Aminoisobutyric acid (AIB) was proved to act as a competitive inhibitor and the inhibition effect of other amino acids has also been studied on the above mentioned enzyme (Charng et al., 1997).

In this work a partially purified apple ACO was used to evaluate the inhibition of CDA, PCCA, MCA, and CCA and the results of inhibition were expressed by comparing the enzymatic activity with and without each inhibitor. Therefore, no further purification of the enzyme was required. The mean value for the activity of the enzyme preparation obtained, was 200 nM of ethylene min^{-1} mg of $protein^{-1}$ (Fig. 1). Meanwhile, the apparent value of K_m for ACO was determined at 25–30 μM (Fig. 2), which is in accordance with the results given for purified apple fruit ACO (Dupille et al., 1993). Preliminary experiments showed that the activity of the partially purified enzyme used in this work was directly proportional to the amount of the enzyme added to the reaction mixture over the range of activities determined (Fig. 1). However, the reaction rate was not linear but decreased as the reaction proceeded (data not shown). A non-linear time course has been observed with many other dioxygenases (John et al., 1991). In addition, we detected a low level substrate inhibition, which affected the initial velocity of the reaction and was more frequently apparent at ACC concentrations higher than 150 μM, even though it varied upon each enzymatic activity (Fig. 3).

CDA was clearly shown to have a non-competitive inhibition and its K_i value was determined at 100±20 μM (Figs. 4 and 5). For PCCA by plotting inhibition data K_i value was determined at 10±2 mM if the competition was non-competitive or 5–6 mM if the competition was competitive. In the double reciprocal plot of ACO in the absence and presence of 10 and 5 mM of PCCA (Figs. 6 and 7) a slight displacement of the lines changes the pattern of the inhibition from non-competitive to competitive (dashed lines in Figs. 6 and 7). Another hypothesis is to have a real intermediate type of inhibition. Unlike to strictly non-competitive inhibition, the inhibitor has different affinities for the two forms of the enzyme (free or bound with ACC) having two dissociation constants K_i. The straight lines obtained at different values of ACC (Fig.7) intersect at the points [PCCA] = $-K_i$ and if at the intersection points $(1/v) > 0$ the inhibition tends to be competitive. Concerning the rest of the compounds, the ethylene produced, when CCA was added to the assay mixture, was much higher compared to the blank assay (data not shown). In addition, CCA, as well as MCA, produced ethylene when reacted with Fenton reagent.

Previous work on the catalytic mechanism of ACC oxidation have shown that catalysis involves an octahedral

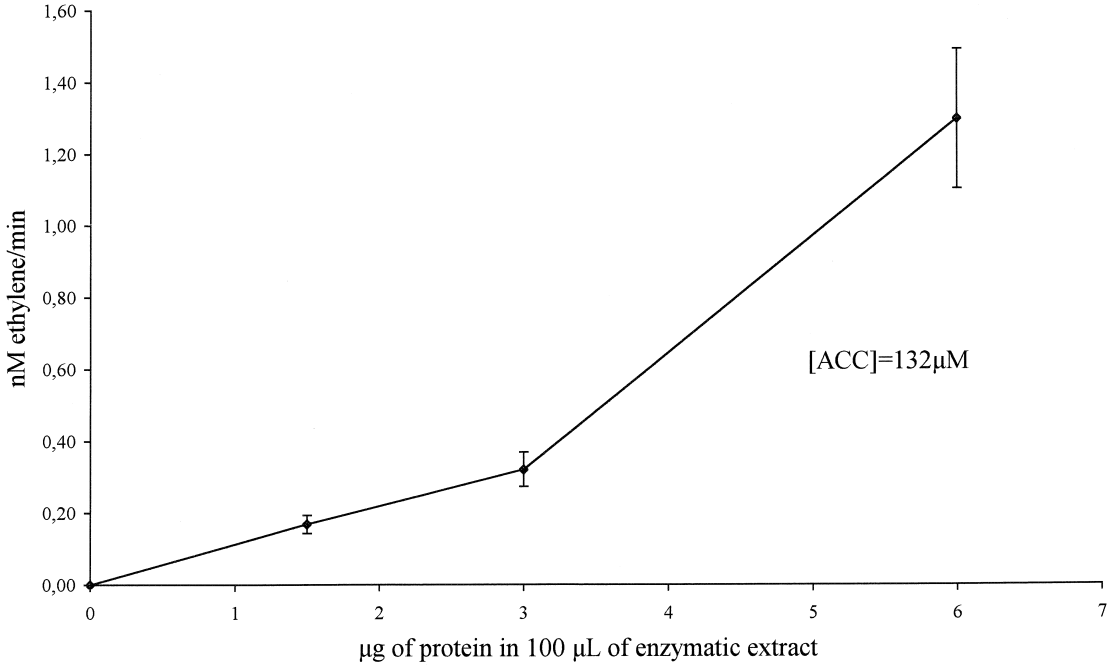

Fig. 1. Effect of different ACO concentrations, on the initial velocity of the reaction.

