Inactivation of 1-Aminocyclopropane-1-carboxylate Oxidase Involves Oxidative Modifications†

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ABSTRACT: 1-Aminocyclopropane-1-carboxylate (ACC) oxidase catalyzes the final step in the biosynthesis of the plant signaling molecule ethylene. It is a member of the ferrous iron dependent family of oxidases and dioxygenases and is unusual in that it displays a very short half-life under catalytic conditions, typically less than 20 min, and a requirement for CO_2 as an activator. The rates of inactivation of purified, recombinant ACC oxidase from tomato under various combinations of substrates and cofactors were measured. Inactivation was relatively slow in the presence of buffer alone ($t_{1/2}$ > 1 h), but fast in the presence of ferrous iron and ascorbate ($t_{1/2}$ ≈10 min). The rate of iron/ascorbate-mediated inactivation was increased by the addition of ACC, unaffected by the addition of CO_2 at saturation (supplied as bicarbonate) but decreased by the addition of catalase or ACC + CO_2 at saturation (supplied as bicarbonate). Iron/ascorbate-mediated inactivation was accompanied by partial proteolysis as observed by SDS−PAGE analysis. The fragmentation pattern was altered when ACC was also included, suggesting that ACC can bind to ACC oxidase in the absence of bicarbonate. N-terminal sequencing of fragments resulted in identification of an internal cleavage site which we propose is proximate to active-site bound iron. Thus, ACC oxidase inactivates via relatively slow partial unfolding of the catalytically active conformation, oxidative damage mediated via hydrogen peroxide which is catalase protectable and oxidative damage to the active site which results in partial proteolysis and is not catalase protectable.

Ethylene is an important plant signaling molecule ("hormone") produced by almost all plant tissues (Abeles et al., 1992; Ecker, 1995; Wilkinson et al., 1995; Schaller & Bleecker, 1995). It is involved in the regulation of plant growth and development and in the wounding response. Its biosynthesis from *S*-adenosyl methionine occurs in two enzyme-catalyzed steps. In the first, ACC synthase catalyzes the cyclization of *S*-adenosyl methionine to give 1-aminocyclopropane-1-carboxylate (ACC).[1] Subsequently, ACC oxidase (also known as the ethylene-forming enzyme) catalyzes the oxidative fragmentation of ACC to give ethylene (Figure 1).

ACC oxidase is a member of the ferrous-dependent family of non-heme oxygenases (Prescott, 1993; Feig & Lippard, 1994; Roach et al., 1995). Like other members of the family it requires ferrous iron as a cofactor and utilizes dioxygen as a co-substrate. However, unlike all other identified members of the family, except isopenicillin N synthase (IPNS) which requires only dioxygen as a co-substrate, it does not utilize a 2-oxo acid co-substrate. Instead, in the case of ACC oxidase, ascorbate acts as a co-substrate being apparently stoichiometrically oxidized to dehydroascorbate (Dong et al., 1992). Uniquely among the family of known ferrous dependent oxygenases and oxidases, ACC oxidase also requires bicarbonate or CO_2 as an activator (Dong et al., 1992; Smith & John, 1993).

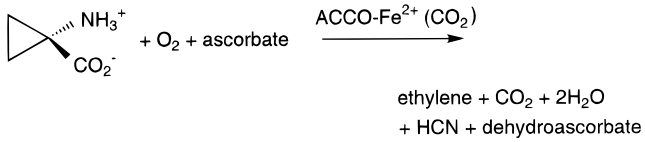

FIGURE 1: ACC oxidase-catalyzed production of ethylene from ACC.

Inactivation of the ferrous dependent oxygenases has been previously reported (Blanchard et al., 1982; Nietfeld & Kemp, 1981). However, purified ACC oxidase is particularly labile with a typical half-life of less than 20 min under optimized catalytic conditions (Smith et al., 1994). The deletion of catalase, BSA, and DTT from the optimal reaction mixture reduces the catalytic half-life of ACC oxidase even further (Smith et al., 1994).

Oxidative inactivation of other enzymes has been well documented. However the number of cases where specific modifications have been identified are limited (Roberts et al., 1989; Stadtman, 1993; Gan et al., 1995; Yao et al., 1996). The iron- and ascorbate-mediated fragmentation and/or inactivation of enzymes has also been reported [glutamine synthase, Levine et al. (1981), Levine (1983), Liaw et al. (1993), and Rivett and Levine (1990); malic enzyme, Wei et al. (1994, 1995) and Chou et al. (1995); propanediol oxidoreductase, Cabiscol et al. (1992, 1994)]. Fragmentation

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[1] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; PA, 1,10-phenanthroline; BSA, bovine serum albumin; DTT, dithiothreitol; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; TRIS, tris(hydroxymethyl)aminomethane; IPNS, isopenicillin N synthase; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; ESIMS, electrospray ionization mass spectrometry; HPLC, reverse phase high-performance liquid chromatography.

