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Kinetic and Mutagenic Evidence for the Role of Histidine Residues in the *Lycopersicon esculentum* 1-Aminocyclopropane-1-carboxylic acid Oxidase

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The ACCO gene from *Lycopersicon esculentum* (tomato) has been cloned into the expression vector PT7-7. The highly expressed protein was recovered in the form of inclusion bodies. ACCO is inactivated by diethyl pyrocarbonate (DEPC) with a second-order rate constant of 170 M^{-1} min^{-1}. The pH–inactivation rate data imply the involvement of an amino acid residue with a pK value of 6.05. The difference UV spectrum of the the DEPC-inactivated versus native ACCO showed a single peak at 242 nm indicating the modification of histidine residues. The inactivation was reversed by the addition of hydroxylamine to the DEPC-inactivated ACCO. Substrate/cofactor protection studies indicate that both iron and ACC bind near the active site, which contains histidine residues. Four histidines of ACCO were individually mutated to alanine and glycine. H39A is catalytically active, while H177A, H177G, H211A, H211G, H234A, and H234G are basically inactive. The results indicate that histidine residues 177, 211, and 234 may serve as ligands for the active-site iron of ACCO and/or may play some important structural or catalytic role.

KEY WORDS: ACC oxidase; DEPC; chemical modification; histidine; mutagenesis.

1. INTRODUCTION

Ethylene, the simplest alkene, is a phytohormone that plays a key role in plant growth and development, including fruit ripening, abscission, and senescence (Abeles *et al.*, 1992; Mattoo and Suttle, 1991). Ethylene is synthesized from methionine via S-adenosylmethionine (SAM).[3] The 2-aminobutanoic acid portion of SAM is cyclized to 1-aminocyclopropane-1-carboxylic acid (ACC) by the pyridoxal-phosphate-utilizing enzyme ACC synthase (Kende, 1993; Yang and Hoffman, 1984). Ethylene-forming enzyme (EFE) [1-aminocyclopropane-1-carboxylic acid oxidase (ACCO)] catalyzes the final step in the biosynthesis of ethylene by the oxidation of ACC to ethylene (Kende, 1993; Yang and Hoffman, 1984).

Until recently, the isolation and biochemical characterization of ACCO was impeded due to enzyme instability during homogenization procedures. Hamilton *et al.* (1990) showed that the pTOM13 antisense gene significantly reduced ACCO activity in tomato fruits, suggesting that the tomato pTOM13 gene product was related to ACCO. The cDNA of ACCO showed sequence homology to flavanone-3β-hydroxylase, which required Fe^{2+}, α-ketoglutarate, and ascorbate for activity (Hamilton *et al.*, 1990). This led Ververidis and John (1991) to demonstrate ACCO activity *in vitro* by the inclusion of Fe^{2+} and ascorbate in the assay of their enzyme. Subsequently, ACCO was obtained in partially

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[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; DEPC, diethylpyrocarbonate; DTT, dithiothreitol; EFE, ethylene-forming enzyme; EDTA, [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; IPNS, isopenicillin-N-synthase; SAM, S-adenosylmethionine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TRIS, tris(hydroxymethyl)aminomethane.

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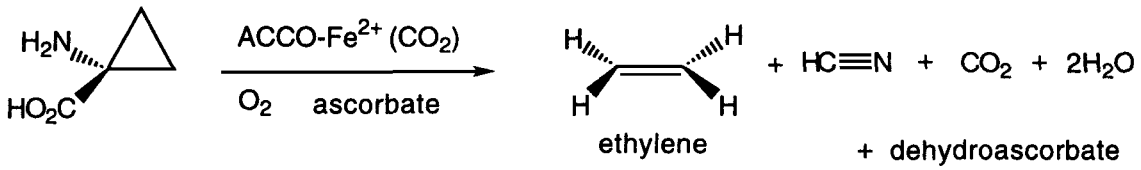

Scheme 1. Reaction catalyzed by 1-aminocyclopropane-1-carboxylic acid synthase.

purified or pure preparation from apple (Dilley *et al.*, 1993; Dong *et al.*, 1992*a*, *b*; Dupille *et al.*, 1992; Fernandez-Maculet and Yang, 1992; Kuai and Dilley, 1992; Pirrung *et al.*, 1993), avocado (McGarvey and Christoffersen, 1992), melon fruits (Smith *et al.*, 1992), and pear (Vioque and Castellano, 1994). ACCO activity has been expressed in yeast (Hamilton *et al.*, 1991) and *Xenopus* oocytes (Spanu *et al.*, 1991) and most recently in *Escherichia coli* (Zhang *et al.*, 1995).

In the present paper, we describe the high-level overexpression of *Lycopersicon esculentum* (tomato) ACCO in *E. coli* in the form of inclusion bodies and the solubilization of ACCO inclusion bodies by sodium deoxycholate or guanidinium hydrochloride. We provide chemical and kinetic evidence for the involvement of the histidine residues in ACCO catalysis as evidenced by the pH dependence of inactivation, substrate protection studies, reactivation of DEPC-inactivated enzyme by hydroxylamine, and detection of *N*-carbethoxyhistidine by UV difference spectra. Finally, results from mutagenic studies are presented that suggest histidine 177 and 234 are possible ligands involved in binding of the iron at the active site of ACCO, while histidine 211 may be involved with either catalysis or structure.

2. MATERIALS AND METHODS

Restriction and DNA modifying enzymes were from Boehringer Mannheim. The pCR-Script SK(+) cloning kit and the Quik-Change Site-directed Mutagenesis Kit were purchased from Stratagene Cloning System, Inc. The *E. coli* strain BL 21 (DE 3) was obtained from Novagen. Imidazole, sodium deoxycholate, and diethylpyrocarbonate were from Sigma Chemical Company. The 1-aminocyclopropane-1-carboxylic acid was purchased from Calbiochem (LaJolla, CA); hydroxylamine from Aldrich Chemical Co.; ferrous sulfate from Alfa (Ward Hill, MA); and guanidinium hydrochloride from Gibco BRL (Gaithersburg, MD). TG1 cells were obtained from Dr. Jack Dixon of the Biological Chemistry Department at the University of Michigan. The pYES plasmid containing the pRC13 insert (pYTE2) was a gift from Dr. Hans Kende of the Plant Biochemistry Department at Michigan State University. The pT7-7 was obtained from Dr. Stanley Tabor of the Harvard Medical School. The PCR amplification was performed using an MJR Research Thermal cycler. Oligonucleotides were synthesized by the University of Michigan Biomedical Research Resources Core Facility. DNA and protein sequencing as well as electrospray mass spectral analysis and total amino acid content were also carried out by the University of Michigan Biomedical Research Resources Core Facility. The GeneAmp kit was from Perkin-Elmer/Cetus, except that recombinant *Pfu* DNA polymerase (Strategene Cloning Systems, Buffer #1) was substituted for *Taq* DNA polymerase. Promega DNA and PCR purification kits were utilized.