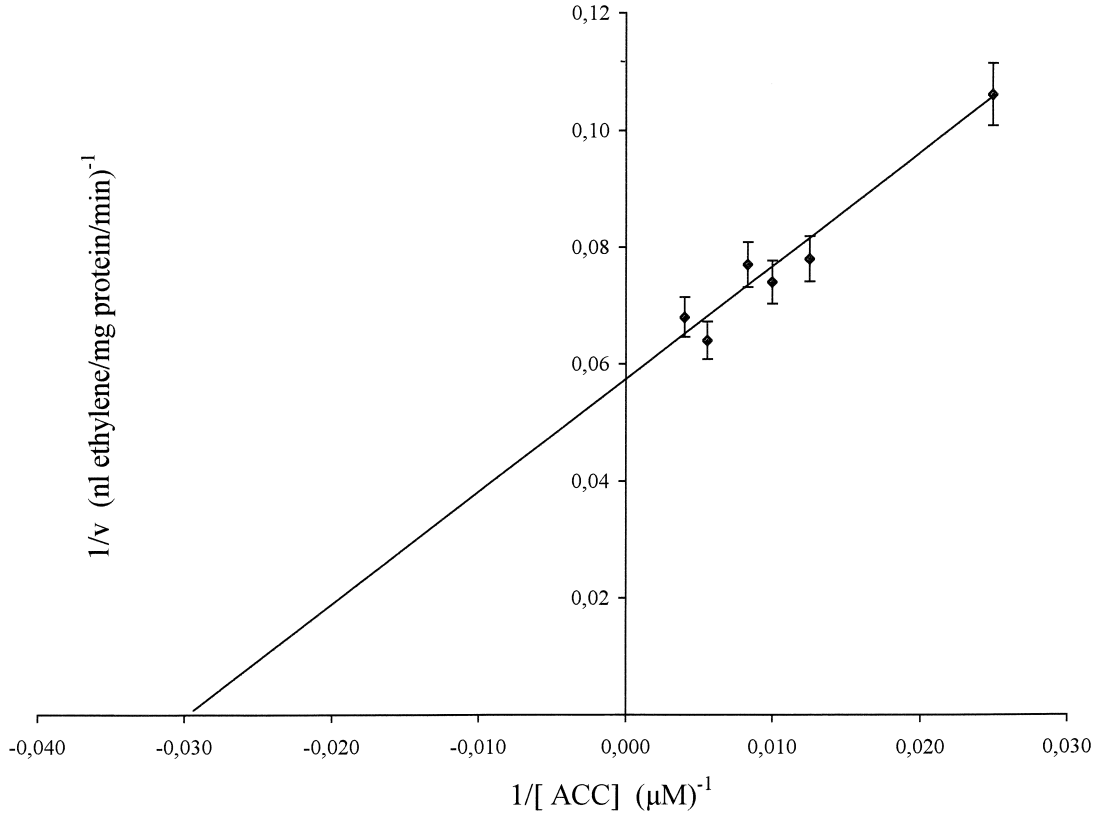

Fig. 2. Double reciprocal plot (Lineweaver–Burk) of ACO activity vs concentration of ACC. $K_m \approx 30$ μM.