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does not necessarily accompany inactivation (Stadtman, 1990) and in the case of iron/ascorbate/dioxygen-mediated modification of malic enzyme lags behind it (Chou et al., 1995) indicating that fragmentation involves minimally a two-stage process. γ-Butyrobetaine hydroxylase, a member of the 2-oxo acid dependent dioxygenase family to which ACC oxidase is related, has also been reported to undergo ascorbate/dioxygen-mediated inactivation (Blanchard et al., 1982). However, the inactivation of γ-butyrobetaine hydroxylase is reportedly independent of the presence of iron, seemingly ruling out the involvement of Fenton chemistry. It should also be noted that ascorbate may also offer a protective role against oxidative inactivation in the case of some 2-oxoglutarate dependent dioxygenases (e.g., prolyl-4-hydroxylase; Nietfeld & Kemp, 1981).

Previously it has been reported that inactivation of ACC oxidase is not due to product inhibition by dehydroascorbate or cyanide and that maximal inactivation of ACC oxidase occurs under catalytic conditions. However, in the absence of ACC a distinct ascorbate dependent inactivation was observed. This inactivation could be partially prevented by the addition of catalase, implying the involvement of hydrogen peroxide (Smith et al., 1994).

Herein, we report the results of a detailed study on ACC oxidase inactivation. These suggest that the catalytic lability of ACC oxidase results from minimally three discrete processes: (i) partial unfolding of the catalytically active conformation; (ii) oxidative damage from hydrogen peroxide in solution, which is catalase protectable; (iii) oxidative damage to the active site, which is not catalase protectable. The active site directed inactivation results in partial proteolysis of ACC oxidase, the fragmentation pattern of which depends on the presence of ACC.

MATERIALS AND METHODS

Protein Purification. Protein concentration was determined by the method of Bradford (1976) using BSA as a standard. Recombinant ACC oxidase from tomato fruit was purified using the method of Zhang et al. (1995) with the following modifications: 1,10-phenanthroline (PA, 0.1 mM) or EDTA (5 mM in extraction buffer, 1 mM in column buffers) was present in all buffers. Glycerol (10%) was also present in all column buffers. Supernatants from centrifugation of cell lysates were diluted by the addition of an equal volume of buffer A [25 mM HEPES-NaOH, pH 7.5, 25 °C, 2 mM DTT, 0.5 mM benzamidine, 10% (v/v) glycerol] before loading onto a prepacked Q-Sepharose (high performance) column. A linear gradient from 0% to 15% buffer B (buffer A plus 2 M NaCl) was run. Fractions containing ACC oxidase were pooled, concentrated using an Amicon concentrator, and frozen in liquid nitrogen before storage overnight at −80 °C. Thawed samples were then loaded onto a Superdex S-75 column equilibrated in buffer C [20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM PA, 10% (v/v) glycerol]. ACC oxidase thus obtained was estimated to be >95% pure by SDS−PAGE analysis. Storage was at −80 °C in buffer D [20 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol] after freezing in liquid nitrogen, at a typical protein concentration of 25 mg/mL.

Enzyme Activity Assay. ACC oxidase activity was measured according to the method described by Zhang et al. (1995) and references therein. For inactivation time course experiments BSA, DTT, and catalase were not included in the reaction mixture. Incubation times of 10 min were used. 1 unit of activity is defined under these conditions as 1 μmol of ethylene produced per mg of enzyme over a 10 min incubation time. Activities for ACC oxidase used in the reported studies varied from 0.7 to 1.3 units (in the absence of catalase, DTT, and BSA) depending on the particular batch of enzyme used. This corresponds to 25−45 mol of ethylene produced per mole of enzyme for 10 min. Some batches of purified ACC oxidase exhibited much lower initial activities of between 0.2 and 0.45 units. Similar or lower (>0.05 units) initial activities were also seen for some aged (>1 month old) enzyme stocks, stock that had been repeatedly freeze−thawed and stock that had been left at ambient room temperature for 3 days. Enzyme activity in the absence of added bicarabonate was <15% of that observed under full assay conditions.

Pre-Incubation Experiments. ACC oxidase was pre-incubated at a protein concentration of 1 mg/mL (27 μM) in buffer E [100 mM HEPES-HCl, pH 7.1, 10% (v/v) glycerol] in a final volume of 100 μL. Combinations of iron(II) sulfate heptahydrate (final concentration 0.4 mM), calcium or sodium L-ascorbate (25 mM), ACC (20 mM), ammonium bicarbonate (10 mM), catalase (0.5−5 mg/mL), and BSA (5 mg/mL) were included as indicated. The order of addition of the components was as follows: iron (II) (5 μL of an 8 mM solution), buffer E, L-ascorbate (5 μL of 500 mM solution), ACC (5 μL of 400 mM solution), bicarbonate (5 μL of 200 mM solution), catalase or BSA (7 and 10 μL, respectively), and finally ACC oxidase (generally 10 μL). Pre-incubations were carried out at 28 °C in a water bath. No precaution was taken to remove CO_2 from buffer solutions. Samples (10 μL) removed at times indicated in the results section and were tested for ACC oxidase activity as described above.