2.1. PCR Cloning of ACCO

Two primers were constructed to correspond to the 5' and 3' ends of the open reading frame previously identified as the pRC13 (corrected pTOM13) (Hamilton *et al.*, 1991). The 5' sequence was

GATTCTAGAATTCATATGGAGAACTTCCCAATTATT

The 3' sequence was

GATTCTGAATTCGGATCCCTAAGCACTTGC

The forward primer incorporated an *Nde*I site (underlined) and the reverse primer a *Bam*HI (underlined) site for cloning into the expression vector pT7-7, constructed by Dr. Stanley Tabor at the Harvard Medical School. The tomato ACCO gene was amplified from the plasmid pYTE2 by PCR. The annealing temperature was 50°C. After 25 cycles, the amplified DNA was purified by the Promega PCR purification kit. The purified PCR product was cloned into pCR-Script following the procedure described by the Stratagene Co. to give pCR-Script-EFE. The desired fragment containing the pRC13 portion of the pCR-Script-EFE was excised by restriction digestion with *Nde*I/*Bam*HI and subcloned into the comparably restricted pT7-7. Chemically competent *E. coli* TG1 cells were transformed with the ligated plasmid, and re-

striction digest of the resulting constructs isolated from the TG1 cells revealed an insert of 0.95 kb in the vector. Plasmid DNA, pT7-7-EFE, purified from one of these transformants was used to transform chemically competent BL21(DE3) cells (Novagen, Inc.). The correct sequence (Hamilton *et al.*, 1991) of pT7-7-EFE was confirmed by automated sequencing on an Applied Biosystems 373A automated DNA sequencer utilizing dye-labeled dideoxy nucleotides, *Taq* polymerase, and double-stranded DNA, purified by the Promega PCR purification kit, at the University of Michigan Biomedical Research Resources Core Facility. A culture of the transformed cells (1 ml, OD_{600} = 0.5) was mixed with 500 µl of 50% glycerol and stored at −85°C.

2.2. Isolation and Solubilization of Recombinant ACCO Inclusion Bodies

An *E. coli* BL21(DE 3) colony harboring the plasmid pT7-7-EFE, selected from a 1-day-old LB-Amp plate, was grown to an OD_{600} = 0.5 in 5 ml of 2 × TY medium. The preparative medium (2 × TY containing 100 µg/ml ampicillin) was inoculated with 1 ml of the above culture per 200 ml of media. The cultures were incubated at 37°C with vigorous shaking (300 rpm) in 2-L baffled flasks (200 ml/flask) to an OD_{600} = 0.8 and IPTG was added (0.2 mM final). The incubation (37°C, 300 rpm) was continued for exactly 4 hr. The cells were harvested by centrifugation (4500 × *g*, 15 min) and stored at −20°C until use. The total wet weight of the cells was 3–4 g/L of 2 × TY. Frozen cells (from 1 L of culture) were suspended in lysis buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA). The cell suspension was sonicated at 4°C using a Model W-220 sonicator (Heat Systems, Ultrasonics Inc.) with three 15-sec bursts, and centrifuged at 27,000 × *g* for 15 min. The pellet was washed twice with lysis buffer and centrifuged as above. To remove contaminating material that would copurify with ACCO, the pellet (ACCO inclusion bodies) was resuspended in 50 ml of lysis buffer containing 0.5% octyl-β-D-glycopyranoside, incubated for 30 min at 4°C, sonicated at 4°C with two 15-sec bursts, and centrifuged at 6000 × *g* for 15 min. The inclusion bodies washing procedure was repeated at least twice. The ACCO inclusion bodies were suspended in lysis buffer containing 6 M guanidinium hydrochloride at a protein concentration of 2 mg/ml. The suspension was incubated for 1–2 hr at 4°C and centrifuged at 5000 × *g* for 10 min to remove insoluble material. The solubilized material (mostly ACCO) was diluted to 300 µg/ml and dialyzed overnight against three changes of 50 mM Tris-HCl (pH 8) containing 100 mM NaCl and 1 mM DTT. The sample was further dialyzed in 10 mM HEPES, pH 7, containing 1 mM DTT and then concentrated by ultrafiltration to a protein concentration of 5 mg/ml and stored at −80°C. Alternatively, the inclusion bodies were suspended in 2% sodium deoxycholate in lysis buffer. The suspension was incubated for 1–2 hr at 4°C with continuous stirring, sonicated at 4°C with two 15-sec bursts, and centrifuged at 5000 × *g* for 10 min. This cycle of resuspension, sonication, and centrifugation was repeated at least twice. The combined solubilized extracts were applied to a Sephadex G-25 desalting column equilibrated with 10 mM HEPES, pH 7, containing 1 mM DTT. The flow through fractions containing ACCO were pooled, dialyzed overnight against three changes of equilibration buffer, and concentrated by ultrafiltration to a protein concentration of 5 mg/ml on an Amicon PM-10 unit and stored at −85°C.

2.3. ACCO Assays

Method 1. The assay mixture contained, in a total volume of 1.0 ml, 100 mM HEPES (pH 7), 1 mM ACC, 1 mM sodium ascorbate, 100 µM ferrous sulfate, 4 mM sodium bicarbonate, and enzyme (10–25 µg protein). The reactions were carried out in 6-ml culture tubes sealed with rubber septa. After 20 min of incubation at 30°C, a 1-ml sample was withdrawn from the headspace of each tube and analyzed on a Varian 2400 gas chromatograph. The GC was equipped with a flame ionization detector and a stainless steel column (18 × 1/8 in) packed with Porapack R 80/100 mesh, operating at oven, injector, and detector temperatures of 60, 180, and 280°C, respectively. The ethylene produced in the headspace of the tube was quantitated by comparison with an authentic ethylene standard (5 ppm/injection). One unit of enzyme activity is defined as 1 nmol of ethylene produced per min (Fernandez-Maculet and Yang, 1992).

Method 2. The assay was performed as described above except that the 1.0-ml assay mixture contained in addition to the above reactants 10% glycerol, 500 mg catalase, 100 mg BSA, and 2 mM DTT (Zhang *et al.*, 1995).

2.4. Modification of ACCO with Diethylpyrocarbonate

Stock solutions of diethylpyrocarbonate (DEPC) were freshly prepared in dry ethanol (100%) immediately before use. The concentration of DEPC was determined spectrophotometrically by measuring the increase

in absorbance at 240 nm in 10 mM imidazole, 100 mM HEPES, pH 7. The extension coefficient is 3200 M^{-1} cm^{-1} (Melchior and Fahrney, 1970). The inactivation of ACCO was performed in a reaction mixture containing 12 μM ACCO (enzyme), 100 mM HEPES (pH 7), and DEPC (10 μl/ml). At time intervals, aliquots were removed and assayed for residual enzyme activity as described above.

2.5. Fluorescence Spectroscopy

Fluorescence spectra were performed on a Perkin-Elmer LS-50 Luminescence Spectrometer. Freshly prepared ACCO solutions (27 μg/ml) in 10 mM HEPES, pH 7, were placed in a 10 × 10 mm^2 quartz cuvettes and the cell temperature maintained at 25°C. The excitation wavelength was 278 nm and the emission spectra were recorded between 300 and 400 nm using a band width of 5 nm.

