Expression, purification and characterization of 1-aminocyclopropane-1-carboxylate oxidase from tomato in *Escherichia coli*

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1-Aminocyclopropane-1-carboxylate (ACC) oxidase catalyses the final step in the biosynthesis of the plant hormone ethylene. The successful overexpression and characterization of active ACC oxidase from tomato has been achieved. PCR was used to insert the corrected cDNA coding for the tomato ACC oxidase into the pET-11a expression vector. Cloning of the resultant construct in *Escherichia coli* BL21(DE3)pLysE gave transformants which expressed ACC oxidase at levels greater than 30% of soluble protein under optimized conditions. When induced by addition of isopropyl- β -D-thiogalactopyranoside (IPTG) at 37 °C the ACC oxidase expressed was less soluble and less active than when induced at 27 °C. The enzyme was purified to near homogeneity by a three-step chromatographic procedure. The specific activity of the purified recombinant ACC oxidase was typically 1.3–1.9 mol of ethylene/mol of enzyme per min, higher than values reported for native enzyme. Like the native enzyme it displayed a requirement for ferrous iron and ascorbate, and CO₂ was an activator. The ability to discriminate between

racemic diastereomers of 1-amino-2-ethyl cyclopropane-1-carboxylic acid was demonstrated. The enzyme was found to have a loose specificity for ascorbate, showing apparent preference for D-ascorbate and 5,6-O-isopropylidene L-ascorbate rather than L-ascorbate. The addition of catalase, dithiothreitol and BSA to incubation mixtures all resulted in significant increases in activity. When treated with diethylpyrocarbonate (DEPC) under mildly acidic conditions, the enzyme rapidly lost activity. Comparison of the rate of inactivation with the increase in absorbance at 240 nm gave results consistent with the modification of two to three histidine residues at the active site, although the possibility of additional modification of other nucleophilic residues cannot be excluded. Inactivation was largely prevented by the addition of substrates and ferrous iron, implying that DEPC treatment results in the modification of active-site histidines, which act as ligands for ferrous iron. CO₂ offered no protection against DEPC inactivation, either in the absence or presence of substrates and/or ferrous iron.

INTRODUCTION

Ethylene is a plant hormone which is important for many aspects of plant physiology including fruit ripening and senescence (for reviews see: Kende, 1993; Yang and Hoffman, 1984). Biosynthesis of ethylene occurs in two steps from S-adenosyl-methionine. In the first step 1-aminocyclopropane-1-carboxylate (ACC) synthase catalyses the cyclization of S-adenosyl-methionine to form ACC. Subsequently, ACC oxidase (formerly known as the ethylene-forming enzyme) catalyses the oxidative fragmentation of ACC to form ethylene (Adams and Yang, 1979; Yang and Hoffman, 1984).

For some time *in vitro* studies on ACC oxidase were hampered by the inability to isolate cell-free extracts containing authentic catalytic activity, but recent developments in the molecular biology of ethylene biosynthesis have led to the isolation of ACC oxidase. cDNA clones from a ripening-related library from tomato, were screened by differential hybridization and the plasmid clone pTOM13 identified (Holdsworth et al., 1987). On the basis of experiments in which the production of pTOM13 mRNA was inhibited by an antisense gene and a consequent reduction in ACC oxidase activity observed, it was proposed that pTOM13 was related to ACC oxidase (Holdsworth et al., 1987; Hamilton et al., 1990). Indeed it was subsequently shown that pTOM13 conferred *in vivo* ACC oxidase activity when expressed in yeast (Hamilton et al., 1991). A cDNA clone for tomato ACC oxidase has also been identified by expression in *Xenopus laevis* oocytes (Spanu et al., 1991). cDNA clones homologous to

pTOM13 have since been identified from apple fruit (Dong et al., 1992b; Wilson et al., 1993), avocado fruit (McGarvey et al., 1990) and carnation flower (Wang and Woodson, 1991).

The deduced amino acid sequence of pTOM13 displays homology with those of flavanone 3-hydroxylase and other 2-oxo acid-dependent and related dioxygenases [for a review see Prescott (1993)], as noted by Hamilton et al. (1990) and Matsuda et al. (1991). This led Ververidis and John (1991) to develop a protocol for the recovery of ACC oxidase activity from melon, based on protocols used for other 2-oxo acid-dependent dioxygenases. Subsequently ACC oxidase has also been isolated from apple fruit (Kuai and Dilley 1992; Dong et al., 1992a; Dupille et al., 1993; Pirrung et al., 1993) and avocado (McGarvey and Christoffersen, 1992). Native ACC oxidase has been reported to exist in a monomeric form with an apparent *M_r* of 35000–40000 (Dong et al., 1992a; Dupille et al., 1992; Smith et al., 1992).

