Metal-Catalyzed Oxidation and Mutagenesis Studies on the Iron(II) Binding Site of 1-Aminocyclopropane-1-carboxylate Oxidase†

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Received July 28, 1997; Revised Manuscript Received October 6, 1997⊗

ABSTRACT: The final step in the biosynthesis of the plant signaling molecule ethylene is catalyzed by 1-aminocyclopropane-1-carboxylate (ACC) oxidase, a member of the non-heme iron(II) dependent family of oxygenases and oxidases, which has a requirement for ascorbate as a co-substrate and carbon dioxide as an activator. ACC oxidase (tomato) has a particularly short half-life under catalytic conditions undergoing metal-catalyzed oxidative (MCO) fragmentation. Sequence comparisons of ACC oxidases with isopenicillin N synthase (IPNS) and members of the 2-oxoglutarate Fe(II) dependent dioxygenases show an aspartate and two of six ACC oxidase conserved histidine residues are completely conserved throughout this subfamily of Fe(II) dependent oxygenases/oxidases. Previous mutagenesis, spectroscopic, and crystallographic studies on IPNS indicate that the two completely conserved histidine and aspartate residues act as Fe(II) ligands. To investigate the role of the conserved aspartate and histidine residues in ACC oxidase (tomato fruit), they were substituted via site-directed mutagenesis. Modified ACC oxidases produced were H39Q, H56Q, H94Q, H177Q, H177D, H177E, D179E, D179N, H177D&D179E, H211Q, H234Q, H234D, and H234E. Among those histidine mutants replaced by glutamine, H39Q, H56Q, H94Q, and H211Q were catalytically active, indicating these histidines are not essential for catalysis. Mutant enzymes H177D, H177Q, D179N, H177D&D179E, H234Q, H234D, and H234E were catalytically inactive consistent with the assignment of H177, D179, and H234 as iron ligands. Replacement of H177 with glutamate or D179 with glutamate resulted in modified ACC oxidases which still effected the conversion of ACC to ethylene, albeit at a very low level of activity, which was stimulated by bicarbonate. The H177D (inactive), H177E (low activity), D179E (low activity), and H234Q (inactive) modified ACC oxidases all underwent MCO fragmentation, indicating that they can bind iron, dioxygen, ACC, and ascorbate. The results suggest that MCO cleavage results from active site-mediated reactions and imply that, while H177, D179, and H234 are all involved in metal ligation during catalysis, ligation to H234 is not required for fragmentation. It is possible that MCO fragmentation results from reaction of incorrectly folded or "primed" ACC oxidase.

The plant signaling molecule ethylene is involved in a diverse range of physiological processes including regulation of plant growth/development, fruit ripening, and in responses to pathogen infection and mechanical wounding [for reviews see Kende (1993), Yang and Hoffman (1984), and Abeles et al. (1992)]. Ethylene is found in most plant tissues and is biosynthesized via two enzyme-catalyzed steps from *S*-adenosyl methionine. ACC[1] synthase catalyzes the cyclization of *S*-adenosyl methionine to 1-aminocyclopropane-1-carboxylic acid (ACC), and subsequently ACC oxidase, previously known as ethylene-forming enzyme, catalyzes the oxidative conversion of ACC to ethylene (Adams & Yang, 1979; Yang & Hoffman, 1984).

ACC oxidase is a member of the iron(II) dependent family of oxidase/oxygenases (Hamilton et al., 1991; Prescott, 1993; Feig & Lippard, 1994; Roach et al., 1995). All known members of this family have an absolute requirement for iron(II) and most use 2-oxoglutarate as a co-substrate. Along with isopenicillin N synthase (IPNS), ACC oxidase is atypical in that it does not require a 2-oxoacid co-substrate. IPNS has no substrates other than dioxygen and the tripeptide [L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine, ACV] which undergoes a four-electron oxidation to give the penicillin nucleus (Roach et al., 1995; Feig & Lippard, 1994). In the case of ACC oxidase the role of a 2-oxoacid as a co-substrate is filled apparently (at least *in vitro*) by ascorbate which is presumably oxidized to dehydroascorbate (Figure 1) (Dong et al., 1992). To the extent of our knowledge no other highly purified member of the family requires ascorbate for catalysis, but it is often added to optimize *in vitro* substrate conversion. ACC oxidase is also unusual since it requires carbon dioxide or bicarbonate, one of the products of the ACC oxidase reaction, as an activator (Grodzinski et al., 1982; Kao & Yang, 1982; Dong et al., 1992; Smith & John, 1993). The nonenzymatic oxidative breakdown of ACC to give ethylene by iron and hydrogen peroxide is also stimulated in the presence of bicarbonate (McRae et al., 1983).

† Supported by grants from the EPSRC, the BBSRC, the MRC, and Zeneca via a DTI LINK award. JNB was supported by a studentship from the AFRC.

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1997.

[1] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; ACV, L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine; 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; MCO, metal-catalyzed oxidation; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

S0006-2960(97)01823-0 CCC: $14.00

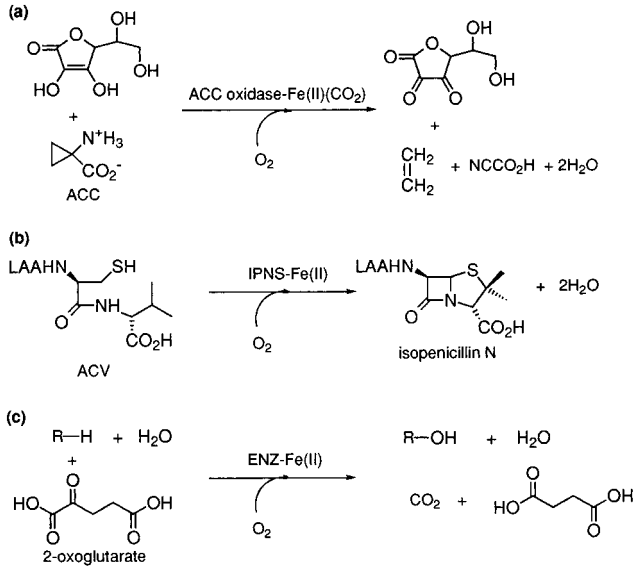

FIGURE 1: (a) ACC oxidase catalyzed biosynthesis of ethylene. (b) IPNS-catalyzed biosynthesis of isopenicillin N. (c) Stoichiometry of a 2-oxoglutarate dependent dioxygenase catalyzed hydroxylation reaction.