2.6. Analytical Procedures

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out under reducing conditions on 12% vertical slab gels according to the method of Laemmli (1970). After electrophoresis, the samples were stained with 0.25% Coomassie brilliant blue R-250. Protein concentrations in cell lysate, inclusion bodies, and soluble enzyme preparations were determined by a dye binding assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard. The molecular weight of the recombinant ACCO was determined by electrospray mass spectra performed by the University of Michigan Biomedical Research Resources Core Facility. The amino acid composition and N-terminal sequence analysis of recombinant ACCO were determined by the University of Michigan Biomedical Research Resources Core Facility utilizing an Applied Biosystems 420 analyzer equipped with a Model 130 HPLC unit. The ten amino-terminal amino acids were sequenced by the University of Michigan Biomedical Research Resources Core Facility via an Applied Biosystems 473 analyzer.

2.7. Site-Directed Mutagenesis

The histidine-to-alanine mutant genes were prepared by a modified PCR method (Chen and Przybyla, 1994) using the following mutagenic primers (designated Pm): H39A, 5'-GTTGGTGAAC**GCT**GGAATTCC-3' (eliminates an *Nco*I site); H177A, 5'-CCGCGCT**GCT**ACAGACGCAGGAGG-3' (introduces a new *Bso*FI restriction site); H211A, 5'-GGTCACCAAGGTTAACCACAATAGA**CGC**GCGCATGGGAGGAACATCG-3' (non-reading frame, introduces a new *BssH*II restriction site); and H234A, 5'-GTGCAATTACTCTG**GCC**AGCACAC-3' (non-reading frame, introduces a new *Msc*I restriction site). The nucleotides replaced from the wild-type (the pRC13 sequence of the plasmid pT7-7-EFE constructed above) gene are in bold. The common forward primer (upstream of the 5' portion of the coding region of ACCO) was the commercially available T7 sequencing primer, 5'-TAATACGACTCACTATAGGG-3' (designated P1). The common reverse primer (non-reading frame) (downstream of the 3' end) was 5'-GCATTGGTAACTGTCAGACC-3' (designated P2). The synthetic mutagenic primer (Pm) containing the desired mutagenic change and P2 for the two mutants H39A and H177A and P1 for mutants H211A and H234A were used for the first round of PCR amplification using wild-type plasmid pYTE2 as template and Vent DNA polymerase. The amplification was performed for 25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. Following the last cycle, the reaction mixture was held at 72°C for 5 min. The proper base-sized fragments from the first round of PCR were purified by electrophoresis on 1% low-melting agarose gel using 0.5 × TAE buffer. The excised bands (minimum amount of gel) were used directly as primers (in the double-stranded form) together with the alternate primer (P1 for the first two mutants and P2 for H211A and H234A) to amplify a second round of DNA synthesis. The wild-type plasmid pYTE2 was again used as template with Vent DNA polymerase. The inclusion of additional $MgSO_4$ to compensate for the EDTA carried over from the LM agarose gel greatly improved the yields in the second amplification step. The newly amplified DNA was purified by LM agarose gel electrophoresis and eluted from the gel. The purified DNA was digested with *Nde*I and *Hin*DIII and ligated into the expression plasmid pT7-7 digested with the same two restriction enzymes. The ligation mixture was used to transform chemically competent BL 21 *E. coli* cells.

The histidine-to-glycine mutant genes were prepared utilizing a new parental strand-only replication methodology (Weiner and Costa, 1994; Weiner, *et al.*, 1994). *Miniprep* wild-type plasmid DNA (pT7-7-EFE) and two oligonucleotide primers, each containing the desired mutagenic replacement codon (one the exact complement of the other), for each mutant were temperature cycled 15 times with high-fidelity Pfu (or Vent) DNA polymerase in the present of buffer and dNTPs utilizing

the cycle program of 94°C for 1 min, 45°C for 1 min, and 72°C for 3.5 min according to the procedure supplied by the company with the kit. The reading frame mutagenic primers for the mutant ACCOs were H177G, CAAGGGACTCCGCGCTGGTACAGACGCAGGAGGC (introduces a new *Rsa*I restriction site); H211G, CGATGTTCCTCCCATGCGCGGCTCTATTGTGGTTAACC (eliminates a *Hph*I site); and H234G, GTACAAGAGTGTGCTGGGCAGAGTAATTGCACAAAC (eliminates a *Bbv*I site). The thermal-cycled reaction mixture containing the mutated plasmid with staggered nicks was treated with *Dpn*I to digest the parental DNA template, pT7-7-EFE. The *Dpn*I digestion reaction mixture, containing the nicked mutagenic DNA, was used to transform supercompetent XL1-Blue *E. coli* cells supplied with the kit.

The isolated purified mutant plasmid DNA from each of the above clones was characterized by first restriction digestion and then DNA sequencing. DNA containing the proper mutagenic sequence was used to transform chemically competent BL21(DE 3) *E. coli* cells for the expression of the desired mutant protein. A culture of the transformed cells (1 ml, OD_{600} = 0.5) was mixed with 1 ml of 50% glycerol and stored at −85°C.

2.8. Mutant Ethylene Preparation and Purification

E. coli containing the H → A and H → G ACCO mutant sequences were grown, and the mutant protein isolated and purified as the apo-protein from the inclusion bodies by the methods described for the recombinant wild-type ACCO. The activities of these mutants were determined as described above in Method 1.

2.9. Imidazole Rescue Studies

The histidine mutants H177A, H177G, H211A, H211G, H234A, and H234G were individually incubated with 100 mM HEPES (pH 7) containing 50 mM imidazole at 4°C for 30 min and then assayed by Method 1 except that the final assay mixture contained 50 mM imidazole. The histidine mutants H211G and H234G were purified using the same procedure as described above (2% sodium deoxycholate in lysis buffer) for wild-type ACCO except that 50 mM imidazole was included in all buffers used in the purification procedure. The H211G and H234G mutants, purified by inclusion of imidazole in the buffers, were assayed by Method 1; the final assay mixture contained 50 mM imidazole.

2.10. Circular Dichroism Studies

The circular dichroism spectra of the wild-type ACCO and the mutant enzymes were obtained by scanning the sample solutions in a 1-mm stoppered strain-free quartz cuvette over the far-UV range (250–185) at 25°C using an Aviv Circular Dichroism Spectrophotometer Model 62 DS containing a water jacket attached to a circulating water bath. All protein samples were desalted on a Sephadex G-25 column equilibrated with 2 mM potassium phosphate buffer (pH 7.5). Protein concentrations in column fractions were adjusted to 0.1 mg per ml. Scans were collected at 1-nm intervals with a band width of 1.5 nm and a dwell time of 1 sec. The dynode voltage remained below 600 V for all samples. A total of five readings for each nm measured were averaged for each scan and a total of five scans were collected for each protein solution, the signal averaged, calibrated to remove the background of the buffer, and smoothed using the program supplied with the spectrometer (the smoothing program gave a value which was similar to the Q test @ 99% and 95% CL). The estimated percentages of secondary structure were calculated from the CD spectra utilizing the self-consistent method (SELCON program) described by Sreerama and Woody (1993).