Anaerobic pre-incubation experiments, at a dioxygen concentration of less than 2 ppm, were performed in a glovebox (Vacuum/Atmospheric Company). All solutions were prepared within the glovebox. Reduced benzyl viologen remained colored over periods greater than 6 h. Frozen ACC oxidase was stored on dry ice while being introduced into the glovebox via a vacuum chamber port. The ACC oxidase solution was then diluted 5× with degassed buffer E. Incubations were carried out at the same temperatures as for aerobic incubations on a hot block at the same concentrations of iron, L-ascorbate, and enzyme as used for aerobic pre-incubations. Samples (10 μL) were then removed at times indicated in the results section and frozen on dry ice. These samples were then removed from the glovebox and individually assayed for ethylene production over a 20 min incubation time. The frozen aliquots were transferred to the assay vials after rapid (<15 s) resuspension in 40 μL of argon-saturated buffer E.

Fragmentation of ACC Oxidase. ACC oxidase was incubated as described for the pre-incubation experiments under aerobic conditions. The temperature of incubations was varied from 28−40 °C (see results). Incubations were quenched by freezing using solid CO_2 or by adding 5× SDS−PAGE sample buffer [250 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.05% w/v bromophenol blue, 5% (v/v) β-mercaptoethanol]. Samples were desalted (if necessary) using a Bio-Spin 6 chromatography column (Bio-Rad). Incubations in the presence of ferrous iron,

L-ascorbate, and hydrogen peroxide were performed using the method of Ettner et al. (1995).

SDS−PAGE and N-Terminal Sequence Analyses. Fragmentation of ACC oxidase was visualized by electrophoresis of samples (10−15 μg) on 15% Tris-glycine SDS−polyacrylamide minigels (Laemmli, 1970) or 12.5% Tris-tricine SDS−polyacrylamide "minigels" (Schägger & Von Jagow, 1987). Samples containing 6 M urea were prepared by addition of SDS to 0.1% before gel loading. All other samples contained SDS−PAGE sample buffer. Gels were run for approximately 50 min at 45 mA (Tris-glycine) or 200 min at 15 mA (Tris-tricine) on Bio-Rad Mini-Protean II systems and stained with Coomassie Blue. Cleaved fragments were blotted on to a Waters Immoblion Membrane in a Bio-Rad Mini-Transblot cell at 0.3 A for 1 h in the presence of 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid in 50% methanol, pH 11, and submitted for N-terminal sequencing by Edman degradation.

Electrospray Ionization Mass Spectrometry (ESIMS) and Reverse Phase High-Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry (HPLC/ESIMS). Mass analyses of ACC oxidase were performed using a VG Instruments triple-quadrupole atmospheric mass spectrometer fitted with an electrospray ionization interface. Protein samples (10 μg in 20 μL, acidified by addition of formic acid to 1%) were injected at a flow rate of 20 μL/min, as a solution in acetonitrile/water (1:1, v/v) containing 1% formic acid. Calibration was performed using horse heart myoglobin (M_r 16951.48). Protein samples not suitable for direct ESIMS analysis due to the presence of salts, were desalted using Bio-Spin 6 chromatography columns into 10 mM Tris-HCl, pH 8.0. Alternatively, samples containing salt were analyzed by HPLC/ESIMS. Samples (20 μL of approximately 1 mg/mL) were injected onto a microbore HPLC column (Synchropak C-18, particle size 6.5 μm) equilibrated with 4% aqueous MeCN containing 0.1% trifluoroacetic acid, connected to Applied Biosystems 140B pumps. Samples were eluted with a 4%−80% MeCN gradient at a flow rate of 80 μL per min.

RESULTS

Preincubation Studies. In order to simplify interpretation of the results preincubation studies were carried out in the absence of BSA, catalase, and DTT, which are present in our optimal catalysis conditions. Exponential decay curves ($A = A_0e^{-\lambda t}$; A = activity at time t, A_0 = activity at $t = 0$, λ = exponential decay constant) were used to fit the inactivation data.