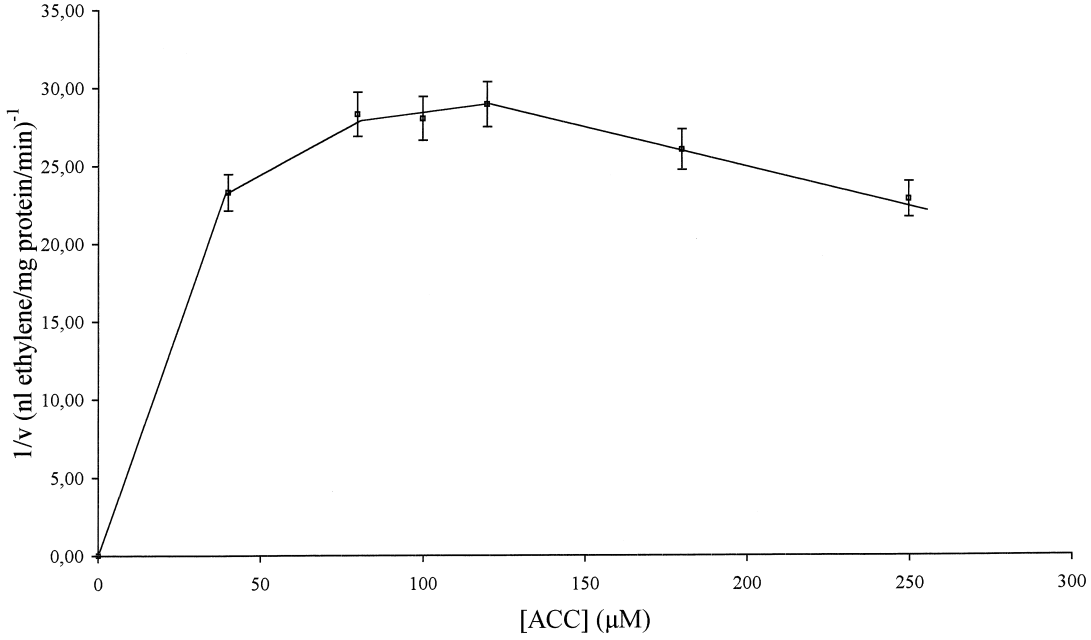

Fig. 3. Time course of ethylene production from apple ACO, at different ACC concentrations.

ferrous iron (Fe^{II}) structure with H_{177}, D_{179} and H_{234} of apple ACO participating in the active center of the enzyme and being ligated to Fe^{II} (Kadyrzhanova et al., 1997; Barlow et al., 1997; Zhang et al., 1997; Gibson et al., 1998) (structure **I**, Fig. 8). In our work, it was necessary to explain the kinetic data obtained by the inhibitors by using a mechanistic approach. The suggested mechanism for the catalytic cycle of ACO involves

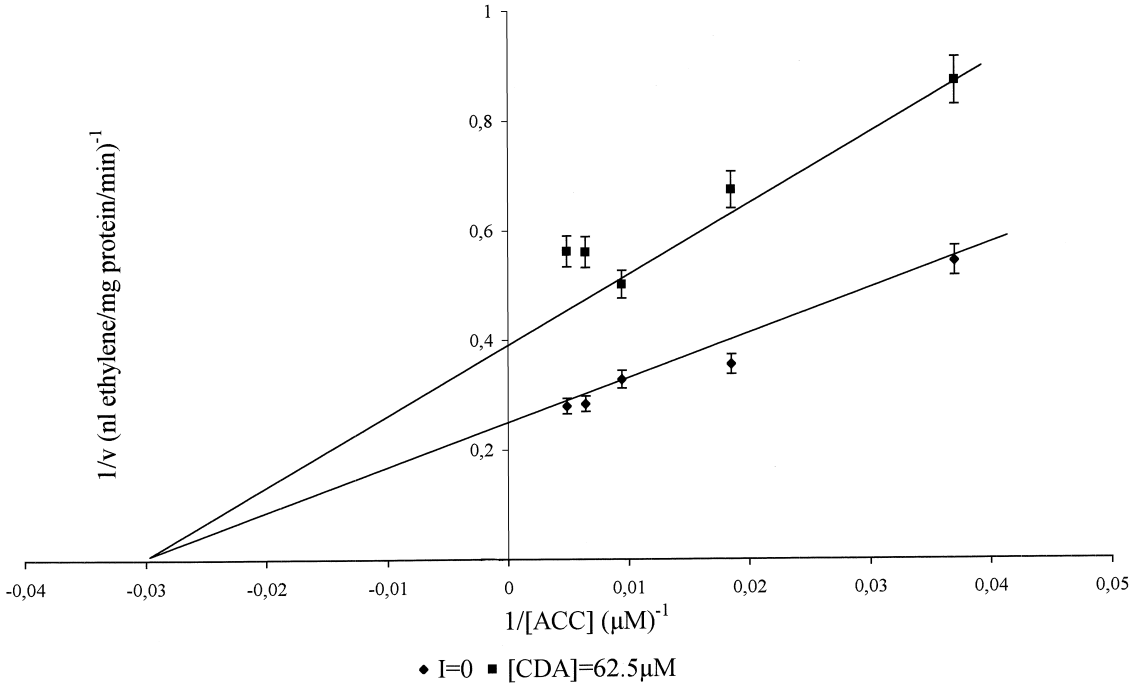

Fig. 4. Double reciprocal plot (Lineweaver–Burk) of ACO activity vs concentration of ACC in the absence and presence of 62.5 μM cyclopropane-1,1-dicarboxylic acid (CDA).