ACC oxidase displays significant sequence similarity with some other members of the ferrous dependent oxygenase/oxidase family (Figure 2) (Hamilton et al., 1990; Matsuda et al., 1991; Prescott, 1993; Roach et al., 1995, 1997). Certain amino acid residues, including H177, H234, and D179 (using the tomato fruit ACC oxidase numbering system), are conserved throughout all members of the iron(II) oxidase/oxygenase family which have significant overall sequence similarity with IPNS (the "IPNS subfamily"). Shaw et al. (1996) reported that substitutions in ACC oxidase (apple fruit) of H177 and H234 with Phe, Ala, and Asn, and D179 with His and Ala resulted in complete loss of catalytic activity in assays of crude cell extracts. Similar conclusions were drawn by Lay et al. (1996) in a study involving modification of H177 and D179 in ACC oxidase (kiwi fruit). Chemical modification has also implicated histidine residues in ferrous iron binding by ACC oxidase and other members of the family (Lamberg et al., 1995; Zhang et al., 1995; Lawrence et al., 1996).

The crystal structure of IPNS complexed to manganese ion at its active site reveals that the conserved aspartate and two histidine residues provide ligands for ferrous iron (Roach et al., 1995, 1997). Similarly, to the ACC oxidase studies, replacement of these residues with hydrophobic residues results in catalytically inactive proteins (Tan & Sim, 1996; Borovok et al., 1996). In the IPNS−Mn(II) structure the side chain of a fourth residue, Gln-330 (the penultimate residue of the polypeptide) is also complexed to the active site metal. However, this residue is not conserved in ACC oxidase or in other members of the subfamily, and site-directed mutagenesis studies have demonstrated that its presence is not essential for IPNS catalysis (Landman et al., 1997; Sami et al., 1997).

Metal-catalyzed oxidative (MCO) modification of enzymes is a biochemical "ageing" process, with modified enzymes being apparently more susceptible to protease mediated degradation (Stadtman & Oliver, 1991; Stadtman, 1990, 1992, 1993). Oxidative modification of proteins mediated by iron and ascorbate is well documented (Stadtman, 1993; Blanchard et al., 1982; Nietfeld & Kemp, 1981). Amino acid residues which are particularly prone to MCO systems include methionine and cysteine and those with basic side chains, i.e., arginine, lysine, and histidine. Site-specific modification of enzymes by MCO systems has, however, been demonstrated in a relatively small number of cases, including 1,2-propandiol oxidoreductase (Cabiscol et al., 1992, 1994) and glutamine synthase (Levine et al., 1981; Levine, 1983; Liaw et al., 1993; Revitte & Levine, 1990), pigeon liver malic enzyme (Wei et al., 1994, 1995; Chou et al., 1995), and alcohol dehydrogenase (*Zymomonas mobilis*) (Cabiscol et al., 1994).

ACC oxidase is particularly sensitive to oxidative damage (Smith et al., 1994; Barlow et al., 1997), making it an interesting case study for mechanisms of oxidative modification to proteins. Highly active recombinant ACC oxidase (tomato fruit) has an *in vitro* half-life of *ca.* 10 min under catalytic conditions. The oxidative damage to ACC oxidase occurs via reaction of hydrogen peroxide, which can be protected against by the addition of catalase, and by oxidative damage to the active site region, which cannot be protected against by the addition of catalase. The latter results in partial fragmentation (Barlow et al., 1997) of the enzyme close to its predicted active site based on the IPNS template structure (Roach et al., 1995). MCO fragmentation has also been used to map the metal/active sites of a variety of enzymes (Wei et al., 1995; Chou et al., 1995; Ettner et al., 1995). Recently, Zaychikov et al. (1996) combined MCO with site-directed mutagenesis to investigate the Mg^{2+} binding ligands of the RNA polymerase of *Escherichia coli*. They converted the three proposed aspartate ligands, to alanine. The resulted mutant was able neither to initiate polymerization of new RNA strand nor to be oxidized, although it still bound the template DNA. Although the previous mapping studies were conducted using Fe(II) and ascorbate, the enzymes of interest utilized Mg^{2+}, rather than Fe(II), as a cofactor. In contrast, ACC oxidase actually requires both Fe(II) and ascorbate for catalysis.

The studies described herein were aimed at identifying the iron ligands of ACC oxidase and determining the functional consequences of their modification for catalysis and oxidative modification. An important aspect of the experimental design was to use MCO fragmentation of ACC oxidase to investigate the metal binding site of the modified ACC oxidases. The results support the predictions that H177, D179, and H234 act as metal ligands during catalysis. They also imply that H234 does not ligate to the iron during fragmentation of the wild-type enzyme, suggesting that (at least in part) active site directed oxidative inactivation results from "incorrect" metal binding. It is also demonstrated that it is possible to replace at least one of the iron(II) binding ligands of ACC oxidase, while retaining some activity, albeit at an extremely low level.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Promega or Stratagene. Chromatography media were obtained from Pharmacia Biotech Inc. DNA sequencing regents were from Amersham. All other chemicals were purchased from Sigma except where stated otherwise.