In addition to being related to the 2-oxo acid-dependent dioxygenases by sequence, ACC oxidase resembles them in requiring ferrous iron as a cofactor and dioxygen as a co-substrate. Unusually ACC oxidase apparently utilizes ascorbate as a co-substrate rather than a 2-oxo acid and CO₂ is an activator (Dong et al., 1992a; Fernandez-Maculet et al., 1993; Poneleit and Dilley, 1993; Smith and John, 1993). *In vivo* mechanistic studies using labelled or modified ACC analogues have demonstrated that the conversion of ACC into ethylene in apple involves a stepwise cleavage of the cyclopropane ring of ACC (Adlington et al., 1982; Baldwin et al., 1988); however, little or no *in vitro* mechanistic or structural studies have been reported.

Abbreviations used: ACC, 1-aminocyclopropane-1-carboxylate; AEC, 1-amino-2-ethylcyclopropane-1-carboxylate; DEPC, diethylpyrocarbonate; PA, 1,10-phenanthroline; IPTG, isopropyl β -D-thiogalactopyranoside.

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In order to facilitate this work large quantities of active ACC oxidase is required. This is difficult from the native plant sources and early attempts at the heterologous expressions of the tomato ACC oxidase in yeast (Hamilton et al., 1991) and *Xenopus laevis* oocytes (Spanu et al., 1991) showed that these systems were unsuitable for this purpose. In this paper, we describe the overproduction of ACC oxidase of tomato in *Escherichia coli* and the subsequent purification and characterization of the recombinant enzyme. The presence of histidine residues at the active site of ACC oxidase was also investigated by chemical modification using diethylpyrocarbonate (DEPC).

EXPERIMENTAL

Materials

All chemicals and enzymes used in this study were analytical grade or higher and were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), Promega (Madison, WI, U.S.A.) or Pharmacia LKB Biotechnology (Uppsala, Sweden) except where stated otherwise.

Plasmid construction and bacterial transformation

Restriction endonuclease digestions, DNA ligations, preparations and transformations of competent cells and other recombinant DNA manipulations were carried out according to standard procedures (Sambrook et al., 1989). pTOM13 was isolated from a tomato fruit ripening-specific cDNA library by differential hybridization (Slater et al., 1985). The nucleotide sequence of the corresponding genomic clone *eth1* (Kock et al., 1991) revealed that the cDNA sequence in pTOM13 (Holdsworth et al., 1987) contained two single nucleotide deletions near the 5' end of the coding region. Hamilton et al. (1991) reconstructed the gene to repair the deletions and, following expression of the pTOM13 cDNA in *Saccharomyces cerevisiae*, confirmed that the gene encoded ACC oxidase. We used the PCR to effect the same repairs to the pTOM13 cDNA and simultaneously introduce restriction sites to facilitate DNA manipulations in order to allow expression of the native ACC oxidase in *E. coli*. The pair of oligonucleotides used as primers were: (1) 5'-TAACATA**TTGGAGAACTTCCCAATTATTA**-ACTTGGAAAAGCTC-3' [which contained the nucleotide sequence at 5' end of the ACC oxidase cDNA, the two missing bases (underlined) and a *Nde* I site (italicized; the translation initiation codon shown in bold) introduced in order to facilitate the following cloning steps] and (2) 5'-CGGATCCTCAAGCACTTGCAATTGGATC-3' [which contained the sequence of the 3' end of the ACC oxidase cDNA on the opposite strand]. PCR reactions were carried out using standard conditions (Sambrook et al., 1989): a DNA thermal cycler (Perkin Elmer Cetus) was used for 30 cycles. The first cycle was 94 °C, 5 min; 55 °C, 2 min; 72 °C, 1 min, followed by 29 cycles of 94 °C, 1 min; 55 °C, 1 min; and 72 °C for 1 min. The program was terminated by 15 min incubation at 72 °C. The obtained product was analysed on an 0.8% agarose mini gel to assess its purity. The DNA fragment was then cloned into pBluescript SK+ (Stratagene) as a blunt-ended fragment. The correct assembly of the resulting plasmid, pZBS113, was confirmed by sequencing using the dideoxy chain termination method (Sanger et al., 1977) and a battery of appropriately spaced synthetic oligonucleotide primers. *E. coli* BL21(DE3)pLysE was the host strain for all expression studies. The insert of the construct plasmid was cleaved with *Nde* I and *Bam* HI and ligated into the expression vector pET-11a (Novagen Inc.) which was predigested with *Nde* I and *Bam* HI. DNA ligations were performed overnight at 16 °C using the T4 ligase

from Promega. *E. coli* strain BL21(DE3)pLysE competent cells were transformed with the recombinant plasmid pZAT6. Transformants containing the corrected cDNA were used in the expression experiments.

Expression of ACC oxidase in *E. coli* BL21(DE3)pLysE

E. coli BL21(DE3)pLysE was grown in terrific broth (TB) supplemented with 50 mg/ml ampicillin and 35 mg/ml chloramphenicol in shake flasks (250 rev./min) to the late exponential phase at 27 °C or 37 °C, and induced for protein production by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Crude cell extracts were prepared from cell pellets resuspended in a buffer containing 50 mM Tris/HCl, 25% glycerol, 2 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulphonyl fluoride (PMSF) and 0.5 mM benzamidine hydrochloride at pH 7.5 and 25 °C. The suspension was then sonicated and spun at 20000 g for 30 min at 4 °C. Protein was analysed by SDS/12.5% PAGE, and the enzyme activity assayed using gas chromatography as described below.