3. RESULTS

3.1. Overexpression and Purification of ACC and ACC Mutants

The synthetic gene, obtained by PCR amplification using a pYES plasmid containing the pRC13 sequence [*Lycopersicon esculentum* (tomato)] as template, was ultimately cloned into the *Nde*I and *Bam* HI restriction sites of the expression vector, pT7-7, designed to utilize T7 DNA polymerase to direct transcription of the target gene. Overexpression of ACCO from the *E. coli* strain BL 21 (DE 3) containing the pT7-7-EFE, after induction with IPTG, allowed the production of 150–250 mg of ACC oxidase per 3–4 g (wet weight) of *E. coli* cells from 1 L of 2 × TY. Recombinant ACCO overexpressed in *E. coli* was isolated in the form of insoluble aggregates or inclusion bodies. The distribution of ACCO in whole cells, supernatant, and pellet fractions was examined with reducing SDS–PAGE (gel not shown). More than 20% of total protein in whole-cell lysate was recovered as ACCO. The enzyme was virtually absent in the supernatant, whereas the pellet fractions contained mostly ACCO, suggesting that the enzyme accumulated in the

Table I. Effect of Preincubating ACCO with Fe^{2+}

Preincubation conditions	Percent activity
Aerobic	
Enzyme	100
Enzyme plus HCO_3^-	96
Enzyme plus ascorbate	98
Enzyme plus Fe^{2+}	22
Enzyme plus Fe^{2+} and ascorbate	9
Enzyme plus Fe^{2+} and ACC	70
Enzyme plus Fe^{2+} and HCO_3^-	20
Anaerobic	
Enzyme	100
Enzyme plus Fe^{2+}	96

form of insoluble aggregates or inclusion bodies. ACCO was obtained at >95% purity after cycles of low-speed centrifugation and resuspension in lysis buffer containing 0.5% octyl-β-D-glucopyranoside to remove contaminating proteins from the inclusion bodies preparation.

The solubilized ACCO inclusion bodies were examined by running the resulting supernatant after centrifugation on reducing SDS–PAGE. The results showed that ACCO inclusion bodies were completely solubilized after denaturation in 6 M guanidinium hydrochloride (data not shown). The soluble material showed no detectable ACCO activity and displayed the emission spectra characteristic of a denatured protein. The unfolded protein was refolded by dialysis. The folding of ACCO was confirmed by the recovery of enzyme activity with a specific activity of 14 units/mg, a value comparable to that previously reported for the native enzyme (Dilley *et al.*, 1993, Dong *et al.*, 1992, Zhang *et al.*, 1995).

The ACCO inclusion bodies were solubilized without denaturation with 2% sodium deoxycholate in lysis buffer by repeated cycles of resuspension in solubilization buffer, sonication, and low-speed centrifugation (5000 × *g* for 10 min). The soluble fractions contained >90% of the ACCO present in the inclusion bodies. The solubilized protein exhibited a specific activity of 14 nmol of ethylene/min/mg protein and an emission spectrum characteristic of a native protein. In addition, the solubilized sample was 95% pure as judged by SDS–PAGE and migrated as a 35-kDa protein (gel not shown).

3.2. Fluorescence Spectroscopy

The unfolding and refolding of ACCO was monitored by intrinsic fluorescence. The emission spectra obtained between 300 and 400 nm were performed at λ_{ex} = 278 nm. When excited, the native protein (solubilized with sodium deoxycholate) and the refolded protein exhibited fluorescence maxima of 336 and 338 nm, respectively, characteristic of a tryptophan side chain partially shielded from the aqueous solvent (data not shown). Unfolding by the addition of 6 M guanidinium hydrochloride resulted in a red-shifted λ_{em} to 352 nm (data not shown).

3.3. Molecular Properties

The amino acid composition, the N-terminal sequence, and the molecular weight of recombinant ACCO were performed at the University of Michigan Biomedical Research Resources Core Facility. The molecular weight was determined by electrospray mass spectroscopy and shown to be 35,802 ± 11 (predicted 35,813, spectra not shown). The molecular weight was 35,000 as determined by SDS–PAGE. The N-terminal sequence obtained, MENFPIINLE, is consistent with that predicted from the DNA sequence.

3.4. Effect of Preincubating ACCO with Fe^{2+} on Enzyme Activity

Incubation of ACCO aerobically with 100 μM Fe^{2+} without substrate and other cofactors at 30°C for 30 min resulted in 78% inhibition of enzyme activity (Table I). Preincubation of the enzyme with Fe^{2+} under similar conditions, except for the addition of ascorbate, resulted in >90% loss of enzyme activity. ACCO was protected from inactivation by Fe^{2+} plus ascorbate either by the inclusion of ACC in the preincubation mixture or by flushing the solution with nitrogen during the preincubation period (Table I). In contrast, ascorbate or HCO_3^- did not inhibit ethylene production when preincubated separately with ACCO (Table I).

3.5. Inactivation of ACCO by DEPC

Since DEPC hydrolyzes in aqueous solutions, the inactivation process is corrected for the hydrolysis of DEPC in buffer and is described by the following equation:

$$\ln(A/A_0) = -(k/k')I_0(1 - e^{-k't}) \quad (1)$$

where A/A_0 is the percent activity remaining at time t, I_0 is the initial concentration of DEPC, k' is the second-order rate constant for the inactivation of ACCO by DEPC, and k' is the pseudo first-order rate constant for the hydrolysis of DEPC (Gomi and Fujioka, 1983). To estimate the value of k', DEPC was incubated in 100

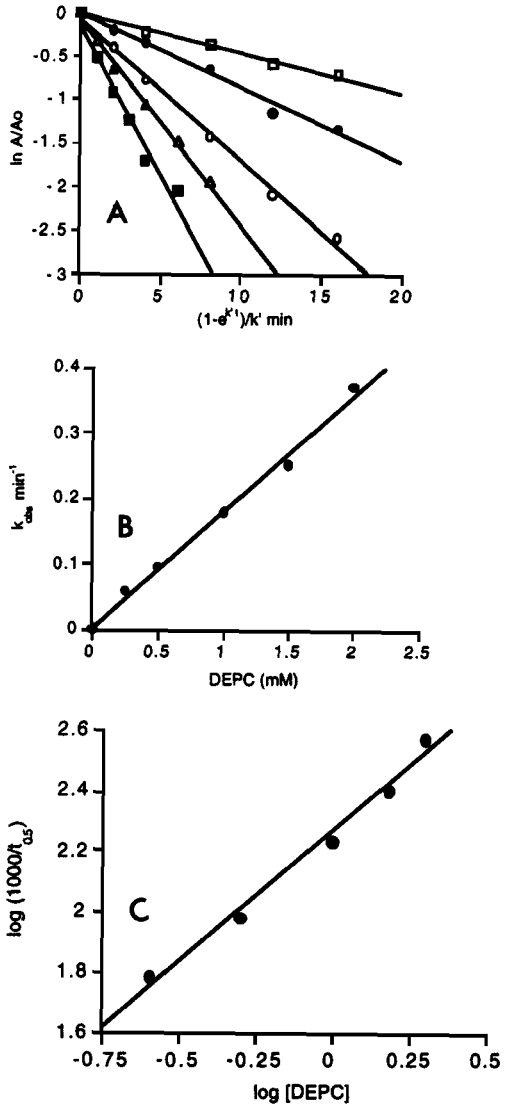

Fig. 1. Kinetics of inactivation of ACCO by diethylpyrocarbonate. (A) ACCO (12 μM) was incubated with 0.25 (□), 0.5 (●), 1.0 (○), 1.5 (▽), or 2.0 (■) mM of DEPC at 4°C. At intervals, aliquots were removed and assayed for residual enzyme activity as described in Section 2. The data were plotted according to Equation (1). (B) Determination of the second-order rate constant. The pseudo first-order rate constant k_{obs} obtained from panel A at different concentrations of inhibitor were plotted against DEPC concentration. (C) A plot of the reciprocal of the half-life of inactivation versus DEPC concentration on a log–log scale. The reaction order was obtained from the slope of this plot, following the procedure of Blanke and Hager (1990).