Most rapid inactivation ($\lambda = 0.12-0.17$ min^{-1}, Figure 2) of ACC oxidase was observed in the presence of ACC, iron, and L-ascorbate, but in the absence of bicarbonate (condition A, Table 1). The rate of inactivation was reduced in the presence of iron and ascorbate, but in the absence of ACC ($\lambda = 0.06-0.09$ min^{-1}, condition D, Table 1, Figure 2). Under these conditions $t_{1/2}$ was typically ca. 10 minutes. Without ACC the rate of inactivation was not significantly affected by the presence of up to 15 mM bicarbonate (condition C, Table 1, Figure 2). However, the rate of inactivation was further reduced under complete assay conditions, i.e., by the inclusion of iron, ascorbate, ACC, and bicarbonate, i.e., $\lambda = 0.04$ min^{-1} (condition B, Table 1). Comparison of conditions A and B most clearly demonstrates the effect of bicarbonate. The inactivation rate was approximately the same with either 10 or 15 mM added bicarbonate. A significant reduction of the inactivation rate (cf., conditions A and B, Table 1) was also seen when using 1 mM added bicarbonate ($\lambda = 0.06$ min^{-1}). For tomato ACC oxidase ca. 15 mM bicarbonate is required for optimal catalytic activity. The observation that the rate of inactivation is the same for conditions C (− ACC + bicarbonate) and condition D (− ACC − bicarbonate) suggests that the protective effect of CO_2 is directly related to catalytic turnover.

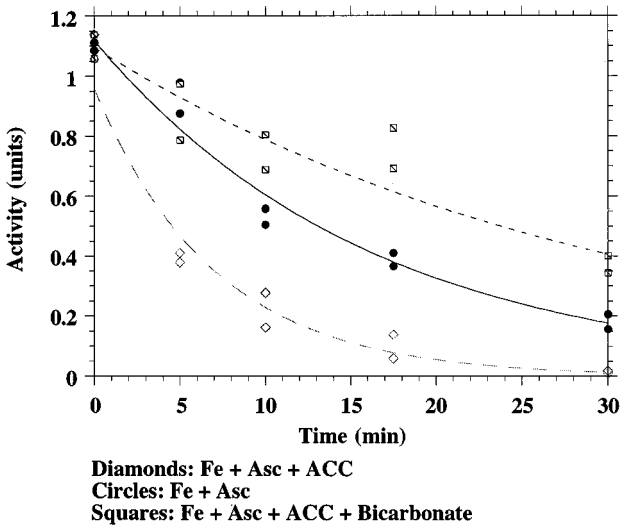

FIGURE 2: Inactivation time course of ACC oxidase under condition A (iron/ascorbate/ACC: diamonds), condition B (iron/ascorbate/ACC/bicarbonate: squares), and condition C (iron/ascorbate: circles).

The rate of inactivation in the absence of all co-substrates and iron was much slower than using conditions A−D ($\lambda = 0.008-0.017$ min^{-1}; condition E, Table 1). No change in rate of inactivation was observed in the presence of ascorbate alone in the preincubation mixture ($\lambda = 0.017$ min^{-1}; condition F, Table 1) or by the addition of DTT (0.3 mM). In the case of iron alone (condition G, Table 1) a slightly increased level of inactivation (relative to condition E) was observed after a 60 min preincubation period (data not shown). Notably, complete activity loss under condition E was not observed even after 72 h at ambient temperature. Typically, a decrease in specific activity of 60%−80% was observed after 72 h, but the observed rates for this non-oxidative inactivation process were batch dependent and some enzyme preparations were relatively stable.

Under anaerobic conditions (<5 ppm dioxygen, condition H, Table 1) the rate of inactivation in the presence of ferrous iron and ascorbate was much lower than that under aerobic conditions (condition D, Table 1). The rate of inactivation was the same (within experimental error) as that observed in the absence of co-substrates and ferrous iron (condition E, Table 1).

Inactivation in the presence of iron and L-ascorbate (condition D, Table 1) was partially prevented by the presence of catalase ($\lambda = 0.03-0.04$ min^{-1}; condition I, Table 1). The addition of BSA at the same concentration had no effect on loss of ACC oxidase activity ($\lambda = 0.09$ min^{-1}; condition J). Inactivation under condition E (absence of all substrates) was not affected by the inclusion of catalase ($\lambda = 0.01$ min^{-1}; condition K, Table 1). Increasing the

Table 1: Preincubation Conditions

	O_2	ASC[a]	Fe^{2+}	ACC	HCO_3^-	catalase	BSA	$t_{1/2}$ (min)[b]	σ (min)[d]	fragmentation
A	+	+	+	+	−	−	−	4; 5; 6	0.6	observed
B	+	+	+	+	+	−	−	15	−	observed
C	+	+	+	−	+	−	−	10; 12; 9	1.2	observed
D	+	+	+	−	−	−	−	10 (8)[c]	1.0	observed
E	+	−	−	−	−	−	−	58 (5)[c]	15.0	not observed
F	+	+	−	−	−	−	−	41	−	observed[e]
G	+	−	+	−	−	−	−	24; 46	−	observed[e]
H	−	+	+	−	−	−	−	−	−	not observed
I	+	+	+	−	−	+	−	21 (4)[c]	3.6	observed
J	+	+	+	−	−	−	+	8	−	observed
K	+	−	−	−	−	+	−	58; 70	−	−

[a] Sodium/calcium ascorbate. [b] Values given to nearest minute with one value per experiment. $t_{1/2} = \ln 2/\lambda$ where λ was obtained from curve fitting using the program Kaleidograph (Macintosh). [c] Values represent the mean with number of repeats indicated in brackets. [d] Standard deviations given to one decimal place. [e] Observed fragmentation greatly reduced by SDS−PAGE analysis.