Fermentation

Typically *E. coli* BL21(DE3)pLysE/pZAT6 was grown at 27 °C in a 12 l fermenter under constant aeration, using a nitrogen-rich, low-caesin medium 'LCM50' (Kara and Hockney, 1991). The cultures were induced while still in the logarithmic phase of growth (absorbance approx. 20 at 550 nm) by the addition of IPTG to 0.4 mM. After a further 2 h of growth following induction, the cultures were harvested by centrifugation at 4 °C. The cell paste was frozen in liquid nitrogen and stored at -80 °C.

Protein purification

Protein concentration was determined by the method of Bradford (1976) using BSA as a standard. Protein purification was carried out (in part) using a Pharmacia FPLC machine. SDS/PAGE analyses were carried out using a Bio-Rad Mini Protean II system, using 12.5% running gels and 3% stacking gels. Chromatographic resins were purchased from Pharmacia LKB. Cell lysis was carried out by a combination of lysozyme treatment and sonication (MSE Soniprep 150 sonicator, power setting 5). Cell pellets were resuspended in a lysis buffer containing 50 mM Tris/HCl (pH 7.5 at 25 °C), 25% glycerol, 2 mM DTT, 0.5 mM PMSF, 0.5 mM benzamidine hydrochloride and 100 mM phenanthroline (PA). Lysozyme was added to the cell suspension (0.4 mg/ml cell suspension) and the mixture was incubated at 4 °C for 40 min with gentle stirring. To the lysate MgCl₂ was added (to a final concentration of 10 mM) and DNA was digested by the addition of DNase I (20 mg per ml of lysate) followed by incubation for a further 20 min. The lysate was then sonicated for 3 × 10 s with 1 min intervals. Unbroken cells and cell debris were removed by centrifugation at 40000 g for 40 min at 4 °C. Supernatants were loaded on to a prepacked DEAE-Sepharose (fast flow) column equilibrated with buffer A (25 mM Hepes/NaOH, 2 mM DTT, 0.5 mM PMSF and 0.5 mM benzamidine, pH 7.5, at 25 °C). After washing the column with two column volumes of the same buffer, proteins absorbed to the column were eluted with buffer B (buffer A plus 1 M NaCl) with a linear gradient from 0 to 20%. B. Fractions containing ACC oxidase, as determined by activity measurements, were pooled and loaded on to a MonoQ column pre-equilibrated with buffer C, containing 25 mM Hepes/NaOH (pH 7.6 at 25 °C), 2 mM DTT, 0.5 mM PMSF, 0.5 mM benzamidine hydrochloride and 10 mM PA. The absorbed proteins were eluted using a step

gradient with buffer D (buffer C plus 1 M NaCl). The fractions containing ACC oxidase, as determined by SDS/PAGE and activity assays, were pooled and concentrated using an Amicon concentrator (YM-10 membrane, WR Grace and Co., Danvers, MA, U.S.A.). The concentrated eluate from the MonoQ column was loaded on to a Superdex S-200 column and the ACC oxidase was eluted with buffer E (20 mM Tris/HCl, 2 mM DTT, pH 7.5). The fractions containing ACC oxidase, as determined by SDS/PAGE and activity assays, were pooled, concentrated and the enzyme activity remeasured.

Enzyme activity assay

ACC oxidase activity was measured essentially according to the method described by Smith et al. (1992). The cell extract or purified enzyme (between 8 and 15 μ g) was added to 1 ml (in a 7 ml bottle) of 0.1 M Hepes, pH 7.2, containing 10% glycerol, 5 mM ascorbate, 0.1 mM ACC, 80 μ M FeSO₄, 10 mM NaHCO₃, 500 μ g of catalase, 100 μ g of BSA, and 2 mM DTT and the reaction mixture was incubated at 28 °C for 20 min. The ethylene produced was measured by GC [Pye Unicam Series 104 machine equipped with a Porapak R column (Phase Sep, Queensferry, Clwyd)] at 80 °C.

Mass spectrometry

Analysis of ACC oxidase was carried out using a VG triple-quadrupole atmospheric mass spectrometer fitted with an electrospray interface.

Samples (10 μ l) were injected into the electrospray source via a loop injector at a flow rate of 2 μ l/min, as a solution in methanol/water (1:1, v/v) containing 0.5% formic acid (1:1, v/v) to give a final protein concentration of approx. 10 pmol/ μ l. The machine was calibrated with horse heart myoglobin (M_r 16951.48).

N-terminal sequencing

Chromatographically purified ACC oxidase was electrophoresed on an SDS/15% PAGE minigel, blotted on to a Waters Immobilon membrane in a Bio-Rad mini-transblot cell at 0.3 A for 1 h in the presence of 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid in 50% methanol, pH 11, and submitted for N-terminal sequencing by Edman degradation.