DNA Manipulation and Site-Directed Mutagenesis. Standard DNA manipulation procedures, competent cells preparation, and *E. coli* transformations were carried out essentially

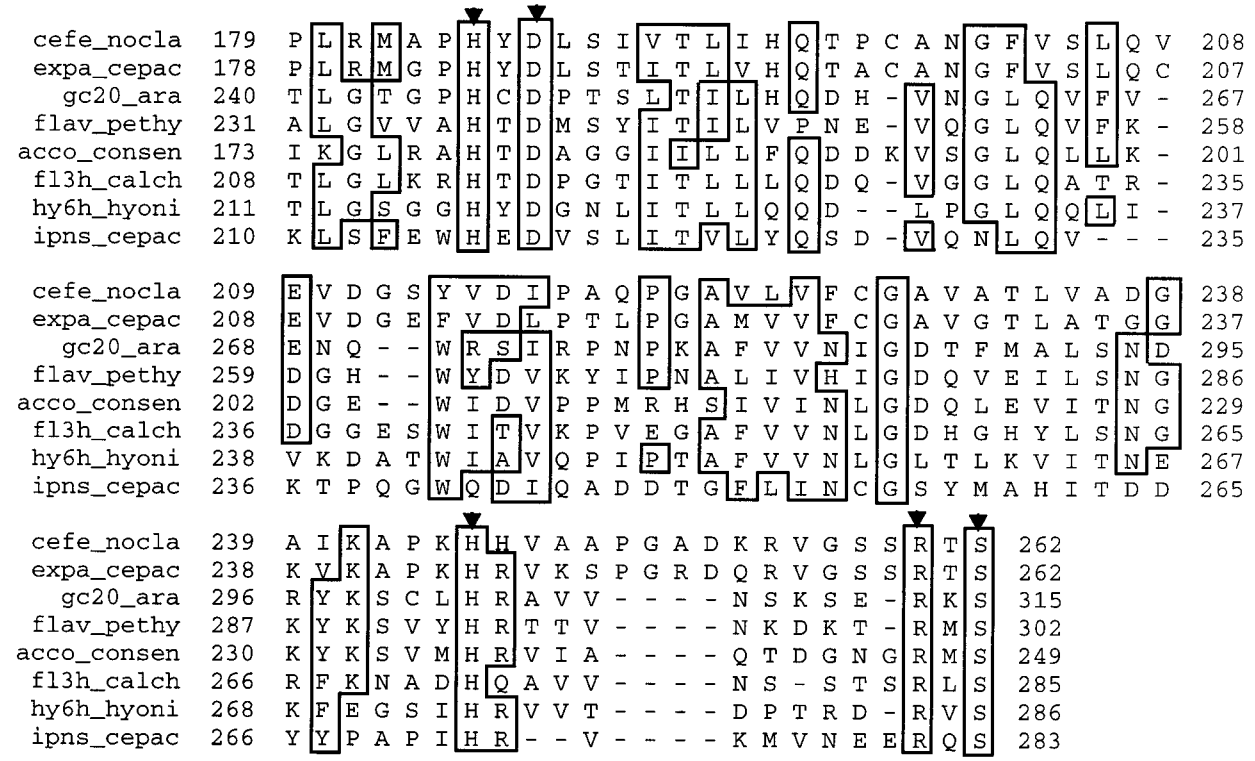

FIGURE 2: Partial comparisons of the consensus ACC oxidase sequence, derived from comparing thirteen ACC oxidase sequences, with sequence related iron(II) dependent oxygenases and oxidases: deacetoxycephalosporin C synthase from *Nocardia lactamdurans* (cefe_nocla), deacetylcephalosporin C synthase from *Cephalosporium acremonium* (expa_cepac), gibberellin C-20 oxidase from *Arabidopsis* (gc20_ara), flavonol synthase from petunia (flav_pethy), ACC oxidase consensus sequence (acco_consen), flavanone-3-hydroxylase from *Callistephus chinese* (fl3h_calch), hyoscyamine 6-hydroxylase from Henbane (hy6h_hyoni) and isopenicillin N synthase from *C. acremonium* (ipns_cepac). Regions of high similarity are boxed, and the conserved aspartate, arginine, serine, and two histidine residues are indicated by arrows. Alignments were made using the GCG software package.

as described (Sambrook et al., 1989). The most frequently *E. coli* used codon was used for substitution in the synthetic oligonucleotides, which were synthesized by Mrs. Val Cooper of the Oxford Centre for Molecular Sciences, University of Oxford. Site-directed mutagenesis processes were performed using the recombinant expression system pZAT6, which contains the full-length cDNA of ACC oxidase (tomato fruit) (Zhang et al., 1995). Mutagenesis was carried out using an *in vitro* oligonucleotide-directed mutagenesis system based on the unique restriction site elimination procedure (Pharmacia Biotech Inc). Mutations were identified by restriction digestions of the resulting plasmids. Sequences of modified ACC oxidases were verified by either manual or automatic dideoxynucleotide sequencing (Sanger et al., 1977). Mutant recombinants were then transformed into *E. coli* strain BL21(DE3) for expression studies. The resultant recombinant modified ACC oxidases were H39Q, H56Q, H94Q, H177D, H177E, H177Q, H177D&D179E, D179N, D179E, H210Q, H234Q, H234D, and H234E.

Expression and Purification of Modified ACC Oxidases. *E. coli* BL21(DE3) was grown in 2× TY medium supplemented with 100 μg/mL ampicillin in shake flasks (250 rpm) to the exponential phase at 27 or 37 °C, and induced for protein production by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Crude cell extracts were prepared from cell pellets resuspended in 25 mM Tris-HCl, pH 7.5, 25% (v/v) glycerol, 2 mM dithiothreitol (DTT), 0.5 mM benzamidine, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 3 mM EDTA. The suspension was then sonicated, and DNase I was added to a final concentration of 20 μg/mL, and the lysate was incubated for 30 min at 4 °C. The lysate was centrifuged at 20000*g* for 30 min at 4 °C. Protein concentrations were determined by the Bradford method (1976) using BSA as a standard.

