Ethylene biosynthesis: processing of a substrate analog supports a radical mechanism for the ethylene-forming enzyme

Michael C Pirrung, Jun Cao and Jrlung Chen

Background: The chemical mechanism of the final step of ethylene biosynthesis (the conversion of 1-aminocyclopropanecarboxylic acid, ACC, to ethylene by ACC oxidase, the ethylene-forming enzyme, EFE) is poorly understood. Two possibilities have been suggested: a radical mechanism and an *N*-hydroxylation mechanism. We investigated reaction pathways available to radical intermediates in this reaction using an ACC analog, 1-aminocyclobutanecarboxylic acid (ACBC) as a substrate.

Results: ACBC was converted to dehydroproline (Δ^1-pyrroline-2-carboxylic acid) by the EFE via a ring expansion process. The possibility that an *N*-hydroxy-aminoacid (produced during two-electron oxidation) acts as an intermediate in this process was eliminated by control experiments. Chemical model reactions involving two-electron oxidants, such as a positive halogen (X^+), which presumably generate *N*-halo derivatives, produce only decarboxylation products. Radical-based oxidants, in contrast, generate dehydroproline. Model reactions involving sequential single-electron transfer mechanisms also produce dehydroproline; thus our results support the proposal that the EFE-catalyzed step of ethylene biosynthesis proceeds using a radical-based mechanism.

Conclusions: Our results provide support for a radical mechanism in the final step of ethylene biosynthesis and refute an alternative *N*-hydroxylation mechanism. This work extends the idea that the intrinsic chemical reactivity of a high energy iron–oxo intermediate can account for the observed products in ethylene biosynthesis.

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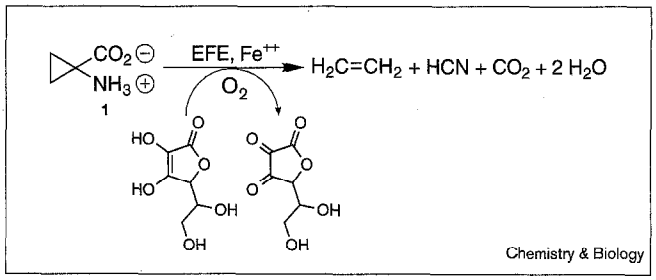
Introduction

The biosynthesis of ethylene from 1-aminocyclopropane-carboxylic acid (ACC) in higher plants is a fascinating process [1]. Its study has been challenging because the enzyme that catalyzes this reaction was for some time difficult to isolate and is very unstable. Consequently, much initial work was conducted using classical methods for the study of biosynthesis [2]; labeled substrates were fed to plants, followed by product analysis using physical methods. Isolation of ACC oxidase, also known as the ethylene-forming enzyme (EFE), in homogeneous form from apple fruit [3–6] has facilitated more rigorous study. We have studied the inhibition of this enzyme with different amino acid hydroxamates, and their varying potency suggests that they act not by simply removing iron from the enzyme, but through both metal chelation and hydrophobic interactions in the active site [7]. The most potent inhibitor, ACC-hydroxamate, has a K_i comparable to the substrate K_m. EFE has now been isolated from a number of sources [8,9], and recombinant bacterial and yeast expression systems have been developed [10–12]. Heterologously-expressed recombinant tomato enzyme, interestingly, behaves differently with hydroxamates than does the native apple enzyme [13].

The availability of purified EFE has allowed the determination of a number of the key characteristics of its reaction (Figure 1). One question of particular interest is the fate of the four oxidizing equivalents of O_2, as the conversion of ACC to ethylene is only a two-electron oxidation process. EFE belongs to a family of non-heme iron oxygenases; in these enzymes, α-ketoglutarate is often a cosubstrate that consumes the remaining two oxidizing equivalents during its conversion to CO_2 and succinate. Ascorbate is required for EFE activity, however, and one group has reported that it is oxidized to dehydroascorbate [5]. On the other hand, inhibition by α-keto acids has also been reported [14]. The enzyme also experiences significant auto-inactivation [15].

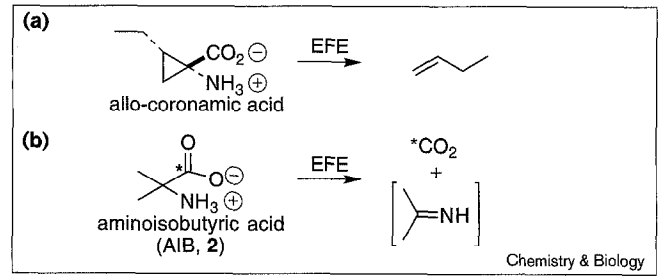
Known alternate substrates for the EFE include 2-alkyl ACC analogues of specific stereochemistry (a cis relation of the nitrogen and alkyl group is required) [16–18] and α-aminoisobutyric acid (AIB; Figure 2) [19]. The former are processed to the corresponding 1-alkenes, whereas the latter is converted to CO_2 and (presumably) acetone imine. Gibson *et al.* [13] have identified a similar oxidative decarboxylation reaction, also catalyzed by the EFE, in the processing of D-valine to isobutyraldehyde.