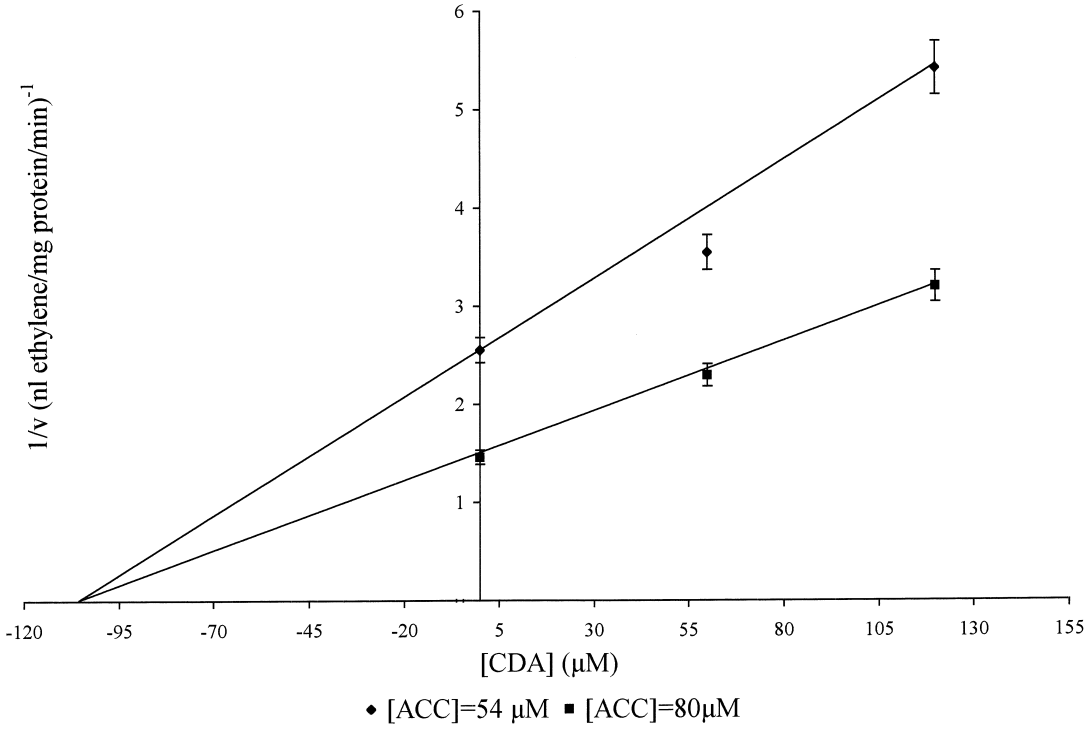

Fig. 5. Effect of different cyclopropane-1,1-dicarboxylic acid (CDA) concentrations on the activity of the partially purified ACO [$1/v = f(\mathrm{CDA})$]. Two different plots at two different concentrations of ACC.

a first step of oxidation using dioxygen and ascorbate and replacement of dehydroascorbate by carbon dioxide or bicarbonate at the Fe^{II} active centre (structure **II**, Fig. 9) (Barlow et al., 1997; Zhang et al., 1997).

At this point, the hypothesis suggested in our work for the following steps of the mechanistic approach, differed from that of previous authors. In particular, we propose that the carboxyl group of ACC can be ligated