Inactivation with DEPC

DEPC was freshly diluted to 10–20 mM with 100% ethanol. Protein solutions at a concentration of 1 mg/ml in 50 mM phosphate buffer, pH 6.8, were treated with DEPC at room temperature. The ethanol (3%, v/v) in the protein solution had no apparent effect on the enzyme activity or stability. The same amount of ethanol as in the reaction mixture was added to the control sample. After incubation for various time periods aliquots were removed to measure the remaining enzyme activity. The stoichiometry of modification was estimated by measuring the difference in absorbance at 240 nm between DEPC-treated samples and control samples. The number of modified histidine residues was calculated using a molar absorption coefficient of 3600 for N-carboxyethylated histidine at 240 nm (Miles and Kumagai, 1974).

Treatment of the inactivated enzyme with hydroxylamine

After incubation with DEPC, the excess DEPC was removed by the addition of imidazole (2.5 mM final concentration) to the reaction mixture. The reaction mixture was then dialysed for 2 h

against three changes of 50 mM phosphate buffer, pH 7.0, containing 2 mM DTT and the enzyme activity was measured. Hydroxylamine was added to a final concentration of 2.5 mM followed by incubation for 2 h at room temperature. The control sample, in which ethanol was added alone, was treated similarly.

RESULTS

Plasmid construction and expression of the ACC oxidase

The 1370 bp cDNA fragment from plasmid pTOM13 contains the entire tomato ACC oxidase coding sequence, with the exception of two bases, a G at position 5 and a C at position 17, missing from the 5' end, due to cloning artefacts introduced during the construction of the cDNA library (Hamilton et al., 1991). To obtain a bona-fide ACC oxidase, ECPCR (Macferrin et al., 1990) was used to correct the two errors. The nucleotide sequence of the PCR product was determined and the two

Figure 1 Construction of plasmid pZAT6 (a) and SDS/PAGE analysis (b) of the expression of ACC oxidase in *E. coli* BL21(DE3)pLysE

(a) The 945 bp cDNA coding for the tomato ACC oxidase, after correction and amplification by PCR, was inserted into the expression vector pET-11a. The direction of transcription is indicated by an arrow. (b) Lane 1, total cell extract from uninduced cells; lanes 2 and 3, total cell extract and soluble fraction of cells grown at 37 °C; lanes 4 and 5, total cell extract and soluble fraction of cells grown at 27 °C; lane 6, molecular-mass markers. The position of the ACC oxidase is indicated by the arrowhead. Cells were grown in shake flasks and harvested 2.5 h after induction with 0.4 mM IPTG.

Figure 2 SDS/PAGE of recombinant ACC oxidase at various stages of purification

Lane 1, soluble fraction of induced cells of *E. coli* BL21(DE3)pLysE/pZAT6; lane 2, pooled fractions after DEAE-Sepharose chromatography; lane 3, pooled fractions after MonoQ chromatography; lane 4, pooled fractions after Superdex S-200 chromatography; lane 5, molecular-mass markers.

missing bases shown to be correctly incorporated into the sequence. The full-length ACC oxidase cDNA of tomato was ligated into the expression vector in the correct orientation, pET11a, yielding plasmid construct pZAT6 (Figure 1a). The initiation codon of the transcript from the construct is the cDNA initiation codon, therefore the induced gene product will be the native ACC oxidase.

The isolated plasmid, pZAT6, was then transformed into the expression host *E. coli* strain BL21(DE3)pLysE which contains the T7 RNA polymerase gene under the control of the *lacUV5* promoter (Studier et al., 1990). Initially cultures were grown at 37 °C until the late logarithmic phase when the expression of ACC oxidase was induced by addition of IPTG. The culture was grown for a further 3 h, when induced cells were sonicated and the cell lysate analysed by SDS/PAGE. An intense band with an apparent M_r of 38000 was observed, which was absent from extracts of control cultures. Laser densitometry-SDS/PAGE analysis indicated that this band corresponded to more than 30 % of total cell protein (Figure 1b). Unfortunately, the protein was largely insoluble and examination by phase-contrast microscopy indicated the presence of highly refractile inclusion bodies, which were not present in uninduced cells. Furthermore, the ACC oxidase activity was very low. When the incubation temperature was decreased to 27 °C the ACC oxidase expressed was almost fully soluble, albeit at a slightly lower level of expression (Figure 1b). The A_{600} of the cells peaked 2 h after IPTG induction and subsequently remained constant. The effect of IPTG concentration (0–1.0 mM) on ACC oxidase production was examined in shake flask experiments over the course of 4 h of post-induction at 27 °C and was monitored by SDS/PAGE and activity measurements (results not shown). The recommended (Novagen, Madison, WI, U.S.A.) IPTG concentration is 1 mM and very little ACC oxidase was expressed without IPTG, indicating that the T7 polymerase gene is under tight control. In the presence of IPTG the level of ACC oxidase was observed to

accumulate steadily, but IPTG concentrations of 0.4–1 mM made little or no observable difference to the level of ACC oxidase produced.