All soluble mutants were purified to near homogeneity (by SDS−PAGE analysis) using a two-column chromatographic procedure employing Q-Sepharose High Performance and Mono Q resins (Zhang et al., 1995). Protein analyses by SDS−PAGE were carried out using a Bio-Rad Protean II gel system. Supernatant obtained as above was loaded on to a Q-Sepharose (high-performance) column pre-equilibrated with buffer A (25 mM Hepes-HCl, pH 7.6, 0.5 mM benzamidine, 0.5 mM PMSF, 2 mM DTT, and 3 mM EDTA). After the column was washed with two column volumes of buffer A, ACC oxidase was eluted with buffer B (buffer A plus 2 M NaCl) in linear gradient from 0−20% of buffer B. Fractions containing the enzyme, as determined by SDS−PAGE analysis, were pooled and concentrated using an Amicon concentrator from Amicon Inc. (WR Grace and Co., Danvers, MA) and loaded on to a Mono-Q column pre-equilibrated with buffer A. The absorbed proteins were eluted with buffer B, using a combination of step and linear gradients. Fractions containing ACC oxidase, as determined by SDS−PAGE, were pooled and concentrated as described above. The enzyme thus purified was about 95% pure (SDS−PAGE analysis). No attempt was made to further purify those active mutants which were expressed in a predominantly insoluble form.

Enzyme Activity Assays. ACC oxidase activity was measured essentially according to the reported method (Zhang et al., 1995, and references therein). Supernatant

from cell extracts (about 100 μg total protein) or pure enzyme (between 8 and 15 μg) was added to 1 mL of 0.1 M Hepes, pH 7.2, containing 10% (v/v) glycerol, 5 mM L-ascorbate, 0.1 mM ACC, 80 μM $FeSO_4$, 15 mM sodium bicarbonate, 100 μg BSA, 500 μg catalase, and 2 mM DTT, and the reaction was incubated at 28 °C for 20 min. Ethylene production was analyzed by gas chromatography [using a Pye Unicam Series 104 machine equipped with a Porapak R column (Phase Sep, Queensferry, Clwyd)] at a column temperature of 80 °C.

Metal-Catalyzed Oxidations (MCO) of ACC Oxidase. MCO of modified ACC oxidases was carried out essentially according to the method of Barlow et al. (1997) with the following modifications. Wild-type or mutant ACC oxidases (1−3 mg/mL) were incubated in a buffer containing 50 mM Tris-HCl, pH 7.2, 0.1 mM $FeSO_4$, 25 mM ascorbate, and 25 mM ACC in a volume of 60 μL. The mixture was incubated at 37 °C for 20 min, when the reaction was terminated by the addition of SDS−PAGE sample loading buffer. Analogous experiments were conducted on purified inactive (D179N, H177D&D179E, H234Q) and active (D179E, H56Q) modified ACC oxidases.

SDS−PAGE and N-Terminal Sequence Analyses. ACC oxidase fragments generated as above by MCO were separated using 16% Tris-tricine SDS−PAGE essentially according to the protocol described by Schägger and Von Jagow (1987). Gels were run at 30 mA for *ca.* 3.5 h using a Bio-Rad Mini Protean II gel system and stained with 0.1% Serva Blue in 10% acetic acid. For N-terminal sequence determinations, gels (not stained) were blotted onto a Waters Immobilon membrane in a Bio-Rad Mini Protean II 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. It should be noted that it is possible that the N-termini of some internal peptide fragments produced by MCO fragmentation of ACC oxidase are blocked (Amici et al., 1989) or that they are poorly stained and invisible by SDS−PAGE analysis.

Circular Dichroism Studies. Wild-type and mutant ACC oxidases were exchanged into 50 mM potassium phosphate buffer, pH 7.3, using Bio-Rad Bio6 Spin columns. CD spectroscopic analyses in the range 190−300 nm (scan rate: 50 nm/min) were performed using a Jasco J-600 spectropolarimeter equipped with a 0.2 mm cell at room temperature and at a protein concentration of 150 μg/mL.

RESULTS AND DISCUSSION

Production and Initial Characterization of Modified Proteins

When grown at 37 °C all modified ACC oxidases were expressed at high levels but as observed for wild-type ACC oxidase, the solubility of the expressed proteins was low. When grown at 27 °C, modified ACC oxidases were also expressed at high levels, but the solubilities of the expressed proteins were variable. At 27 °C the H56Q, H177D, H177E, H177Q, H234Q, D179E, D179N, and H177D&D179E modified ACC oxidases were expressed solubly at ca. the same level as wild-type ACC oxidase and were purified to near homogeneity (by SDS−PAGE analysis). The H39Q, H94Q, H211Q H234D, and H234E ACC oxidases were expressed in a predominantly insoluble form at 27 °C. Assays of these modified ACC oxidases were performed on crude cell extracts.

Table 1: Activities of Purified Wild-Type and Modified ACC Oxidases (See Text for Comments)

	apparent K_m (μM):			
enzyme	ACC	Fe(II)	V_{max}[a] (units/mg)[a]	apparent k_{cat} (s^{-1})[b]
wild-type	83 ± 2.2	0.43 ± 0.02	355 ± 4.4	5.9 ± 0.1
H177E	42 ± 3.1	0.29 ± 0.12	0.57 ± 0.03	0.0095 ± 0.0005
D179E	132 ± 21	4.6 ± 1.8	0.52 ± 0.07	0.0087 ± 0.0012
H56Q	121 ± 13	0.47 ± 0.02	310.2 ± 18	5.17 ± 0.3

[a] Unit is defined as 1 nmol of ethylene produced per min (over 20 min incubation). [b] Calculated using parameters determined for ACC.

Activity Analyses

The H177Q, H177D, H234Q, D179N, and H177D&D179E proteins were catalytically inactive while the H39Q, H56Q, H94Q, H211Q, H177E, and D179E proteins were active. However, the activity of the latter two was very low. Circular dichcroism analyses on wild-type and each of the purified modified ACC oxidases (H177Q, H177D, H177E, D179E, D179N, H177D&D179E, H234Q, H56Q) indicated that all were similar in overall structure to the wild-type enzyme (data not shown) and that ACC oxidase contains a significant amount of α-helical secondary structure. Ferrous iron was found to be an essential cofactor for all the active ACC oxidase mutants; as for wild-type ACC oxidase; its replacement with other metals (data not shown) led to loss of activity. Similarly, all the active modified ACC oxidases studied were activated by carbon dioxide (supplied as bicarbonate). Bicarbonate at 30 mM increased the specific activity of the H177E, H56Q, and D179E modified ACC oxidases, by ca. 3−4-fold, about the same level of activation as observed for the wild-type enzyme. Results on the cell-free extracts of the less soluble mutants, H39Q, H94Q, and H210Q also showed a *ca.* 3−4-fold increase in specific activity by the addition of 30 mM bicarbonate.