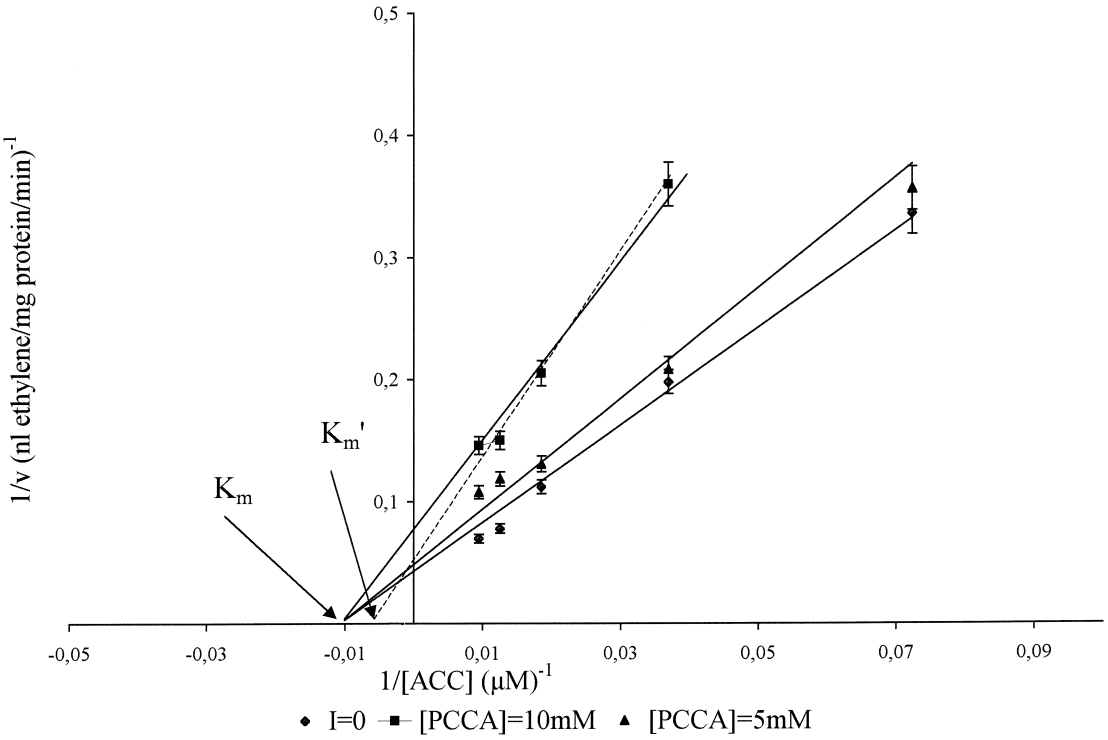

Fig. 6. Double reciprocal plot (Lineweaver–Burk) of ACO activity vs concentration of ACC in the absence and presence of 10 mM and 5 mM of *trans*-2-phenylcyclopropane-1-carboxylic acid (PCCA). K_m and K'_m for non-competitive and competitive inhibition pattern.

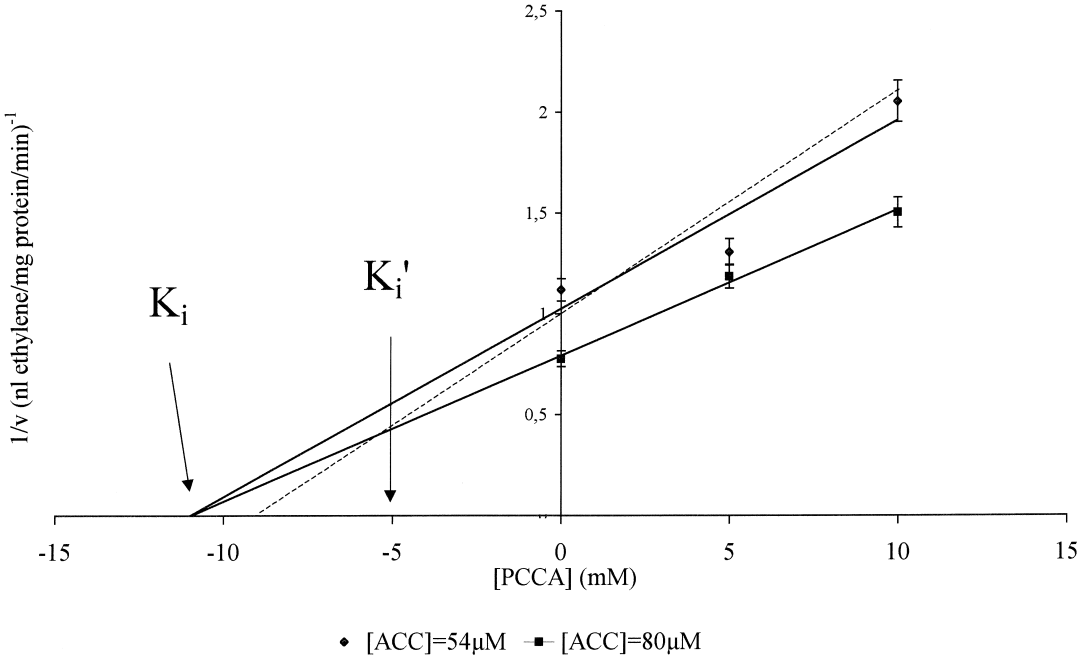

Fig. 7. Effect of different *trans*-2-phenylcyclopropane-1-carboxylic acid concentrations on the activity of the partially purified ACO [$1/v = f$(PCCA)]. Two different plots at two different concentrations of ACC). K_i and K'_i for non-competitive and competitive inhibition pattern.

to Fe^{IV} replacing a molecule of water with subsequent formation of Fe^{III} (structure **III**, Fig. 8). At the next step of catalysis, the amino group of ACC replaces the hydroxyl ligand of Fe^{III} by forming a bidantate five atom ring between Fe^{III} and ACC (structure **IV**, Fig. 8). This bidentate coordination has been suggested by Dourtoglou et al. (1999) and recently confirmed independently by Rocklin et al. (1999) binding alanine