mM HEPES (pH 7) at 4°C. At time intervals, aliquots were removed and assayed for DEPC by reaction with imidazole as described in Section 2. Although it has been reported (Miles, 1977) that DEPC is most stable in phosphate buffer, HEPES was used instead because phosphate interferes with ACCO activity. Unfortunately, earlier studies involving the inactivation of the histidines of ACCO performed the experiments in phosphate buffer (Zhang *et al.*, 1995). The value of k' in HEPES was estimated to be 0.012 min^{-1}. Plots of ln (A/A_0) versus $(1 - e^{-k't})/k'$ at various DEPC concentrations were linear (Fig. 1A). The inactivation process followed pseudo first-order rate kinetics (Fig. 1A). The second-order rate constant k for the inactivation was obtained from the slope of the plot of k_{obs} versus DEPC concentration (Fig. 1B), yielding a k value of 170 min^{-1} M^{-1}. The k_{obs} was obtained from the slopes of the linear plots in Fig. 1A. The linearity of the plot with zero origin (Fig. 1B) is indicative of an irreversible complex formation between the enzyme and DEPC (Gomi and Fujioka, 1983).

In order to determine the number of essential histidine residues required for ACC oxidation, the log of the reciprocal of half-life of inactivation $(1000/t_{0.5})$ was plotted against the log of DEPC concentration, where k_{obs} is substituted for by the reciprocal of half-life (Church *et al.*, 1985; Blanke and Hager, 1990). As shown in Fig. 1C, the plot was linear with a slope of 0.86, indicating that approximately 1 mol of histidine was modified per mol of DEPC.

3.6. pH Dependence of Inactivation

DEPC inactivation of ACCO was studied in the pH range of 5.5–7.8 in HEPES buffer according to the following equation:

$$k_{obs} = k_{obs(max)}/(1 + [H^+]/K_a) \quad (2)$$

Equation (2) is rearranged to the following linear form:

$$k_{obs}[H^+] = K_a k_{obs(max)} - K_a k_{obs} \quad (3)$$

where K_a is the acidic dissociation constant of the reacting group and $k_{obs(max)}$ the pseudo first-order rate constant of the unprotonated group (Takeuchi *et al.*, 1986). The pseudo first-order rate constant of inactivation k_{obs} at various pH values is obtained from the plots of residual enzyme activity versus time. The experimentally determined k_{obs} values were plotted against pH (Fig. 2A, closed circles) according to Equation (2). The k_{obs} values at different pH values were used to plot Fig. 2B according to Equation (3). A pK_a value of 6.05 and a $k_{obs(max)}$ value of 0.186 min^{-1} were obtained from the slope of the line and from the ordinate intercept, respectively (Fig. 2B). The pK_a and $k_{obs(max)}$ values were fit to Equation (2) to construct the theoretical pH dependence curve (Fig. 2A, solid line, open circles). This pH dependence result, a pK_a value of 6.05, strongly suggests

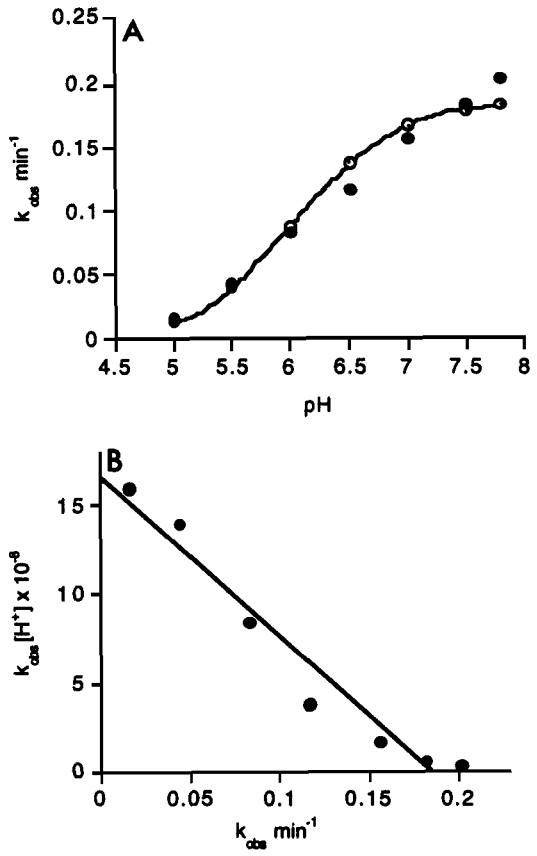

Fig. 2. The pH dependence of inactivation. (A) ACCO (12 μM) was incubated with 1.0 mM diethylpyrocarbonate at various pH values. The k_{obs} was determined as described in the Section 3 and plotted against pH. The solid line (with open circles) represents the pH theoretical curve and closed circles represent the experimental data (see text for details). (B) The k_{obs} values shown in panel A were plotted according to Equation (3). The experimentally determined K_a value (obtained from the slope of the plot) and $k_{obs(max)}$ (obtained from the x intercept) were used to plot the pH theoretical curve shown in panel A according to Equation (2).

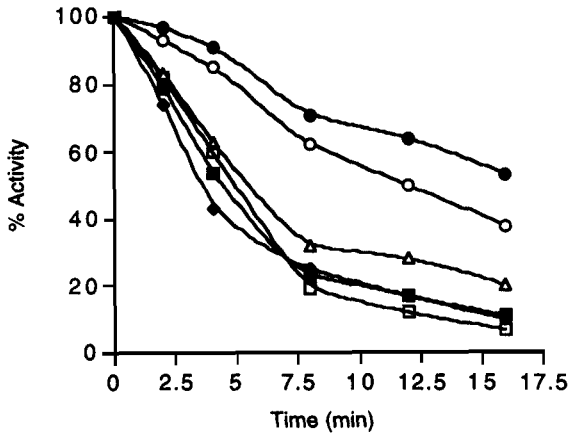

Fig. 3. Substrate protection against DEPC inactivation of ACCO. ACCO (12 μM) was incubated with 1.0 mM diethylpyrocarbonate in the presence of different substrates in 100 mM HEPES, pH 7, at 4°C. (■) No substrates added, (♦) 1.0 mM ascorbate, (□) 1.0 mM $NaHCO_3$, (○) 0.1 mM Fe^{2+}, (▵) 1.0 mM ACC, and (●) Fe^{2+} and ACC. At intervals, aliquots were removed and assayed for ACCO activity as described in Section 2. Activities were expressed as percentage of the initial activity of untreated enzyme.

that DEPC inactivation of ACCO is due to the modification of histidine residues.

3.7. Substrate Protection of ACCO against Inactivation with DEPC

The ability of substrates and cofactors to protect against inhibition of ACCO by DEPC was evaluated and the results are shown in Fig. 3. Neither ascorbate nor $NaHCO_3$ provided any protection against inactivation by DEPC. ACC alone after a 16-min pretreatment, provided little protection. Iron(II) alone afforded protection against inactivation. The best protection was obtained with ACC and Fe^{2+} together. These results suggest that Fe^{2+} or Fe^{2+} plus ACC binds to a histidine(s) at or near the active site of the ACCO.