The observation that the H39Q, H56Q, H94Q, H211Q modified ACC oxidases were active, whereas the H177Q, D179N, and H234Q modified ACC oxidases displayed no catalytic activity, supports the assignment of H177, D179, and H234 as iron(II) ligands in ACC oxidase. H56Q modified ACC oxidase was the only one of the four active histidine−glutamine substitutes to be expressed in a predominately soluble form and was purified for comparison with the wild-type enzyme. Its specific activity was similar to that of wild-type ACC oxidase. In contrast, the specific activities of the H177E and D179E modified enzymes were very low, being, <0.2% that of wild-type ACC oxidase (Table 1). Apparent K_m and k_{cat} values were determined for wild-type and three of the modified ACC oxidases (Table 1). As noted in studies concerning IPNS (Sami et al., 1997) the interpretation of such kinetic data is complex in the case of many iron(II) dependent oxygenases/oxidases. In the case of ACC oxidase, complications include the lability of the enzyme under catalytic conditions (Barlow et al., 1997), the nonenzymatic consumption of dioxygen and ascorbate (in particular via Fenton/Udenfriends's type reactions occurring in solution), irreproducibility in the behavior of ACC oxidases from different fermentation/purification batches, the possibility of more than one active conformation of ACC

oxidase (Barlow et al., 1997), the necessary use of an assay time (20 min) only double the half-life of the enzyme (typically <10 min), and the low levels of activity observed for some of the modified ACC oxidases (H177E, D179E). It should also be noted that *in vitro* apparent k_{cat} values for ACC oxidase are probably significantly less than the true values of k_{cat}, due to the instability of the enzyme. However, the apparent k_{cat} values reflect the efficiency of differently modified ACC oxidases under the same conditions. These considerations require any interpretation of the results in Table 1 be treated with caution. The differences in apparent K_m values for ACC, which range from 83−132 μm,[2] may well fall within experimental error. Interpretation of the differences in k_{cat} values for the wild-type and H56Q enzymes with the H177E and D179E enzymes is probably more significant since the values for the former pair are >500 times those of the latter pair.

It was anticipated that substitution of glutamine for the H177 and D179 ligands would very much reduce the iron binding capacity of the modified enzymes relative to the wild-type enzyme. The substitutions in which H177 and H234 were replaced with aspartate or glutamate residues were made in the hope that substitution of carboxylate for imidazole ligands might still allow for productive iron binding. D179 was converted to a glutamate residue as this was considered probably the least dramatic change in iron-(II) coordination chemistry that could be readily made.

As observed for substitution with glutamine, substitution of H177 with aspartate resulted in an inactive protein (H177D), but substitution with glutamate resulted in a protein (H177E) displaying ACC oxidase activity, albeit at a very low level, <1% that of the wild-type enzyme. A similar level of activity was observed for the D179E enzyme.[3] Although interpretation of the apparent K_m values is problematic, it is perhaps notable that the apprent K_m values for ACC and Fe(II) both increase for the D179E protein and decrease for the H177E protein. Since the apparent K_m for Fe(II) is ca. 10-fold higher for the D179E enzyme than the wild-type or H177E enzymes, it is tempting to suggest that the D179E enzyme binds Fe(II) less strongly than the other two enzymes analyzed. However, the relationship between the apparent K_m for Fe(II) and its binding constant is not straightforward, and even this suggestion may be an over-interpretation.

That replacement of H177 with glutamate produced an active enzyme, whereas its replacement with an aspartate residue yielded an inactive protein suggests that side chain length is important in the residues supporting productive metal binding. It is likely that the imidazole ring of H177 binds the iron(II) via its $N^{\epsilon 2}$ nitrogen. That H177E is active and H177D is not may reflect from the fact that the carboxylate oxygen of glutamate is approximately the same distance from the α-position as the $N^{\epsilon 2}$ of histidine, whereas that of aspartate is one methylene short.

When H234 was replaced with aspartate or glutamate residues the resultant proteins (H234D, H234E) were expressed in predominately insoluble forms and no activity could be detected in crude cell extract assays of these proteins. In the case of recombinant apple ACC oxidase, H234 was also converted to an aspartate residue and the crude extracts were shown to be inactive (Shaw et al., 1996). However, it was not reported if protein was produced in a soluble or insoluble form. It is possible that the activities of the H234D and H234E proteins are too low to be detected due to the low solubility of the modified proteins.

Myllyharju and Kivirikko (1997) recently reported mutagenesis studies on the iron binding site of prolyl-4-hydroxylase. They concluded that H412, D414 and H483, which are in regions of weak sequence similarity with the IPNS subfamily correlating with H177, D179, and H234 of ACC oxidase, act as iron ligands. H412E, D414A, D414Q, and D414E modified enzymes were made. All were inactive except the D414E enzyme which possessed ca. 15% the activity of wild-type enzymes, significantly higher than that observed for the analogous substitution with ACC oxidase (D179E). Also in contrast to the H177E modified ACC oxidase, no activity above background was observed for the H414E modified prolyl-4-hydroxylase.

MCO Fragmentation Experiments

Significant fragmentation was observed in the case of the wild type, H177E, H177D, D179E, H234Q, and H56Q modified ACC oxidases. In these cases the observed patterns were distinctly different dependent on the presence or absence of ACC. Very much reduced fragmentation was observed in the cases of the D179N and H177Q modified ACC oxidases, and it was difficult to distinguish fragmentation from minor impurities present before incubation. (It cannot be ruled out that no fragmentation occurred since the minor impurities may result from fragmentation occurring during production of the proteins.) For the H177D&D179E doubly-modified ACC oxidase no significant fragmentation was observed.