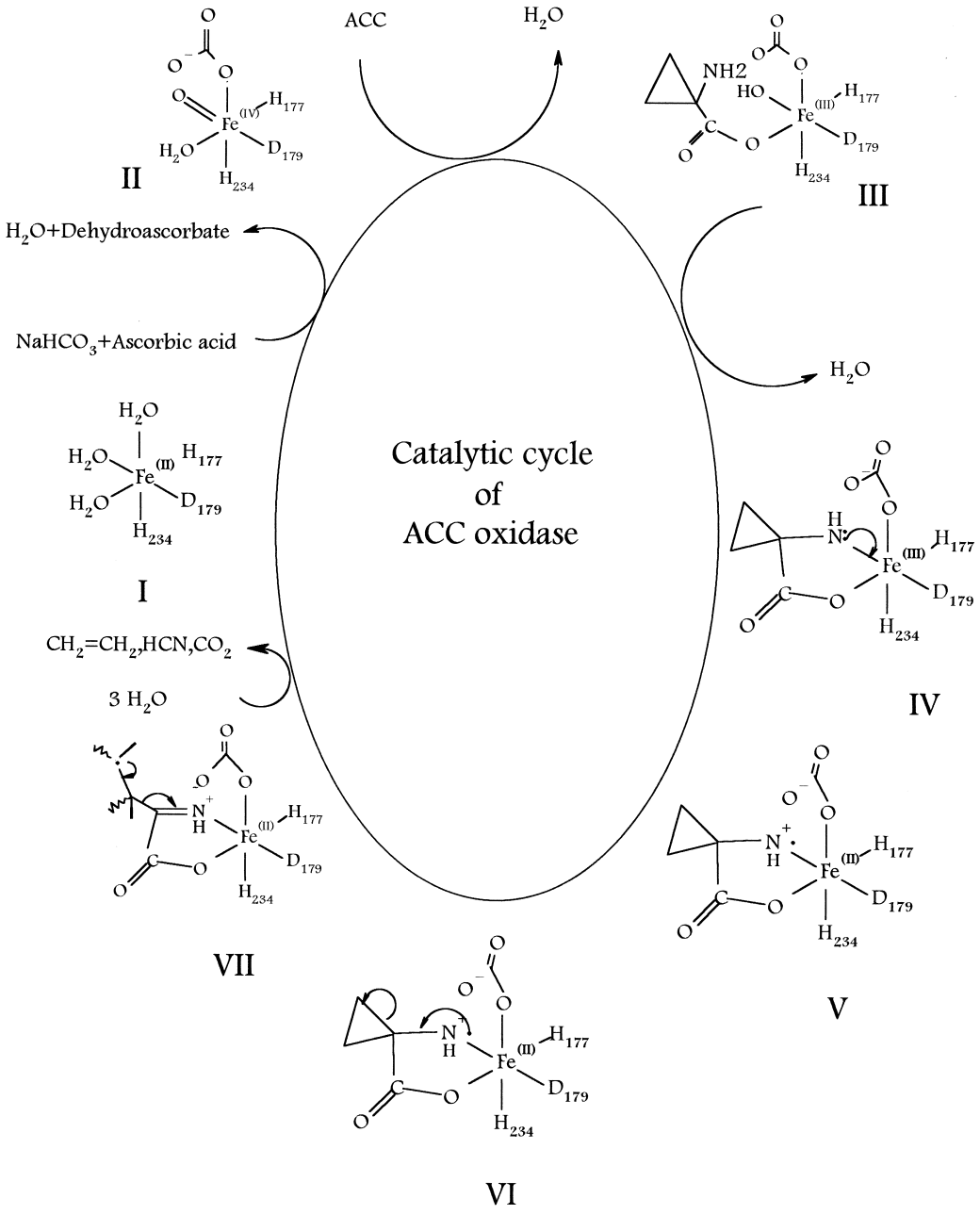

Fig. 8. Proposed catalytic cycle of ACC oxidase.

specifically labelled with ^{15}N and examined by using Q-band electron nuclear double resonance.

In addition ACO's stereospecificity for (1*S*, 2*R*)-2-ethyl-ACC (Hoffman et al., 1982) can be explained by this structure. In this rigid five atom bidantate ligation of the above substrate to iron, the preferential transformation of 1*R*, 2*S* diastereoisomer of 2-ethyl-ACC to 1-butene can be explained by the absence of steric hindrance of the ethyl group when it is at a trans position to the carboxylic acid. The steric hindrance of the ethyl group of (1*S*, 2*R*), (1*S*, 2*S*), (1*R*, 2*R*) diasteroisomers of 2-ethyl-ACC mainly affects the stabilization of the positively charged amino group of the substrate by the bicarbonate anion attached to the iron. This hypotesis is also supported by the observed ACO catalysis of D-valine to isobutanal conversion (Gibson et al., 1998). It is also supported by the fact that the enzyme exhibits a much higher activity with D-amino acids than with their L-enantiomers, when using the same substrate concentration and recombinant apple ACO (Charng et al., 1997). The final step of catalysis, according to our hypothesis, involves two electron transfers. The first

electron is transferred from the amino group of ACC to Fe^{III} resulting to the formation of a Fe^{II} complex (structure **V**, Fig. 8). The second electron transfer takes place within the ACC molecule (structures **VI** and **VII**, Fig. 8).