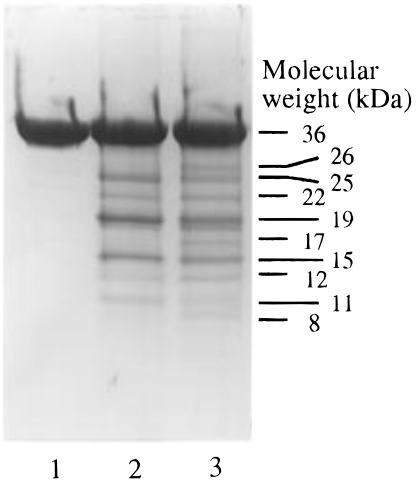

FIGURE 3: Fragmentation of ACC oxidase. Lane 1 (ACC oxidase incubated under condition E [buffer only]); lane 2 (ACC oxidase incubated under condition D [iron/ascorbate], Pattern I); lane 3 (ACC oxidase incubated under condition A [iron/ascorbate/ACC], pattern II).

concentration of catalase above 0.5 mg/mL did not offer increased protection.

Fragmentations of ACC Oxidase. In the presence of ferrous iron and ascorbate under aerobic conditions (condition D) minimally eight major oligopeptide fragments (pattern 1, lane 2, Figure 3) were produced by SDS−PAGE analysis. Fragmentation was apparently complete after 5 min by SDS−PAGE analysis, with the estimated extent of fragmentation of ACC oxidase less than 10%. The addition of hydrogen peroxide at 0.1% (v/v) did not increase the extent of fragmentation but did alter the relative intensities of some bands. 1% hydrogen peroxide (v/v) resulted in the production of smeared bands by SDS−PAGE, making analysis difficult. Inclusion of catalase or BSA did not alter the pattern or extent of fragmentation as observed by SDS−PAGE analysis (data not shown), cf. conditions D and I (Table 1).

No fragmentation was observed when 6 M urea or 5× SDS−PAGE sample buffer was added to the ACC oxidase stock immediately prior to the addition of iron and ascorbate. This suggests a correctly folded enzyme is required for fragmentation. Approximately the same apparent level of fragmentation was seen using iron/ascorbate concentrations of 0.04 mM/25 mM or 0.4 mM/2.5 mM (data not shown). Reduced fragmentation was observed at iron/ascorbate concentrations of 0.4 mM/0.25 mM (data not shown). These limited data apparently suggest fragmentation is more sensitive to changes in ascorbate rather than iron concentrations.

Incubation of ACC oxidase with ACC, iron, and ascorbate produced a different pattern of fragmentation in which additional bands were observed (pattern II, lane 3, Figure 3) compared to that observed after incubation with iron and ascorbate alone. Extra bands at ca. 26, 17, and ca. 8 kDa were present and the ca. 11 and 25 kDa bands were fainter by SDS−PAGE analysis (Figure 3). Incubation with iron, ascorbate, ACC, and bicarbonate (condition B, Table 1) gave approximately the same fragmentation pattern as that obtained with iron, ascorbate, and ACC only (condition A, Table 1) (data not shown).

The N-terminal sequence of the ca. 15 kDa fragment was determined to be FQDNKVS. N-terminal sequencing of the ca. 26, 25, 22, and 19 kDa fragments revealed them to contain the same N-terminus as intact ACC oxidase.

Electrospray Ionization Mass Spectrometry. Masses of 35 813.4 ± 1.5 Da and 35 813.2 ± 1.0 Da respectively were obtained for ACC oxidase before and after a 24 h incubation under condition E (Table 1, buffer only). Accurate masses for samples incubated with iron and/or ascorbate (conditions A−D, F, and G) were not obtained because of peak broadening in the mass spectra. Peak broadening was also seen in the HPLC/ESIMS mass spectra of ACC oxidase obtained from these incubations, suggesting that the broadening is not caused by salt contamination but instead results from covalent modifications to ACC oxidase. The following masses were measured for ACC oxidase by HPLC/ESIMS: condition E (no substrates) 35 816.0 ± 4.5 Da (implying no covalent modification); condition F (ascorbate only) 35 878.4 ± 15.0 Da; condition G (iron only) 35 864.0 ± 13.5 Da; condition B (all substrates) 35 934.96 ± 16.56 Da. HPLC/ESIMS of fragmented ACC oxidase (condition B, Table 1) gave a mass of 14 600 Da for the ca. 15 kDa fragment observed by SDS−PAGE (calculated for the C-terminal fragment resulting from cleavage between Leu-186 and Phe-187 the mass is 14 568 Da. Despite the relatively high errors in the mass analyses of enzyme after incubation with cofactors, there is evidence for mass increases. This evidence is seen most clearly in the case of preincubation under condition B (all substrates). The resolution of the data, however, was insufficient to specify a specific mass shift, and the broad peaks probably reflect a coalescence of similar masses resulting from more than one modification.