Table II. Reactivation of DEPC-Inactivated ACCO with Hydroxylamine[a]

Protein	Incubation time[b] (min)	Activity (%)
ACCO/NH_2OH	0	100
DEPC-modified ACCO	4	53
DEPC-modified ACCO/NH_2OH	4	92
DEPC-modified ACCO	8	24
DEPC-modified ACCO/NH_2OH	8	42

[a] ACCO (12 μM) was incubated with 1 mM DEPC for 4 and 8 min. DEPC-treated and untreated enzymes were incubated with 0.3 M hydroxylamine solution (pH 7). All incubations were performed at 4°C. After 20 hr of incubation, aliquots were removed and assayed for ACCO activity.

[b] Time of incubation of ACCO with DEPC in 100 mM HEPES buffer (pH 7) at 4°C.

3.8. Reaction with Hydroxylamine

Addition of excess hydroxylamine to DEPC-treated ACCO could reverse the inactivation of ACCO by DEPC, depending on the time of addition or degree of inactivation. As shown in Table II, the majority of enzyme activity could be recovered by treatment with hydroxylamine when ACCO had only lost 50% of enzyme

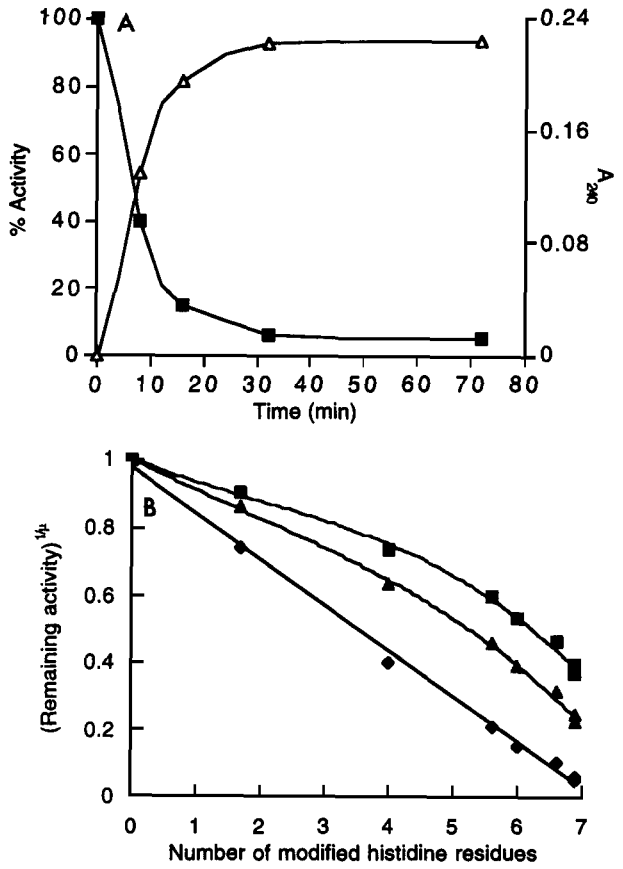

Fig. 4. Correlation of the number of histidine residues modified with enzyme activity. (A) ACCO (10 μM) was incubated with 1.0 mM DEPC (diluted in absolute ethanol) in 100 mM HEPES, pH 7. A reference sample contained an equivalent amount of ethanol without DEPC. The residual enzyme activity (■) and A_{240} (□) were plotted as a function of time. (B) Correlation between the number of modified histidine residues and the remaining activity of the enzyme. The data are presented in the form of a Tsou plot where μ = 1 (◆), 2 (▲), and 3 (■).

activity. However, only 42% of enzyme activity could be recovered by hydroxylamine, when only 76% of residual activity had been lost.

3.9. Correlation between Enzyme Activity and Histidine Residues Modified by Diethylpyrocarbonate.

ACCO contains seven histidine residues in its primary structure as predicted from its cDNA sequence (Hamilton *et al.*, 1991) and as determined from the total amino acid analysis performed on the recombinant ACCO (data not shown). The number of histidine residues modified by DEPC as determined by the difference spectra at 240 nm (data not shown) correlates with the loss of enzyme activity (Fig. 4A). The results indicate that total loss of enzyme activity is associated with the modification of *all* seven histidine residues. This means that all histidine residues are accessible for the DEPC. Therefore, to determine the number of essential histidine residues, the method developed by Tsou (1962) was used. The relationship for remaining activity as a function of histidine modification is given by

$$a^{1/\mu} = (p - m)/p \qquad (4)$$

where a is the fractional activity remaining when m histidines have reacted, p is 7 (the total number of histidines in ACCO), and μ is the number of critical histidines. The results of such a treatment of the data are shown in Fig. 4B. The number of essential histidine residues was found to be one.

3.10. Site-Directed Mutagenesis of ACCO

The four histidine-to-alanine ACCO mutants were constructed using the three-primer, two-step PCR-based mutagenesis procedure previously reported (Chen and Przybyla, 1994). The three histidine-to-glycine ACCO mutants were constructed using the method recently reported by Weiner (the QuikChange Site-directed Mutagenesis Kit marketed by Stratagene contains instructions and everything needed for this procedure except the mutagenic primers) (Weiner and Costa, 1994; Weiner *et al.*, 1994). The incorporation of the mutation into the target DNA regardless of the method of production was first confirmed by restriction digestion (see experimental section) and then by DNA sequencing. The histidine-to-alanine/glycine mutagenic proteins were overproduced from the pT7-7-based vectors containing the desired DNA mutagenic sequence as previously described for the overproduction of wild-type recombinant ACCO, and purified to >95% homogeneity, as assessed by SDS–polyacrylamide gel electrophoresis. The mutants were isolated as inclusion bodies as was the wild-type ACCO. The H39A mutant protein had an activity similar to that of wild-type ACCO (Table III). The activity of the H177A, H177G, H211A, H211G, H234A, and H234G mutants was greatly reduced or totally eliminated, as can be seen in Table III. Since *E. coli* does not produce ethylene, there is no problem of contamination of the mutant ACCO with background wild-type ACCO.

3.11. Imidazole Rescue Studies

The activity of the H177A, H177G, H211A, H211G, H234A, and H234G mutants was determined,

after preincubating each mutant with 50 mM imidazole for 30 min at 4°C, utilizing Method 1 except that 50 mM imidazole was included in the assay mixture. The ethylene-forming activity of the mutants preincubated with imidazole was the same as the corresponding mutants that had not been preincubated or assayed in the presence of imidazole. To determine if the addition of exogenous imidazole into the buffers used during the purification scheme would lead to incorporation of the imidazole (thus the restoration of ethylene-forming activity) by the mutant ACCO, 50 mM imidazole was added to all buffers (breaking, washing, and lysis buffer) for the purification of the mutants H211G and H234G from the frozen *E. coli* pellets. The enzymatic activity of H211G and H234G remained the same as the activity of H211G and H234G purified in the absence of imidazole.