As far as the inhibitors PCCA and CDA are concerned, the kinetic data reveals a pattern of non-competitive inhibition for CDA and still unclear for PCCA for which and intermediate type should be proposed with slightly differences on the value of K_i. It can be concluded that the carboxyl group of the above inhibitors can be ligated to the active site prior to the ACC ligation, (Fig. 9, intermediate **InIII** for PCCA and CDA) or replace the carboxyl group of ACC at the bidantate five atom ring (Fig. 9, intermediate **InIV**). The last is true with certitude only for CDA while in PCCA the model requires further work to be proved.

In any case, we adopt the mechanism proposed by Zhang et al. (1997), in which the iron-ligated bicarbonate may serve to orientated the amino group of ACC towards the ferryl group effectively, in order to stabilize the positively charged amino group (Fig. 9, intermediate **InIV**) or (Fig. 8, intermediates **IV**, **V**, **VI**, **VII**).

In the above mentioned mechanistic hypothesis, the carboxyl group of the inhibitor is ligated to the ferryl or ferrous iron and the principal characteristic of its kinetic behaviour is the addition of the acid to the same, as that of ACC, intermediate (Fig. 9). We assume that the inhibitor does not replace the residues H_{177}, D_{179}, H_{234} of the active site since the catalysis takes place according to kinetic data.

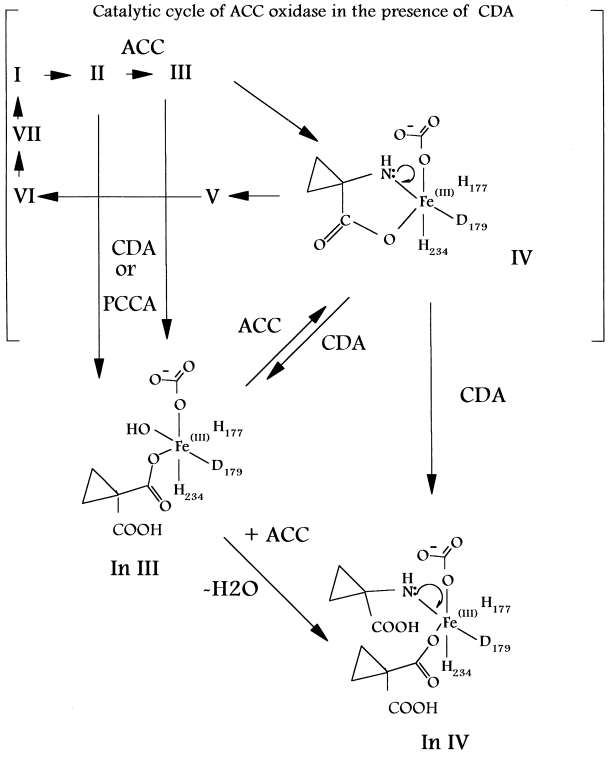

Fig. 9. Proposed catalytic cycle of ACC oxidase in the presence of PCCA and CDA.

Moreover the absence of an amino group and the presence of a carboxyl group attached to a cyclopropane ring within the structure of the inhibitor (PCCA) is necessary for this type of inhibition.

Studies of Charng et al. (1997) with α-aminoisobutyric acid (AIB), as well as other studies with amino acids, reveal a competitive inhibition. As far as amino acids are concerned, there is competition between their amino groups and those of ACC. This leads to the conclusion that amino acids can be ligated to ferrous or ferryl iron both with their amino and carboxyl groups prior to ACC ligation and the resulted type of inhibition is competitive.

In both cases, ACO can oxidize amino acids or AIB and the obtained products are iso-butanal and aceton when valine or AIB were employed as substrates (Gibson et al., 1998; Charng et al., 1997). Consequently, we can conclude that amino acids are also ligated at a bidantate five-atom ring. This explains the stereoselectivity of the enzyme and the fact that they are competitive inhibitors replacing both the amino and the carboxylic group of ACC in the active centre.

The octahedral iron at the active site allow us to proceed to another hypothesis, that explains the steroselectivity of ACO, when D-amino acids are assayed as substrates. Two of three residues (H_{177}, D_{179}) of ACO are placed on the same median plane, while the oxygen of the carbon dioxide and the other residue (H_{234}) occupy, respectively, the two opposite summits of the octahedral bi-pyramidal structure. Amino and carboxylic groups of amino acids occupy the other two sites of the median plane.

This configuration also explains why the ethyl substituent of the pro-*R* methylene which is at a *trans* position to the carboxyl group of (1*R*, 2*S*)-AEC (1-amino-2-ethyl-cyclopropane-1-carboxylic acid) causes very little or no interference with the enzyme when the above substrate was assayed (Charng et al., 1977).