DISCUSSION

The data presented here clearly suggest that minimally three different mechanisms of ACC oxidase inactivation occur. Evidence for these is discussed below. All the inactivation time courses could be fitted assuming first-order or pseudo-first-order kinetics, suggesting that concurrent apparently first-order pathways lead to inactive or low-activity enzyme. The observed rates of aerobic inactivation of ACC oxidase are dependent on the particular combinations of ACC, iron, ascorbate, bicarbonate, and catalase present in the preincubation mixture.

Non-Oxidative Inactivation of ACC Oxidase. Isolated operation of the slowest inactivation mechanism occurs in the absence of all co-substrates and iron (condition E). Since care was taken to remove metals, it is unlikely that this "slow" inactivation results from metal contamination. ESI-MS analysis also demonstrated that the mass of ACC oxidase did not change upon standing in buffer alone (condition E) for 24 h (data not presented). It thus seems likely that this inactivation mechanism results from a conformational change of fully active ACC oxidase to give an inactive or substantially less active enzyme. Near and far UV CD spectroscopy of ACC oxidase which had been allowed to stand in buffer (condition E) for 2 h did not show any substantial changes relative to the fully active enzyme (data not shown). However, substantial conformational changes may not be detected using this technique. Inactivation via the proposed unfolding process did not result in complete loss of activity and it is possible that the partially unfolded protein is in a catalytically suboptimal conformation which retains a lower activity. This may partially account for the differences in kinetic parameters reported for ACC oxidases from different sources.

Oxidative Inactivation. Analysis of the experimental data suggests the operation of at least two oxidative inactivation processes for ACC oxidase, both of which require iron, dioxygen, and ascorbate (Table 1). Reaction of dioxygen, ascorbate, and iron (Udenfriend's reagent) is known to generate reactive oxidative species, including hydrogen peroxide, which may further react to generate hydroxyl radicals (Udenfriend et al., 1954; Stadtman, 1993). Unlike the other case studies on iron/ascorbate-mediated inactivation of enzymes (see introduction) ACC oxidase actually uses both ascorbate and dioxygen as substrates and iron as a cofactor in its catalytic cycle. This makes separation of inactivation processes resulting from the generation of oxidizing species in solution (via Udenfriend/Fenton type reactions) from those that occur via active site located processes difficult. Furthermore, it is possible that the two processes are linked, e.g., hydrogen peroxide may be generated in solution and react at the active site.

Iron/ascorbate-mediated inactivation is only partially prevented by the addition of catalase. This suggests the operation of two oxidative inactivation processes. The catalase sensitive inactivation process differs from the catalase insensitive process in that it must operate, at least in part, outside the active site via catalase accessible hydrogen peroxide. This result contrasts with the cases of glutamine synthase (Levine et al., 1981), propanediol oxidoreductase (Cabiscol et al., 1992), and γ-butyrobetaine hydroxylase (Blanchard et al., 1982), where the inclusion of at least 0.1 μM catalase gave total protection against iron/ascorbate-mediated inactivation. Catalase insensitive iron/ascorbate-mediated inactivation has, however, been reported for creatine kinase (Levine, 1983).

Inactivation of ACC oxidase via the catalase sensitive process thus probably occurs via generation of peroxide(s) in solution. These may further react in solution to generate more reactive oxidizing species, such as hydroxyl radicals which may effect non-specific damage to the enzyme, causing the peak broadening of intact ACC oxidase observed by ESIMS after oxidative inactivation. However, since ACC oxidase contains a metal binding site it may be that solution-generated peroxide can bind to the active site and effect oxidative inactivation, but not fragmentation (see below). This proposal may also explain the sensitivity of other iron dependent non-heme oxygenases to iron/ascorbate-mediated inactivation, i.e., the enzyme bound metal serves to concentrate deleterious oxidative reactions resulting from Udenfriend/Fenton chemistry at the active site. The use of a reducing agent which fulfills the cofactor role of ascorbate, but which does not generate reactive oxidizing species in solution, may be a way of increasing the *in vitro* half-life of ferrous dependent oxygenases employing ascorbate as a cofactor. It may also be beneficial to minimize the presence of CO_2/bicarbonate (if not required for activity as in the case of ACC oxidase) in order to minimize protein damage via Fenton chemistry (Stadtman, 1993).