Purification

The recombinant ACC oxidase was purified to near homogeneity in approx. 43 % activity recovery using a three-step chromatographic procedure, summarized in Table 1 and Figure 2. Thus, crude cell-free extracts were subjected to DEAE-Sepharose, followed by further anion-exchange chromatography using MonoQ resin. Further purification was achieved by gel filtration using Superdex S-200. Although the specific activity was not increased by the latter step, several impurities were removed as indicated by SDS/PAGE analysis. The enzyme obtained was > 95 % pure (by SDS/PAGE–laser densitometry) and had a high specific activity (typically 1.3–1.9 mol of ethylene/mol of enzyme per min), compared with published values for native enzyme (e.g. Dong et al., 1992a).

The M_r of the purified recombinant ACC oxidase of tomato measured by electrospray ionization m.s. was 35817 ± 6 (calc. M_r 35813), indicating that the N-terminal methionine was not cleaved (Figure 3). This deduction was confirmed by Edman sequencing of the first 10 amino acids of the N-terminus of the purified ACC oxidase (MENFPIINLE), which were shown to match those derived from the cDNA sequence (Hamilton et al., 1990).

Functional properties of the ACC oxidase

Dependence on substrates and cofactors

The apparent K_m for ACC in the presence of L-ascorbate and 10 mM HCO_3^- was 23.2 μM , compared to reported values of 32 μM for avocado fruit (in the absence of added HCO_3^- (McGarvey and Christofferson, 1992), 28 and 121 μM (at 0.03 % and 4 % CO_2 , respectively) for enzyme from apple fruit (Fernandez-Maculet et al., 1993), and 62 μM for melon fruit under similar conditions to those used by us (Smith et al., 1992). Whether the wide variation in K_m values reported actually reflect real differences in substrate binding to ACC oxidases from different plants is unclear, but high apparent K_m values in the millimolar or submillimolar range can be indicative of non-enzymic turnover of ACC to ethylene (McKeon and Yang, 1984). It has also been demonstrated that the addition of $\text{CO}_2/\text{HCO}_3^-$ to incubation mixture increases both V_{max} and K_m for the substrate ACC (Fernandez-Maculet et al., 1993).

The ability of the purified recombinant tomato ACC oxidase to discriminate between mixtures of the 1*R*,2*S*- and 1*S*,2*R*-amino-2-ethylcyclopropane-1-carboxylate (AEC), and 1*R*,2*R*- and 1*S*,2*S*-AEC was demonstrated (Table 2). The tomato ACC oxidase effectively converted the mixture of 1*R*,2*S*- and 1*S*,2*R*-AEC into 1-butene and also effected less efficient conversion of the mixture of (1*R*,2*R*- and 1*S*,2*S*-AEC) to 1-butene. As in the conversion of ACC into ethylene the conversion of the racemic diastereomers to 1-butene was also stimulated by the addition of $\text{CO}_2/\text{HCO}_3^-$ (Table 2).

Replacement of ferrous iron with other metals (results not shown) led to no increase in the production of ethylene over background levels and we conclude that ferrous iron is essential for ACC oxidase activity, consistent with the work of Smith et al. (1992). The apparent K_m for ferrous iron in the presence of L-ascorbate and 10 mM HCO_3^- was 5.3 μM . The K_m of ferrous iron was reported to be 0.4 μM for the apple enzyme, in the absence of added bicarbonate (Dupille et al., 1993). The catalytic turnover of ACC was found to be completely dependent upon

Table 1 Purification of recombinant ACC oxidase

The cell pellet (150 g) was lysed and the recombinant ACC oxidase was purified as described in the Experimental section.

Purification step	Total protein (g)	Total activity (nmol of ethylene/min)	Specific activity (nmol of ethylene/mg of protein per min)	Activity recovery (%)	Purification (fold)
Supernatant	7.2	25 780	3.58	100	—
DEAE-Sepharose	0.9	23 060	25.7	89.4	7.2
Mono-Q	0.45	14 770	32.8	57.3	9.2
Superdex S-200	0.315	110	35	42.7	9.8

Figure 3 Electrospray ionization mass spectrum of tomato ACC oxidase

$M_t = 35817 \pm 6$, predicted $M_t = 35813$.

Table 2 Relative conversion of racemic AEC diastereomers into 1-butene

Numbers are means $\pm$ S.E.M., $n = 3$.