Compared to PCCA, CDA is a much more effective inhibitor. This is probably due to its double carboxyl function. Compounds such as MCA, which is also a substrate analogue, demonstrate varying behaviour depending on their final concentration. Inhibition was observed at a MCA concentration of 20 mM, 15 mM corresponds to the control assay, and 10 mM induces a higher ethylene production according to Dourtoglou et al. (1999).

Similarly, a smaller molecule such as CCA can form a loose connection with the active site and consequently cannot act as an inhibitor. Thus, the fact that more ethylene was produced when this molecule is used, than that produced at the blank assay, may be due to a partial breakdown of the molecule.

3. Experimental

3.1. Plant material

"Golden delicious" apples were purchased from the local market and stored at 4°C.

3.2. Chemicals

Potential inhibitors were purchased from ACROS and FLUKA. The compound *trans*-2-phenylcyclopropane carboxylic acid (PCCA) was also synthesized in VIORYL S.A. Dithiothreitol (DTT) and polyvinylpyrolidone (PVP) were purchased from SIGMA.

3.3. ACC oxidase extraction and partial purification

Unless otherwise stated, all operations were carried out at 4°C. One hundred grammes of apple (cut in four to five pieces) were turned into thin powder in a thermomixer that contained liquid nitrogen, and a spoonful of sea sand. The powder was homogenized in two volumes (v/w) of the extraction buffer A (0.1 M Tris–HCl (pH 7.4), 10% (w/v) glycerol, 30 mM sodium ascorbate, 5 mM DTT, 1% (w/v) PVP 40000). The slurry was centrifuged at 13000 rpm for 60 min and the supernatant was adjusted to 30% saturation with $(NH_4)_2SO_4$. After removal of precipitated material by centrifugation (13000 rpm, 60 min), the supernatant was brought to 90% saturation with $(NH_4)_2SO_4$ and centrifuged for 60 min at the same conditions. Precipitated protein was collected by centrifugation (13000 rpm, 60 min). The resulting pellet was thawed and resuspended in 5 ml of buffer B (20 mM Tris–HCl (pH 7.4), glycerol 10% (w/v), sodium ascorbate 3 mM, DTT 1 mM), containing 1M $(NH_4)_2SO_4$. Two millilitres of the dissolved pellet were desalted by passage through a Sephadex-G25 column (33.5 cm long, radius: 0.75 cm) equilibrated with buffer B. Finally, 30 fractions of 3.5 ml were collected. The fractions used for the determination of the inhibitory effect, were those with the highest activity, which in general was found in fractions 6–10.

3.4. Assay for enzymatic activity and protein determination

Enzymatic activity was measured as follows: 100 μl of enzyme extract was added in a 2 ml vial, equipped with a silicon septum cap, which contained the reaction mixture. The reaction mixture, contained: 10 mM $NaHCO_3$, 200 μM $FeSO_4$, 0,25 mM ACC, and was brought to 900 μl with buffer B. The enzymatic extract was always added last and the vial was immediately closed. ACO activity was assayed by measuring the ethylene produced after 10 min of incubation at 30°C. A gas tight syringe of 100 μl was used to take samples from the vial headspace.

Ethylene was measured using the Varian 3800 gas chromatograph, equipped with a capillary RT-Q-PLOT column (RESTEC) (0.53 mm i.d., 15 m, 0.5 μm) with a Helium flow rate of 8 ml/min at 50°C. Column temperature was stable at 50°C (isotherm), and the detector was maintained at 150°C. The GC was also equipped with an autosampler (Varian 8200) which, by using a special gas tight syringe, would withdrawn 100 μl samples from the headspace of the vials through the septum and injects them directly to the GC.

The initial velocity of the reaction was calculated by measuring the produced ethylene both after 10 and 20 min of incubation. Initial velocity (v) was expressed as the slope of the line joining the points corresponding at ethylene production at 10 and 20 min using the curve fitting facility of the Excel program (Microsoft) and the data of each experiment. Each ethylene production value at the corresponding time point represents the mean value of the measurements of three relevant experiments. An error of 5% was attributed to all the values of each curve (Y-axis error bars). The error attributed to these values accounts for all the possible sources of error. Experimental data with error exceeding 5% was rejected. As it was mentioned above, for some experiments the initial velocity was measured at 10 and 20 min time points using the same enzyme preparation for both the blank and the inhibitor. The activity of the purified enzyme was not the same and differs from one experiment to the other.

When studying the effect of the substrate on the enzyme and the reaction with the inhibitors varying concentrations of ACC were used, whereas the concentrations of $NaHCO_3$, $FeSO_4$ and sodium ascorbate remained the same.

Proteins were determined according to the Bradford method (1976) using the Bio-Rad (GmbH Laboratories, Munich, Germany) reagent and bovine serum albumin as a standard.

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