With ACC present in the incubation mixture wild-type enzyme produces 10 major fragments (a−j, Figure 3, Table 2) of which a to g were readily detectable by SDS−PAGE (16% gel) and h to i could only be detected when samples were heavily overloaded (e.g. D179E, Figure 3). In the absence of ACC only seven major fragments were visible with fragments a, f, and i missing. The N-terminal sequences of bands a, b, and d have previously been shown to be the same as that of intact ACC oxidase. Band g was shown to be a ca. 15 kDa C-terminal part fragment of ACC oxidase with the N-terminal sequence: FQDNKVS (Barlow et al., 1997). The N-terminal sequences of the remaining major fragments were determined as part of the present study. The results demonstrated that bands c, e−h, and j contain the same N-terminal sequence as intact ACC oxidase and that the N-terminal sequence of band i was VNLGD. Comparison with the IPNS structure (Roach et al., 1995) reveals the cleavage position is located in one of the β-strands (β-12) forming the "jelly roll" barrel of IPNS and, like the previously reported internal sequence, is within 8 Å of the predicted metal binding site based on the IPNS structure. Differences with the wild-type pattern were observed for all the other ACC oxidases which fragmented, e.g., bands c and

[2] Note also that, for the H56Q mutant which shows a ca. 50% increase in apparent K_m for ACC, comparison with the IPNS structure suggests H56 is probably located at the end of an α-helix (α-3) which is relatively exposed and separated from the active site by β-strands of the "jelly roll" motif (Roach et al., 1995). It is thus unlikely that substitution of H56 directly influences substrate binding.

[3] Previously it has been reported that replacement of D179 of the kiwi fruit ACC oxidase with glutamate resulted in an inactive enzyme (Lay et al., 1996).

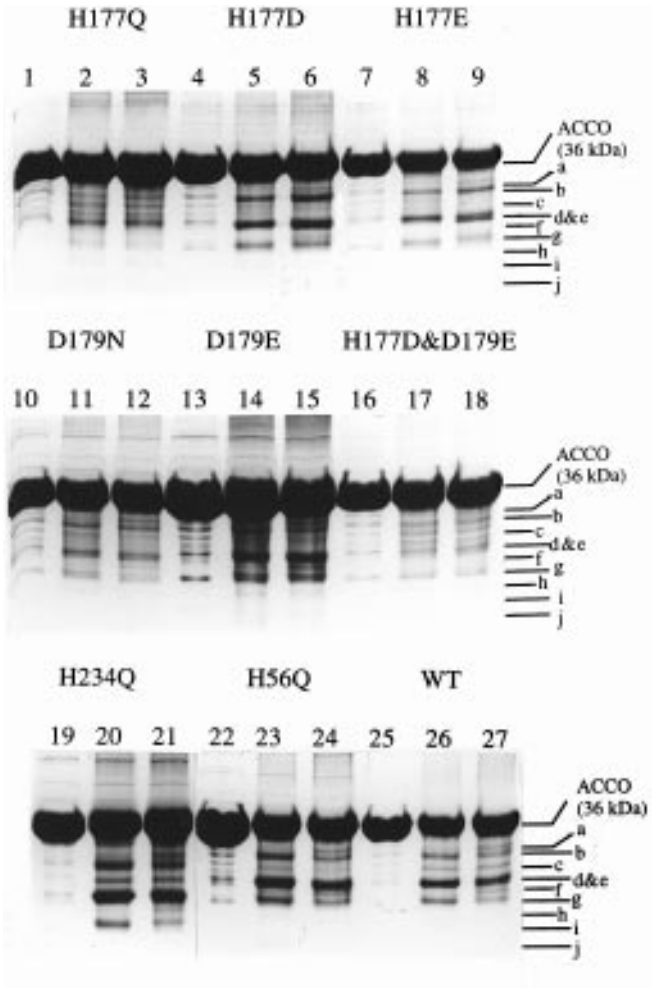

FIGURE 3: SDS−PAGE analysis of metal-catalyzed oxidative fragmentation of wild-type and mutant ACC oxidases. Experimental details were as described in Materials and Methods. Lanes 1, 4, 7, 10, 13, 16, 19, 22 (mutant ACC oxidases), and 25 (wild-type ACC oxidase) were incubated without added Fe(II), ascorbate, or ACC. Lanes 2, 5, 8, 11, 14, 17, 20, 23 (mutant ACC oxidases) and 26 (wild-type ACC oxidase) were incubated with Fe(II) and ascorbate. Lanes 3, 6, 9, 12, 15, 18, 21, 24 (mutant ACC oxidases), and 27 (wild-type ACC oxidase) were incubated with Fe(II) and ascorbate and ACC. Lanes 19−21 were run on a different gel than lanes 22−27.

f were not present when the H177D and H177E proteins were fragmented in the presence of ACC.

The observation that the H56Q modified ACC oxidase displayed an almost identical fragmentation pattern to the wild-type enzyme either in the presence or absence of ACC is consistent with its lack of involvement in iron binding. In the case of the H177Q and D179N modified ACC oxidases, fragmentation was very much reduced relative to the wild-type enzyme consistent with the roles of H177 and D179 as iron ligands. It was difficult to detect any specific fragmentation bands, and the observed patterns were almost identical in the presence or absence of ACC. SDS−PAGE analysis did display some evidence of "smearing", possibly reflecting nonspecific modifications resulting from Fenton/Udenfreind type reactions occurring in solution (Udenfreind et al., 1954). There was no evidence for MCO fragmentation of the inactive H177D&D179E doubly substituted ACC oxidase.

SDS−PAGE analysis of MCO fragmentation of the H234Q modified enzyme led to the unexpected observation that its fragmentation occurred with almost identical patterns to those observed for the wild-type enzyme, either in the presence or absence of ACC. This suggests that metal, ascorbate and ACC binding sites are present at the "active site" of this inactive modified ACC oxidase. The metal binding properties of the side chains of histidine and glutamine residues at residue 234 will differ significantly, with the latter probably ligating less well to iron. Thus, it seems iron ligation by H234 is required for catalysis, but not for fragmentation. In contrast the results obtained for the H177Q and D179N ACC oxidases imply that iron ligation by H177 and D179 is requisite both for catalysis and fragmentation.