Substrate	ACC oxidase activity			
	(mol of 1-butene/mol of enzyme per min)		(mol. of ethylene/mol of enzyme per min)	
	$-HCO_3^-$	$+HCO_3^-$	$-HCO_3^-$	$+HCO_3^-$
1 <i>S</i> , 2 <i>R</i> and 1 <i>R</i> , 2 <i>S</i> -AEC	$0.14 \pm 1.5 \times 10^{-2}$	$0.92 \pm 3.9 \times 10^{-2}$	—	—
1 <i>S</i> , 2 <i>S</i> and 1 <i>R</i> , 2 <i>R</i> -AEC	$2.9 \times 10^{-2} \pm 3.2 \times 10^{-3}$	$7.8 \times 10^{-2} \pm 6.4 \times 10^{-3}$	—	—
ACC	—	—	$0.95 \pm 7.2 \times 10^{-2}$	3.7 ± 0.28

Figure 4 Effect of CO₂ on the ACC oxidase activity

the presence of ascorbate, which could not be replaced by other reductants such as DTT and NADH (results not shown). Three analogues, D-ascorbate, 5,6-*O*-isopropylidene L-ascorbate and L-galactono- γ -lactone, were examined as replacements for L-ascorbate. Under the standard incubation conditions the following order of activity was found: 5,6-*O*-isopropylidene L-ascorbate > D-ascorbate > L-ascorbate. L-Galactono- γ -lactone was completely inactive. The following apparent K_m values were determined: L-ascorbate, 16.7 mM; D-ascorbate, 1.7 mM; and 5,6-*O*-isopropylidene L-ascorbate, 1 mM. It cannot be ruled out that the latter undergoes (partial) hydrolysis under the reaction conditions.

It has been reported that CO₂ acts as an activator for ACC oxidase both *in vivo* (Grodzinski et al., 1982; Kao and Yang, 1982) and *in vitro* (Dong et al., 1992a; Smith and John, 1993). The activity of the recombinant tomato ACC oxidase was found to increase dramatically when CO₂ was introduced into the head space of the reaction bottle (Figure 4), reaching a maximum when the CO₂ concentration was increased to 10% of the head space volume. At this concentration the enzyme activity was five times higher than that observed in the presence of ambient levels of CO₂ (0.03%). The effect of the bicarbonate concentration on the observed enzyme activity was not simple, as has been previously observed for the native apple ACC oxidase (Pirrung et al., 1993). A linear correlation between bicarbonate concentration and enzyme activity was observed between 0.01 and 0.1 mM bicarbonate. However, the observed increase in stimulation by bicarbonate decreased slowly at higher concentrations and reached a maximum at 10 mM.

We previously reported that the addition of catalase to reaction mixtures stimulates the activity of a glutathione S-transferase-ACC oxidase fusion protein (Smith et al., 1994) and these results were confirmed using the non-fusion recombinant tomato ACC oxidase described here. Catalase at 500 μ g/ml increased the enzyme activity by 36%. The addition of DTT (1 mM) and BSA (100 μ g/ml) also increased the observed enzyme activity by 38% and 41% respectively (in separate experiments). In the presence of catalase, DTT and BSA an approx. 60% increase in activity (c.f. in the absence of all three) was observed. It should be noted that in the presence of CO₂, DTT has been reported to (re)activate largely inactivated ACC oxidase by a factor of 20-fold (Poneleit

Figure 5 The effects of DEPC treatment on the spectra (a) and on the activity and histidine residues of ACC oxidase (b)

ACC oxidase (at 0.85 mg/ml in 0.05 M phosphate buffer, pH 6.8) was treated with 0.4 mM DEPC in a Perkin-Elmer spectrophotometer at 22 °C for 1 h as described in the Experimental section. Difference spectra between untreated ACC oxidase in the reference cell and DEPC-treated ACC oxidase in the sample cell were recorded at regular intervals. Aliquots of the treated enzyme were removed for ACC oxidase activity assays immediately after their spectra had been recorded. (a) Curve 1 is the spectrum obtained after DEPC treatment, curve 2 is the spectrum obtained before DEPC treatment and curve 3 shows the difference spectrum recorded after DEPC treatment. (b) This shows the correlation between enzyme activity and the number of histidine residues modified by DEPC treatment. The enzyme was treated with DEPC as above for various times before determination of the activity and the number of histidyl residues modified from the difference absorbance at 240 nm.

and Dilley, 1993). Typically specific activities of 1.3–1.9 mol of ethylene/mol of enzyme per min were obtained for purified enzyme, although activities of up to 3.7 mol of ethylene/mol of enzyme per min were observed in some preparations.

Treatment of the ACC oxidase with DEPC

Treatment of the recombinant ACC oxidase with 0.1–2 mM DEPC led to rapid loss of enzyme activity. Complete inactivation was observed within 20 min at 1.5 mM DEPC and pH 6.8. In an attempt to identify the type of residues modified by DEPC, the change in protein absorbance in the u.v. region upon DEPC modification was determined. As shown in Figure 5(a) the

difference spectrum at 240 nm is characteristic of the presence of N-carboxyethylated histidine residues (Miles and Kumagai, 1974) and the absence of change in the difference spectra at 270 nm indicates that no modification of tyrosyl residues occurred (Miles and Kumagai, 1974), since reaction of tyrosyl residues with DEPC results in a decrease in absorbance at this wavelength. The ACC oxidase of tomato contains nine histidine residues (Holdsworth et al., 1987) and the difference spectroscopy indicated that approximately two of these react rapidly with DEPC (0.4 mM) at pH 6.8, leading to a rapid loss in enzyme activity (Figure 5b). At higher concentrations (0.8 mM), modification of approx. three histidine residues was observed (results not shown).