The catalase insensitive process is largely responsible for the fast inactivation seen in the presence of catalase, iron, and ascorbate (condition I, Table 1). The increase in inactivation rate seen upon inclusion of ACC with iron and ascorbate (condition A, Table 1) is probably caused by a corresponding increase in the rate of this second process and suggests this process occurs at the active site. Fragmentation of ACC oxidase is probably associated with this process, since fragmentation was not prevented by the addition of catalase. The pattern of fragmentation was altered (from pattern I to II, Figure 3) by the inclusion of ACC. This observation suggests that ACC can bind to ACC oxidase in the absence of added bicarbonate. With other enzyme systems, the effects of including substrates with iron and ascorbate varies from total protection against inactivation (e.g., pyruvate kinase with ATP and phospho(enol)pyruvate; Levine, 1983), to stimulation (e.g., glutamine synthase with glutamic acid, ATP or ammonia; Levine, 1983), to no effect (e.g., malic enzyme with L-malate and NADP; Wei et al., 1994). With glutamine synthase, ATP and glutamate together stimulate inactivation of the adenylated form but inhibit the inactivation of the non-adenylated form (Levine, 1983). A possible explanation for these effects is the exposing of sensitive regions of the enzyme via conformational change upon substrate binding. The change of fragmentation pattern that was seen with ACC inclusion is in accordance with this explanation.

The integrity of the iron binding site of ACC oxidase is apparently essential for fragmentation to occur. ACC oxidase denaturation with either SDS−PAGE sample buffer or urea totally inhibited fragmentation. Similar fragmentation patterns were observed after preincubation under complete assays conditions (condition B, Table 1) or at a limiting CO_2 level (condition A, Table 1), suggesting that fragmentation is not directly related to catalytic turnover. The extent of fragmentation in the presence of iron and ascorbate (condition D) did not correlate with activity loss of ACC oxidase;

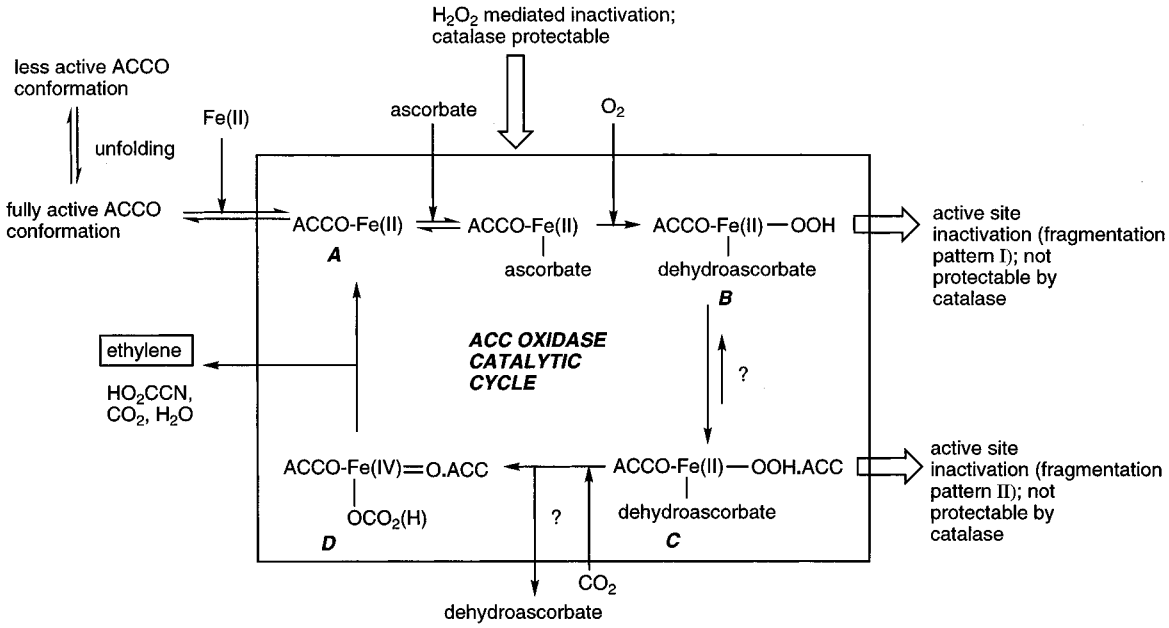

FIGURE 4: Proposed mechanism for the catalytic cycle and inactivation of ACC oxidase.

fragmentation appeared to occur in an initial burst within the first 5 min while enzyme activity decreased with a half-life of 10 min. The reason for the initial burst of fragmentation is unclear, but may be related to incorrectly folded or "primed" enzyme. Additionally, the failure of catalase to prevent fragmentation makes it unlikely that non-enzymatically generated peroxide causes cleavage.