3.12. Circular Dichroism Studies

The conformational integrity of the mutant ACCO was probed by circular dichroism spectroscopy. The CD spectra of the wild-type ACCO and the eight mutants indicate modest but significant conformational changes in some of the mutant enzymes. Since ACCO is isolated in the apo form from the inclusion bodies, the CD spectra obtained were with the apo enzyme. The CD of the two mutants isolated in the presence of imidazole was identical to the CD of the corresponding mutants isolated in the absence of imidazole. The deconvolution of the spectral data by the self-consistency method of Sreerama and Woody (1993) is shown in Table III. The results of analysis of the CD data with other deconvolution programs were consistent with these values (data not shown).

Table III. Deconvoluted CD Data for ACCO, H → A, and H → G Mutants[a]

Mutant	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil	Activity (% of wild-type)
WILD	25.2	27.2	26.1	22.0	100
H39A	23.6	27.5	26.0	20.6	104
H177A	23.1	33.0	27.5	17.3	6
H177G	27.6	19.3	29.3	24.9	0
H211A	32.2	24.2	29.2	15.1	10
H211G	28.4	20.3	24.3	27.9	10
H234A	29.1	21.8	26.6	21.8	0
H234G	26.0	18.7	26.2	27.1	0

[a] Determined by the method of Sreerama and Woody (1993).

4. DISCUSSION

In the present report, we describe the isolation of *Lycopersicon esculentum* (tomato) ACCO from *E. coli* BL21(DE3) containing pT7-7-EFE. The ACCO, which accounts for >25% of the total cellular protein, is recovered in the form of inclusion bodies. Cell breakage followed by low-speed centrifugation results in the total recovery of ACCO-containing inclusion bodies in the pellet fraction, while contaminating soluble protein and nonproteinaceous material that remain in the supernatant are easily removed. It has been previously reported that heterologous proteins, when highly expressed in *E. coli*, accumulate in the form of aggregates or inclusion bodies (Marston, 1986; Marston and Hartley, 1990). At a concentration of 0.5%, the detergent octyl-β-D-glucopyranoside solubilized the remaining contaminating proteins, but not ACCO. The ACCO inclusion bodies could therefore be isolated in >95% purity by repeated cycles of washing with 0.5% octyl-β-D-glucopyranoside and low-speed centrifugation. It seems quite advantageous for ACCO to accumulate as inclusion bodies in its apo form since purification of ACCO for biochemical characterization does not require the laborious three-column chromatographic procedures recently reported (Zhang *et al.*, 1995), and in the absence of Fe^{2+} one does not have to perform the enzyme purification anaerobically or in the presence of various metal chelators in all the buffers. In addition, it has been reported that holo-ACCO is quite unstable in the presence of oxygen.

Solubilization of highly overexpressed proteins from inclusion bodies utilizing mild conditions, such as extraction with sodium deoxycholate, has previously proven to be unsuccessful; however, sodium deoxycholate at concentrations of 0.5% has been employed for the extraction of lipids, nucleic acids, and minor protein contaminants from these inclusion bodies (Marston, 1989). In the present study, ACCO inclusion bodies have been successfully solubilized with 2% sodium deoxycholate. Approximately 60 mg of pure soluble ACCO/g of *E. coli* cells containing the pT7-7-EFE plasmid is obtained on a routine basis. Even at 0.5% deoxycholate, 40% solubilization of ACCO can be achieved. To completely free the sample of deoxycholate, it is necessary to perform both Sephadex G-25 chromatography and dialysis.

The results obtained from the analytical analyses of the solubilized recombinant ACCO are consistent with those from studies involving native ACCO with one notable exception. In contrast to native ACCO obtained from apple, the mature recombinant tomato ACCO retains the N-terminal methionine (Pirrung *et al.*, 1993). The somewhat enhanced specific activity of the present ACCO could be due to the fact that the ACCO contained in inclusion bodies is apo (i.e., it does not contain Fe^{2+}) and

that such large quantities of ACCO are present in the inclusion bodies. It has been shown recently that ACCO is quite unstable in the presence of oxygen, ascorbate, and iron(II) due to oxidative damage (Barlow *et al.*, 1997).

To explore the role of histidine(s) at the active site of various enzymes, the active-site modification reagent diethylpyrocarbonate (DEPC) has been employed (Bhattacharyya *et al.*, 1993; Cheng and Nowak, 1989; Church *et al.*, 1985; Fukumori and Hausinger, 1993*a*, *b*; Lemaire *et al.*, 1994; Lundblad and Noyes, 1984; Saluja and McFadden, 1980, 1982; Takeuchi *et al.*, 1986). In the present work, we have attempted to identify the role of histidine in ACCO catalysis by both chemical and kinetic studies using DEPC. Chemical studies have shown that ACCO is inactivated by DEPC in a time-dependent manner. Kinetic studies revealed that the reaction between DEPC and ACCO is bimolecular (Church *et al.*, 1985) and followed pseudo first-order kinetics (Fig. 1A and 1B). The second-order rate constant k for inactivation by DEPC is 170 M^{-1} min^{-1}. This value is in the range of rate constants obtained for other proteins containing essential histidine residues. These rates vary widely from a low of 0.67 M^{-1} min^{-1} (Bhattacharyya *et al.*, 1993) to a high of 368 M^{-1} min^{-1} (Bhattacharyya *et al.*, 1993; Cheng and Nowak, 1989; Church *et al.*, 1985; Fukumori and Hausinger, 1993*a*, *b*; Lundblad and Noyes, 1984; Miles *et al.*, 1993). The chemical modification studies with DEPC reveal that while only one histidine residue may be essential for activity, all seven histidines are ultimately modified by DEPC (Fig. 4B).

DEPC is known to react with lysine and tyrosine residues as well as histidine residues; therefore, in order to establish unequivocally that DEPC has reacted only with the histidine residues in ACCO, a number of criteria must be demonstrated. DEPC is known to modify selectively histidine residues in the pH range of 5.5–8 (Miles, 1977). The rate of inactivation of ACCO by DEPC exhibited a pH dependence (Fig. 2A) and the pK_a value of the modified amino acid residue was calculated to be 6.05 (Fig. 2B). This value is consistent with the pK_a values for histidine modification (Lundblad and Noyes, 1984; Miles, 1977). Reactivation of DEPC-inactivated proteins by treatment with hydroxylamine is indicative of either an ethoxyformylated histidine or tyrosine residue, but not an ethoxyformylated lysine (Melchior and Fahrney, 1970; Miles, 1977). Since hydroxylamine reactivates DEPC-treated ACCO, the involvement of lysine residues in the modification reaction can most likely be excluded (Table II). This reversibility of inactivation by DEPC has been observed with a number of other enzymes (Bhattacharyya *et al.*, 1993; Church *et al.*, 1985; Daron and Aull, 1982; Lemaire *et al.*, 1994; Saluja and McFadden, 1980, 1982). In some enzyme systems where DEPC inactivation is known to occur via modification of only histidine, inactivation cannot be reversed by hydroxylamine (Bloxham, 1981; Bond *et al.*, 1981; Cheng and Nowak, 1989; Cromartie, 1981; Saluja and McFadden, 1982). The explanation most commonly presented for this lack of reactivation of the DEPC-modified enzyme in these examples is the inaccessibility of hydroxylamine to the carbethoxyhistidine-modified moiety or that hydroxylamine itself directly inactivates the enzyme. DEPC-modified tyrosine and histidine residues display several distinct spectral differences. The modification of tyrosine residues by DEPC can be monitored by spectral changes at A_{278} (Miles, 1977). In the present study there is no change in A_{278}, indicating that the inactivation of ACCO by DEPC is not caused by the modification of tyrosine residues (data not shown). The difference ultraviolet spectrum of DEPC-treated ACCO versus untreated ACCO showed an increase in absorbance at 240 nm, which is a characteristic of protein-bound ethoxyformyl histidine (Lundblad and Noyes, 1984; Miles, 1977) and not an ethoxyformyl tyrosine. Finally, protection against DEPC modification of ACCO by the cofactor Fe^{2+} and one of the substrates, ACC, suggests that the reactive-site histidine residues are most likely at or near the Fe^{2+}-binding site, indicating that histidine residues in ACCO are ligands for the Fe^{2+} binding and are the amino acid residues being modified (Fig. 3). The enhanced protection by ACC suggests that ACC may also act as a ligand to Fe^{2+}. None of the other cofactors or substrates afforded protection against inactivation (Fig. 3). Zhang *et al.* (1995) failed to detect protection against DEPC inactivation by just Fe^{2+}, although they found that Fe^{2+} did provide protection, but only in the presence of ACC and ascorbate. The discrepancy between results is probably due to the difference in the assay conditions used for ACCO. The Schofield group (Zhang *et al.*, 1995) used phosphate buffer for the inactivation studies; however, we have found that phosphate interferes with ACCO activity assays and thus we used HEPES instead of phosphate. The pH dependence of inactivation, the reactivation of DEPC-inactivated ACCO by hydroxylamine, the UV spectral results, and the substrate protection studies strongly suggest that DEPC modified only histidine residues in ACCO and that these histidines and ACC are ligands to the active-site iron.