Fragmentation and other active site mediated oxidative modifications of ACC oxidase are observed to occur (at least predominately) as a "burst" after mixing of enzyme and cofactors/substrates (unpublished results). It is possible that fragmentation is related to incorrectly folded or "primed" enzyme. The present study is consistent with this proposal and indicates incorrect iron ligation as a reason for fragmentation, i.e. in not all of the ACC oxidase present at the initiation of reaction is the iron(II) ligated by H234. Once the first catalytic cycle is complete the enzyme is left in the correctly primed iron complex, which explains the observed burst of fragmentation.

MCO fragmentation experiments with the weakly active H177D and H177E modified ACC oxidases revealed that they both fragmented with a similar pattern, which was slightly different to that observed for the wild-type enzyme, perhaps reflecting a different mode of Fe(II) binding relative to wild-type enzyme. The level of fragmentation for these modified ACC oxidases was reduced relative to the wild-type enzyme.

CONCLUSIONS

The evidence presented implies that H177, D179, and H234 act as iron ligands during ACC oxidase catalysis but that metal ligation by H234 is not essential for fragmentation. The study demonstrates that use of the MCO fragmentation technique with both weakly active and inactive mutants can lead to insights into the chemistry of the iron binding site that simple turnover assays could not provide. Screening for MCO fragmentation of enzymes by electrophoresis is facile and may be readily applied to other non-heme oxidases/oxygenases, possibly with a view to identifying active site residues where there is little or no three-dimensional information.

Figure 4 summarizes proposals for the processes occurring during the inactivation of ACC oxidase (tomato). At room temperature in catalytic buffer purified apo-ACC oxidase (conformer A) may undergo a conformational change leading to conformer B, which binds Fe(II) to give a partially active enzyme (conformer B-Fe) (Barlow et al., 1997). The conversion of conformer A to B does not require Fe(II). Conformer A binds Fe(II) to give an "incorrectly primed" form (conformer A-Fe) in which H234 is not ligated to the iron. Conformer A-Fe may then undergo change to give conformer C-Fe, which is "fully" active, a process which could involve release of Fe(II). The fully active conformer C then binds and reacts with ascorbate and dioxygen to produce a ferryl (or equivalent) intermediate and dehydroascorbate (**I**, Figure 4). ACC then may react with this intermediate **I** displacing dehydroascorbate from the metal

Table 2: Summary of MCO Fragmentation Experiments in the Presence (+) or Absence (−) of ACC[a]

	WT		H56Q		H234Q		H177D & D179E †		D179E		D179N †		H177E		H177D		H177Q †	
	ACC		ACC		ACC		ACC		ACC		ACC		ACC		ACC		ACC	
	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
a	-	•	-	•	-	•	-	-	-	•	-	•	-	•	-	•	?	?
b	•	•	•	•	•	•	-	-	•	•	•	•	•	•	•	•	?	?
c	•	•	•	•	•	•	-	-	?	?	-	-	-	-	-	-	?	?
d	•	•	•	•	•	•	-	-	•	•	•	•	•	•	•	•	?	?
e	•	•	•	•	•	•	-	-	•	•	•	•	•	•	•	•	?	?
f	-	•	-	•	-	•	-	-	-	•	-	•	-	-	-	-	?	?
g	•	•	•	•	•	•	-	-	•	•	•	•	•	•	•	•	?	?

[a] The presence of a fragment by SDS−PAGE analysis (see Figure 3) is indicated by (•), absence or very much reduced level of a fragment by (−). Fragments h, i, and j were not included because they were not stained sufficiently well. Fragmentation for D179N and H177Q was much reduced, cf wild-type enzyme.

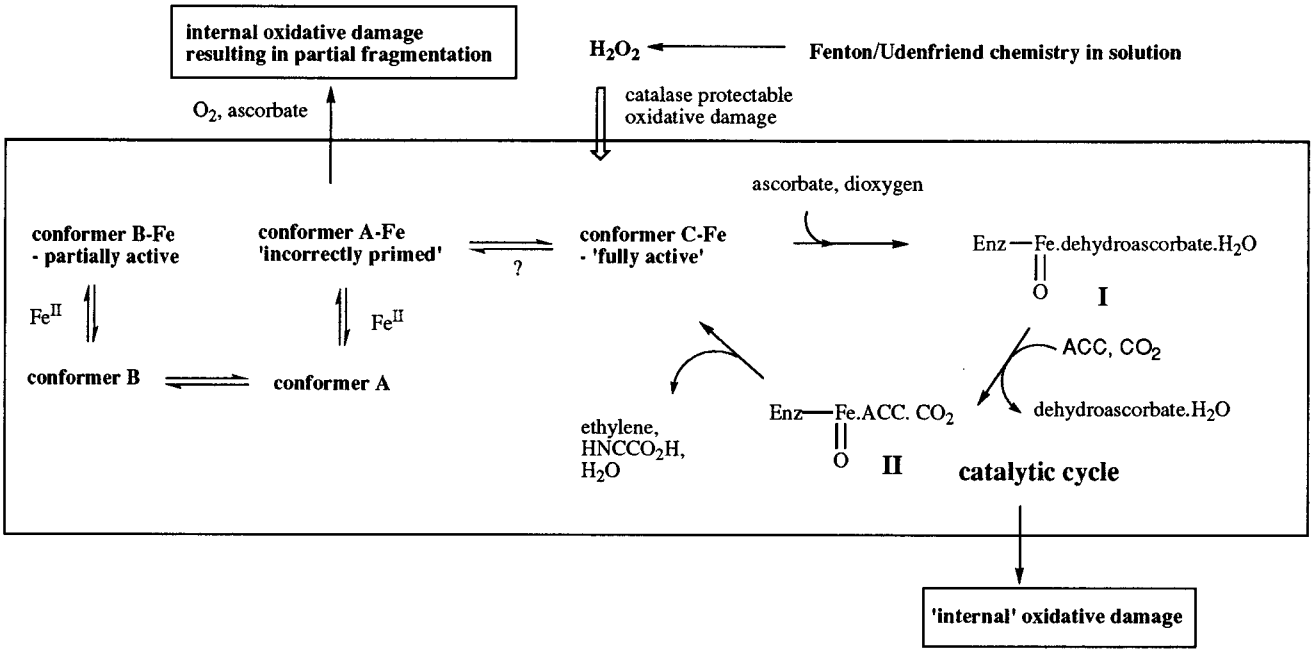

FIGURE 4: Summary of ACC oxidase inactivation processes.

to generate a second ferryl intermediate (**II**, Figure 4), which effects the two-electron oxidation of ACC to give ethylene, H_2O, CO_2, and HCN. The HCN and CO_2 may be released as cyanoformic acid (Peiser et al., 1984). At the end of this catalytic cycle conformer C-Fe is regenerated.