One cleavage site in tomato ACC oxidase was determined by N-terminal sequencing (and supported by HPLC/ESIMS analysis) of the 15 kDa fragment. This was located between Leu-186 and Phe-187. Sequence alignments indicate that the tertiary structure of ACC oxidase resembles that of IPNS (Prescott, 1993; Roach et al., 1995) and three of the ligands to the catalytic iron in ACC oxidase are provided by the side chains of His-177, Asp-179, and His-234. The oxidative cleavage site of tomato ACC oxidase between Leu-186 and Phe-187 is predicted to be located close to the end of a β-sheet and within 8 Å of the active site iron according to the predicted structural similarity of ACC oxidase with IPNS (Roach et al., 1995). The peptide sequence immediately following the cleavage point, FQDD, is similar to the cleavage sequence FNDD reported for malic enzyme (Wei et al., 1994), but the cleavage point in malic enzyme occurs immediately after this sequence, not before. The similarity between the two sequences may be coincidental, but it is possible that the polar side chain residues in them provide ligands for iron binding sites, i.e., in ACC oxidase there are two iron binding sites. Iron may be displaced from its catalytic ligands to a second proximate binding site, or it is possible that the two sites may be simultaneously occupied.

The N-terminal sequences of other peptide fragments obtained were identical to those of intact ACC oxidase. Their SDS−PAGE estimated masses included fragments with estimated masses of ca. 19, 22, 25, and 26 kDa, consistent with cleavage close within the predicted active site region of ACC oxidase (Roach et al., 1995).

The mechanism of bicarbonate or CO_2 activation of the ethylene-forming reaction in ACC oxidase is unknown. Stadtman and Berlett (1991) have reported that amino acid oxidation by Fenton's reagent (Fe, EDTA, hydrogen peroxide) is dependent upon the presence of bicarbonate and have suggested that oxidation proceeds via a bicarbonate complex of iron. Non-enzymatic oxidative breakdown of ACC to give ethylene by iron and hydrogen peroxide (Fenton's reagent) is also greatly stimulated in the presence of bicarbonate (McRae et al., 1983).

The rate of inactivation in the presence of ACC, iron, and ascorbate (condition A, Table 1) was slowed down significantly upon inclusion of bicarbonate (condition B, Table 1). This rate of inactivation (with all substrates present) was even slower than that with just iron and ascorbate present (cf. conditions B and D, Table 1 and Figure 2). However, the addition of 15 mM bicarbonate did not significantly affect the rate of inactivation of ACC oxidase in the presence of iron and ascorbate only (cf. conditions C and D, Table 1). It is likely that this protective effect of bicarbonate against inactivation in the presence of all cofactors/substrates is directly related to its role as a catalytic activator.

Since the (i) addition of bicarbonate does not affect the rate of iron/ascorbate-mediated inactivation in the absence of ACC, but (ii) significantly slows it in the presence of ACC and (iii) the fragmentation pattern of iron/ascorbate-mediated inactivation in the absence of bicarbonate is altered by the addition of ACC, the data presented here suggest that during the catalytic cycle ACC binds prior to bicarbonate.

Mechanistic Proposals. A proposal for the catalytic cycle and inactivation of ACC oxidase is shown in Figure 4. By analogy with proposals for the 2-oxo acid dependent dioxygenases (Hanauske-Abel & Günzler, 1982) and IPNS (Roach et al., 1995) it is proposed that ascorbate and dioxygen bind and react with an ACC oxidase−Fe(II) complex (A, Figure 4) to form an iron(II)-linked peroxide intermediate (B, Figure 4) concomitant with production of dehydroascorbate. It is possible that peroxide formation proceeds via initial single-electron transfer from iron to form a superoxide intermediate (not shown) and the formation of an ascorbate ligated iron(IV) peroxide intermediate also cannot be ruled out. ACC may then bind to the peroxide intermediate to give complex C (Figure 4). CO_2 or bicarbonate then binds causing formation of a ferryl intermediate which oxidizes ACC to

ethylene and cyanoformic acid, resulting in completion of the catalytic cycle. We propose that bicarbonate displaces dehydroascorbate as a ligand for the active site iron. It is possible that the change from a putative bidentate (dehydroascorbate) to monodentate ligand (bicarbonate) allows ACC (possibly via its amino group) to ligate productively to the ferryl species prior to its oxidation.

Inactivation may occur via the following processes. Fully active ACC oxidase may undergo partial unfolding to give a less active conformation(s). The peroxide intermediate(s) (B, C, Figure 4) may break down, resulting in ACC oxidase inactivation and fragmentation. Catalase protectable inactivation is mediated via hydrogen peroxide binding to active site iron. The hydrogen peroxide may be generated in solution or at the active site. Clearly the proposals within this working hypothesis require experimental testing and may also need to accommodate the possibility of more than one iron binding site in ACC oxidase.

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