Figure 1



Possible stoichiometry for ethylene biosynthesis from 1-aminocyclopropanecarboxylic acid (ACC; **1**). The reaction is catalyzed by ACC oxidase, also known as the ethylene-forming enzyme (EFE).

Figure 2



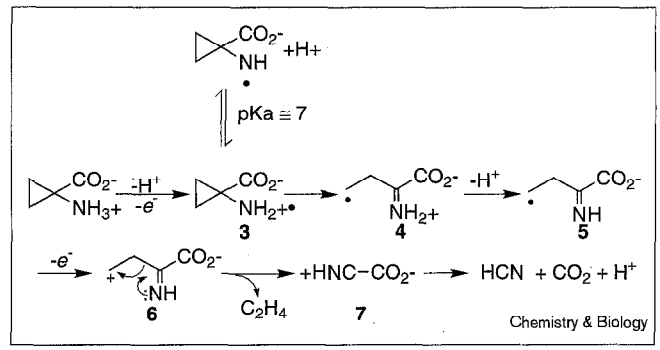
Alternative EFE substrates and their products. **(a)** Allo-coronamic acid, a 2-alkyl ACC analogue, is processed by the EFE forming a 1-alkene. **(b)** Aminoisobutyric acid (AIB; **2**) can also be processed by the EFE to generate carbon dioxide and acetone imine.

As a result of previous mechanistic studies, including an electrochemical oxidation study, a sequential single-electron transfer mechanism for ethylene biosynthesis has been proposed [20], which is shown in Figure 3. We suggested that a primary role of the EFE is to generate a substrate radical, specifically radical cation **3** (Figure 3); a precedent for this kind of radical generation was established in earlier studies on the inactivation of cytochrome P450 (iron–oxo) enzymes by cyclopropylamines [21,22]. Because the pH environment of these enzyme active sites is unknown and the aminyl radical and radical cation differ only by protonation, with an approximate $pK_a = 7$ for the dialkylaminyl radicals [23], it is difficult to exclude the corresponding aminyl radical as a possible early intermediate in ethylene biosynthesis. On the other hand, studies by electron paramagnetic resonance (EPR) spectroscopy in a frozen matrix of inert gas of the radical cation and aminyl radical of cyclopropylamine and their ring-opened isomers [24] have shown that opening of the aminyl radical leads quickly to internal hydrogen-atom transfer from the N–H species to generate an iminyl radical. This species is not consistent with the requirements for ethylene production as it bears an ethyl unit; an aminyl radical mechanism is not favored because of this.

The intrinsic reactivity of **3** and subsequent intermediates (shown in Figure 3) should lead readily to ethylene production (see below) [2]. We have proposed that the ACC-radical cation preferentially undergoes ring opening in order to release cyclopropane ring strain. The production of the radical **3** is evidently the rate-determining step [25], as the ring opening is an irreversible and exothermic reaction [23] (calculated to be 7 kcal mol^{-1}) [26] and, based on the processing of a radical clock substrate [27], which provides an internal kinetic competition after the rate-determining step, the conversion of **4** to ethylene cannot be faster than 10^8 s^{-1}.

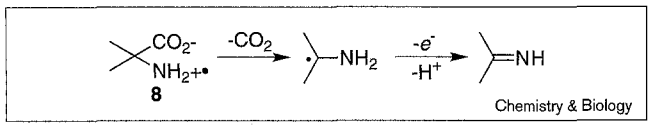
That the EFE is closely related to a group of non-heme iron proteins that catalyse hydroxylations, epoxidations, and oxidative ring closure in a number of biosynthetic pathways found in microorganisms and higher plants further suggests that it uses an iron–oxo intermediate; that the required radical cation could be generated by electron transfer from the amino group of ACC provides further support for this idea. Kinetic measurements on cyclopropylamine radical cations are not available, but it is possible to estimate their reactivity. The 2-phenylcyclopropylaminyl radical is known to open to a stabilized radical with a rate constant $>10^{11}$ s^{-1} [28] and, based on earlier studies and the known effects of the phenyl group on ring-opening rates, the unsubstituted cyclopropylaminyl radical would be expected to open at a rate of $\sim 10^8$ s^{-1}. Amine cation radicals have been shown to undergo rearrangements up to 10^3-fold faster than their cognate aminyl radicals [23], which suggests a rate constant for ring opening of $>10^{11}$ s^{-1} (to produce **4**; Figure 3). One inconsistency in the kinetic picture, currently under investigation, is that hydroxyl rebound to **4** might occur if its conversion to ethylene is no faster than 10^8 s^{-1}. Oxygen rebound rates in non-heme iron enzymes are addressed below.