Oxidative inactivation may occur by a variety of pathways. (i) Generation of hydrogen peroxide in solution followed by protein oxidation: this process is catalase protectable, and may result in damage to both active site and non-active site residues. It does not result in significant fragmentation. (ii) Oxidative modification to conformer A-Fe: this process is dependent upon the presence of iron, dioxygen, and ascorbate and is the predominant process by which fragmentation occurs. It is not protected against by the addition of catalase, bicarbonate, or ACC. However, binding of ACC to the incorrectly primed enzyme modifies the oxidative process. (iii) Oxidative damage to fully active and correctly primed ACC oxidase (conformer C-Fe): this process is not protectable by the addition of catalase and is dependent upon the presence of iron, dioxygen, and ascorbate but does not result in significant fragmentation. Since the addition of bicarbonate only protects against this inactivation mechanism in the presence of ACC it seems possible that this mechanism results from incorrect reaction of a catalytic intermediate or a complex with a closely related structure.

Clearly a better understanding of the ACC oxidase mechanism will require insights into the structures of oxidizing intermediates such as **I** and **II**. A possible structure

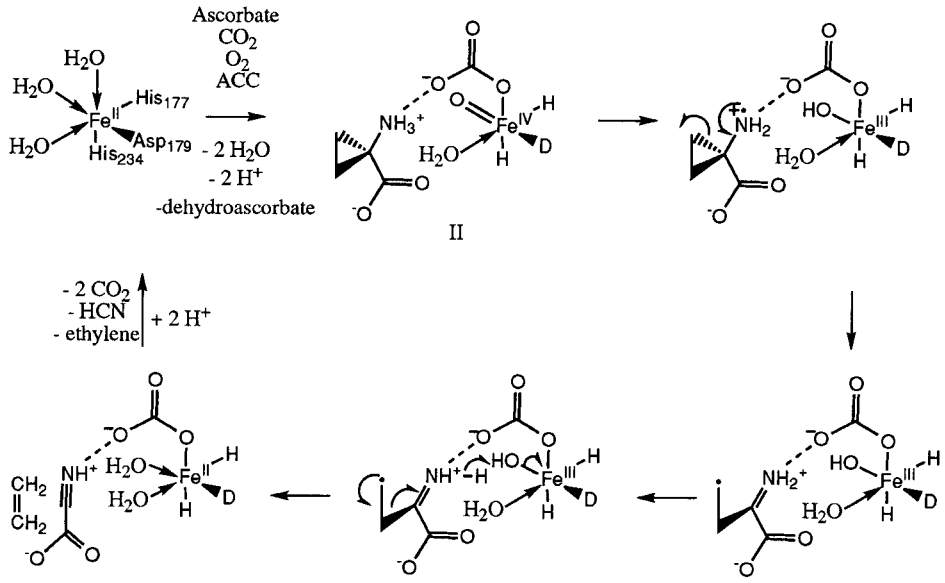

FIGURE 5: Partial mechanism for ACC oxidase. Although dehydroascorbate is shown being displaced from the active site in formation of intermediate II, in this and subsequent intermediates dehydroascorabte may in fact remain ligated to the iron in a monodentate fashion replacing the water opposite H_{177}.

for intermediate **II** is shown in Figure 5, which shows subsequent reaction via a radical mechanism (Baldwin et al., 1985). We propose that the ACC carboxylate binds to the side chains of R244 and S246. These residues form part of a conserved RXS motif and bind to the carboxylate of the valine of ACV during IPNS catalysis (Roach et al., 1997). Support for this proposal comes from the observation that ACC oxidase catalyzes the conversion of D- but not L-valine to iso-butanal (unpublished results). The proposed coordination chemistry is based partially upon that proposed for a monocyclic β-lactam/ferryl intermediate in IPNS catalysis (Roach et al., 1997). The orientation of the ferryl is based upon the observed binding of NO (a dioxygen analogue) to the holo-IPNS−substrate complex. The carbon dioxide activator is shown as bicarbonate ligating to the iron opposite H234 in a position analogous to that occupied by the thiol of ACV in the holo-IPNS−substrate structure (Roach et., 1997). This is also the proposed ligation position of the 1-carboxylate group of the 2-oxoglutarate in 2-oxoglutarate dependent dioxygenases within the ACC oxidase/IPNS subfamily (Roach et al., 1997). Oxidative fragmentation of 2-oxoglutarate in these enzymes results in conversion of the 1-carboxylate to carbon dioxide together with production of succinate. Hence, the proposed iron ligation by CO_2/bicarbonate provides a mechanistic link between ACC oxidase and the 2-oxoglutarate dependent enzymes. The proposal is consistent with available mutagenesis and kinetic studies which indicate the iron, ascorbate, CO_2/bicarbonate, dioxygen, and ACC binding sites are closely linked. It is also supported by the observation that bicarbonate only protects against inactivation in the presence of ACC and with the MCO fragmentation experiments which demonstrate that reactive intermediates can be formed in the absence of ACC or CO_2/bicarbonate, but that productive catalysis only occurs in the presence of both. During ACC catalysis iron-ligated bicarbonate may serve to orientate the amino group of ACC toward the ferryl group in a productive manner and/or to deprotonate the protonated amine of ACC.

ACKNOWLEDGMENT

We thank A. C. Willis for amino acid sequence analyses and Drs. P. L. Roach, P. Thomas, and A. Prescott and Profs. P. John, J. Hajdu, M. Sami, and T. Brown for discussions and encouragement.

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BI971823C