Cofactors and substrate were studied for their ability to protect the ACC oxidase from inactivation by DEPC. A marked protection of the enzyme activity (60–80%) was observed when inactivation was carried out in the presence of ferrous iron, ACC and ascorbate. The effects of individual components in the reaction mixture were systematically studied. Neither ACC, ascorbate nor ferrous iron displayed any protective ability alone (up to concentrations of 3 mM, 3 mM, and 25 mM respectively). The addition of bicarbonate or CO₂ offered no protection alone, nor did it produce any additional protective effect in the presence of the other components. Ferrous iron appeared to be the most effective cofactor in protecting the enzyme from inactivation, since omission of it from the reaction mixture containing DEPC and all other components led to a decrease in observed enzyme activity to about 20% of the level of that found in the presence of ferrous iron, ACC and ascorbate during inactivation. A combination of ACC and ferrous iron increased the observed enzyme activity after DEPC treatment from 2% to 32%, and a combination of ascorbate and ferrous iron led to an increase in the enzyme activity from 2% to 22%. Treatment of inactivated enzyme with 2.0 mM hydroxylamine at room temperature for 1 h in the presence of DTT and BSA led to the increase in the observed activity by 50% (results not shown). Use of higher concentrations of hydroxylamine and/or longer time treatments were ineffectual in restoring activity, since irreversible inactivation of ACC oxidase by hydroxylamine was observed.

DISCUSSION

The corrected pTOM13 cDNA was cloned into the *E. coli* expression vector pET11a. In this system the strong T7lac promoter allowed the production of the soluble, active ACC oxidase at levels in excess of 120 mg/l cells, facilitating a convenient three-step chromatographic purification procedure for the production of large quantities of pure protein.

The N-terminus of the apple fruit ACC oxidase is apparently blocked and analysis by m.s. indicated its *M_r* to be approx. 50 higher than that calculated from the cDNA sequence (Dong et al., 1992a; Pirrung et al., 1993), consistent with N-terminal modification. The expression and purification of the largely soluble tomato ACC oxidase indicates post-translational N-terminal modification is not a prerequisite for catalytic activity.

The native ACC oxidases that have been purified, or partially purified, from apple fruits and melon have low specific activities. Dong et al. (1992a) suggested that the assay conditions had not been properly optimized, e.g. an unidentified cofactor might be required for full activity, or that partial inactivation occurs during purification. It has been reported (Dupille et al., 1993) that inclusion of PA, a metal-ion chelator, in the purification buffers significantly improves the recovery of ACC oxidase activity, probably by preventing the enzyme from undergoing oxidative damage in the presence of iron. The purification of the recombinant tomato ACC oxidase was therefore executed en-

suring that PA was present in the buffers. DTT, BSA and catalase were all found to be beneficial for ACC oxidase activity and were included in the assays.

Under these conditions the purified recombinant tomato ACC oxidase typically had a specific activity of 1.3–1.9 mol of ethylene/mol of enzyme per min, significantly higher than the values of 0.7 mol of substrate/mol of enzyme per min reported by Dong et al. (1992a) for the native ACC oxidase from apple. The differences may result from the intrinsic properties of the tomato and apple enzymes, but may result either from an improved purification protocol, facilitated by the availability of large quantities of enzyme or from the avoidance of interference by plant secondary metabolites. The reported specific activities for ACC synthase (Yip et al., 1991) are significantly higher than those for ACC oxidase, possibly accounting for the relatively large amount of the latter apparent in ripe apple fruit. The tomato ACC oxidase activity was readily inactivated during catalytic turnover and specific activity was typically observed to decrease by about 50% after approx. 8 min under the standard incubation conditions.

Hoffman et al. (1982) have reported that one of the four stereoisomers of an ACC analogue, AEC, namely (1*R*, 2*S*)-AEC, is preferentially (> 40:1) converted into 1-butene by apple fruit and mung bean hypocotyl tissues. It was suggested that the stereochemical discrimination of the diastereomers of AEC indicated that their conversion into butene was catalysed by the same enzyme which converts ACC into ethylene. Our studies utilized racemic mixtures of 1*R*,2*S*- and 1*S*,2*R*-AEC, and 1*R*,2*R*- and 1*S*,2*S*-AEC, and as would be predicted (Hoffman et al., 1982) the former mixture was preferentially converted, but the latter mixture was also converted at a significant rate (Table 2). As in the reaction with ACC, the enzyme activity with the substrate analogues was significantly stimulated by the addition of CO₂/HCO₃⁻ and it is of interest that the degree of discrimination in favour of the 1*R*,2*S*- and 1*S*,2*R* pair of AEC diastereomers was apparently increased in the presence of bicarbonate.