In order to determine which histidine(s) is (are) potentially involved in the binding of the iron at the active site of ACCO, several histidine residues were chosen for mutagenic studies. Which of the seven histidines in ACCO to mutate was based on amino acid sequence

homology comparisons with members of the nonheme ferrous-dependent oxidase/oxygenase superfamily such as flavanone-3β-hydroxylase, isopenicillin-*N*-synthase (IPNS), maize A2 locus gene, hyoscyamine-6β-hydroxylase, and deacetoxycephalosporin-C-synthetase as previously described (Hamilton *et al.*, 1991; Matsuda *et al.*, 1991) as well as with 13 known ACCO amino acid sequences (alignments not shown). Histidine 177 and 234 were obvious choices based on these homologies as well as the X-ray structure of one of its well-studied members, IPNS (Roach *et al.*, 1995, 1997). Histidine 39 was chosen based only on homology with the superfamily, whereas histidine 211 was chosen based on its absence from the superfamily, but its conservation within the ACCO family. During the characterization of these tomato histidine mutants, a report (Shaw *et al.*, 1996) appeared indicating that H177, D179, and H234 of the apple fruit ACCO may be putative ferrous-binding ligands. These conclusions were based on mutation studies of each of the respective amino acids. The fact that H177 and H234 in both apple and tomato ACCO are potentially involved in ferrous binding is not surprising based on their sequence homology with IPNS. The X-ray crystal structure of IPNS clearly demonstrates that two histidines, H214 and H270, corresponding to H177 and H234 in ACCO, are involved in the ligation of the metal.

The concept of restoration of catalytic activity by addition of exogenous small organic molecules, originally reported by Toney and Kirsch (1989) for restoration of activity to the K258A mutant of aspartyl aminotransferase by the addition of amine, was first utilized to restore catalytic activity to a histidine mutant by addition of imidazole in the case of the H117G mutant of *Ps. aeruginosa* azurin (den Blaauwen and Canters, 1993) and since by others (DePillis *et al.*, 1994; Wilks *et al.*, 1995). Although the methyl group of the alanine may be too large to allow the replacement with the exogenous organic molecule to exactly "fit" into the same space occupied by the original side chain of the amino acid replaced, it has been reported to work (Wilks *et al.*, 1995). Unfortunately, in the case of H177A, H211A, and H234A, there was no restoration of ethylene-forming activity by the preincubation of the mutants with imidazole and inclusion of imidazole into the assay mixture. Assuming the reason for lack of recovery of activity was due to the fact that the methyl group was too big, the corresponding histidine-to-glycine mutants were prepared. The results were the same: no restoration of activity by preincubation with imidazole and inclusion of imidazole into the assay mixture. In some cases activity could only be recovered if the exogenous ligand was included during the isolation procedure, the idea being that the ligand would be bound into the missing cavity during purification. The H211G and H234G were purified in the presence of imidazole and assayed in the presence of imidazole with the same disappointing results, no recovery of activity. These results may not be surprising if one examines the crystal structure of IPNS with its sequence homologies to ACCO in mind. Histidines 214 and 270 of IPNS (equivalent to H177 and H234 of ACCO) are located inside the jellyroll motif of IPNS on a loop region and at the start of a β-sheet, respectively. Imidazole may be able to chelate with iron and the mutant ACCOs, but the imidazole is not tethered to the protein backbone; therefore, the movement of the exogenous imidazole may not be able to bring about the necessary structural changes at the active site to allow a catalytically active site to form. This movement or lack of movement would not be detected by CD measurements since the replaced histidines are in a variable region of ACCO. The role of histidine 211 maybe somewhat more complicated. There is no corresponding "H211" in IPNS, so its structural location is harder to predict based on the structure of IPNS. It is clear from the data presented here, however, that both H211A and H211G have only 10% the catalytic activity of wild-type ACCO and that the addition of exogenous imidazole does not restore activity to either mutant. This seems to imply some role for H211 similar to the other histidines.

The DEPC chemical modification studies, when analyzed utilizing the Tsou (1962) method, revealed that the modification of only one histidine is essential for enzymatic activity. Utilizing site-directed mutagenesis, it was demonstrated that two other histidines may be necessary as structural units and that their elimination rather than simple ethoxyformylation by the DEPC impedes enzyme function. From the strong sequence homology with IPNS, it can be inferred that H177 and H234 are most likely involved with metal binding, while H211 may be involved mechanistically or structurally in ACCO activity. In support of this hypothesis, it has been reported by Zhang *et al.*, (1997) that H211Q is active. Since glutamine contains functional groups capable of hydrogen bonding (like histidine) as opposed to either glycine or alanine (prepared in this study), H211 may well be involved in either a structural or catalytic role.

In this article, we present evidence that histidine residues are important for ACCO activity as demonstrated by the DEPC inactivation studies. From site-directed mutagenesis, we have demonstrated that H177, H211, and H234 are important residues for ACCO activity. Ming *et al.* (1991) and Randall *et al.* (1993) have shown that two or three histidine residues are involved in Fe(II) binding in IPNS, which has a high extent of

homology with ACCO. Histidines 177 and 234 in ACCO are homologous with H214 and H270 in IPNS, which were shown by an X-ray crystal structure to be the metal-binding ligands in IPNS. The fact that ACCO mutants H177 and H234 mutants are inactive supports their proposed role in ACCO based on sequence homology with IPNS. For the first time, we were able to demonstrate the importance of H211 for ACCO activity either structurally or catalytically. The exact role of the three histidines 177, 211, and 234 is presently under investigation in our laboratory utilizing NMR methodologies.

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