Ascorbate is a common cofactor for the family of 2-oxo acid-dependent dioxygenases and in many cases apparently prolongs the lifetime of active enzyme under catalytic conditions. Previous kinetic studies using L-ascorbate analogues with prolyl-4-hydroxylase have led to proposals that ascorbate binds to the active site, but that the side chain of the cofactor does not make a significant contribution to binding (Majamaa et al., 1986; Tschank et al., 1994). The ability of three analogues, D-ascorbate, 5,6-*O*-isopropylidene L-ascorbate and L-galactono-γ-lactone, to serve as substitutes for L-ascorbate in the ACC oxidase reaction was studied and from the *K_m* values obtained it is clear that in the case of ACC oxidase the side chain does play an important role in binding to the enzyme. Possibly the apparent difference in the importance of side-chain binding of ascorbate to ACC oxidase compared with prolyl-4-hydroxylase reflects the fact it is a 'primary' substrate in the normal catalytic reaction of ACC oxidase, rather than an alternative substrate, as in the latter case. It should be noted, however, that ascorbate has not been unequivocally demonstrated to be an efficient substrate for ACC oxidase *in vivo* (John, 1994).

Comparison of the cDNA-derived amino acid sequence of tomato ACC oxidase with some other members of the 2-oxo acid-dependent dioxygenase has revealed two regions of high homology close to the C-terminus (Matsuda et al., 1991; Myllyla et al., 1992) and several amino acid residues which are fully conserved, including two histidines (residues 177 and 211). These histidines, which are also conserved through all 13 known ACC oxidase sequences (data not shown), may form part of the iron-

binding/catalytic site, since spectroscopic studies on isopenicillin N synthase have revealed that two or three histidine residues provide ligands for binding to the ferrous iron (for reviews see Baldwin and Schofield, 1992; Feig and Lippard, 1994). Furthermore, derivatization of mammalian prolyl hydroxylase has provided further evidence for the location of histidines at the active site of 2-oxo acid-dependent and related dioxygenases. The treatment of ACC oxidase with DEPC caused rapid loss of ACC oxidase activity together with the modification of two to three histidine residues. The presence of cofactors/co-substrates effected substantial protection of ACC oxidase from inactivation by DEPC. Although DEPC can react with the nucleophilic side chains of a number of amino acids, the group most efficiently modified in neutral or slightly acidic media is the imidazole ring of histidine residues (Miles, 1977). To provide evidence for the modification of histidines of ACC oxidase we monitored the absorbance change in the UV spectrum upon DEPC modification and found it to be characteristic of histidine modification, i.e. an increase in absorbance at 240 nm without change at 280 nm was observed. The failure to observe complete reactivation of inactivated ACC oxidase does not necessarily mean that modification of amino acid residues other than histidine has occurred, because full reactivation reaction could not be effected due to inactivation by the hydroxylamine at high concentrations.

It is difficult to evaluate the precise role of individual cofactors/co-substrates in the protection of ACC oxidase against inactivation by DEPC. The present experiments demonstrate that ferrous iron, ACC and ascorbate together offer almost total protection of the enzyme against inactivation by DEPC. Partial protection could be obtained by the combination of ferrous iron and ACC or ascorbate, but the combination of ACC and ascorbate did not offer protection against DEPC modification. While the addition of ferrous iron alone offered no apparent protection to DEPC modification, it was found to be indispensable in the protection of ACC oxidase from DEPC modification by the use of cofactors/co-substrates. However, it should be noted that the addition of ferrous iron to ACC oxidase results in inactivation under aerobic conditions. The importance of ferrous iron to the protection of the histidine modification is unsurprising since histidine residues are likely to provide ligands for the metal. Based on these experiments, it is therefore suggested that the binding of ACC and ascorbate to ACC oxidase requires pre-binding of ferrous iron and the three binding sites are intimate. Since CO₂ offered no extra protection to DEPC modification it is unlikely to be bound to a residue close to the iron-binding site. The possibility that CO₂ forms a carbamate with ACC cannot be ruled out and it is of interest that the non enzymic conversion of ACC into ethylene is also stimulated by bicarbonate/CO₂ (McRae et al., 1983). Furthermore the results imply that if a lysyl side chain (or another protein nucleophile) reacts with CO₂ it was not efficiently modified by DEPC, since the residual activity after inactivation was still stimulated by CO₂.

In conclusion the high level expression of active tomato ACC oxidase in *E. coli* should facilitate future mechanistic and structural studies on ethylene biosynthesis. The purified recombinant ACC oxidase displays similar characteristics to the native enzyme, including activation by CO₂; however, it is significantly more active. Inactivation experiments using DEPC have uncovered evidence for the presence of histidine residues at the active site of ACC oxidase, thereby providing a structural link between ACC oxidase, which uses ascorbate as a co-substrate, and prolyl hydroxylase, which in common with most members of the 2-oxo acid-dependent and related dioxygenase family utilizes a 2-oxo acid co-substrate. It will be fascinating to investigate the

structural and mechanistic links between the members of this family which catalyse a wide range of oxidative chemistry.

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