Figure 3



The sequential single electron transfer model for ethylene biosynthesis.

Figure 4



A possible mechanism for the processing of an AIB-derived radical (**8**) by the EFE.

The mechanism in Figure 3 can be applied to the known enzymatic processing of the alternate substrate AIB (Figure 4). There is no driving force for a radical or radical cation derived from AIB (**8**; Figure 4) to release a methyl radical, unlike the situation with ring opening of the ACC radical cation; thus **8** may be decarboxylated to provide the α-amino radical. Because conversion of ACC to ethylene is a two-electron oxidation process, and production of **8** requires only one oxidizing equivalent, an additional oxidizing equivalent remains available to convert the radical to the imine.

Following up these leads, we considered how a reactant expected to be intermediate between ACC and AIB in its rate of C–C cleavage would behave. Aminocyclobutanecarboxylic acid (ACBC) has slightly lower ring strain than ACC, and we suspected that it would serve as an adequate substrate. At least three reaction pathways are available to the radical cations derived from these three amino acids (ACC, AIB and ACBC; Figure 5). Partitioning of the radical cations between these competitive pathways (OH rebound, decarboxylation, ring opening) should determine the reaction course. For ACC (X= nil; Figure 5), we have suggested the ring-opening path (Figure 5c) [20], although hydroxyl rebound and subsequent hydroxylation (Figure 5a) are still advocated [29]. This proposal does not address how conversion of the *N*-hydroxyaminoacid to ethylene would be promoted. The hydroxyl rebound rate for a non-heme iron enzyme has not been determined, but is likely to be slower than the 10^{13} s^{-1} observed for cytochrome P-450 [30] (see below), making it at least comparable to ring opening (> 10^{11} s^{-1}, see above). For AIB (X=H_2), we suggest the decarboxylation pathway (Figure 5b) as shown in Figure 4. The purpose of the study reported here was to determine the path for ACBC (X=CH_2); ring opening for ACBC should be about 10^3 s^{-1} slower than for ACC, whatever its rate, and we expected *N*-hydroxylation to be more favored.

Recent data from Silverman and co-workers [31] on the mechanism-based inactivation by, and processing of, substrate analogues of monoamine oxidase (MAO) give further impetus to experiments with ACBC. As shown in Figure 6, 1-phenylcyclobutylamine (**9**) is converted to phenylpyrroline by a mechanism explained as electron transfer, ring opening, ring closure at nitrogen, and further oxidation and proton loss steps. 1-Benzoylcyclobutylamine (**10**) follows a similar pathway except that intermediate **11** is stabilized by captodative substitution, a phenomenon whereby the presence of both electron-withdrawing and electron-donating groups at a radical center lowers the energy of the singly occupied orbital. The longer lifetime of **11** (compared to **9**) permits it to inactivate the enzyme and to be trapped by a nitrone. This experiment represents the first direct observation of a radical intermediate in MAO [31].

We can predict, based on the previous experiments outlined above, that ring opening of the ACBC radical cation **12** (Figure 7), and not decarboxylation, should occur in the EFE reaction, but it should be slower than with ACC. The ring-opened radical has few reaction pathways available, and it might covalently modify the active site of the EFE, as has been proposed for **11**. Alternatively, ring closure on the nitrogen atom followed by oxidation would yield dehydroproline; hydroxyl rebound could lead to 5-hydroxy-2-ketovalerate.

Results and discussion

We wished first to address the possibility that *N*-hydroxylated amino acids could be intermediates in the processing of ACC, AIB, and ACBC by the EFE. Unfortunately, considerable efforts (R. Ludwig, unpublished observations) expended towards synthesis of *N*-hydroxy-ACC have proved fruitless. The Strecker route to *N*-hydroxy-ACC is not available because cyclopropanone oxime exists as the nitrosocyclopropane tautomer, although efforts were made to trap a transient oxime tautomer with cyanide. Various methods based on selective oxidation of ACC also failed to

Figure 5

Reaction pathways for amino acid radical cations. **(a)** Hydroxyl rebound. **(b)** Decarboxylation. **(c)** Ring opening.

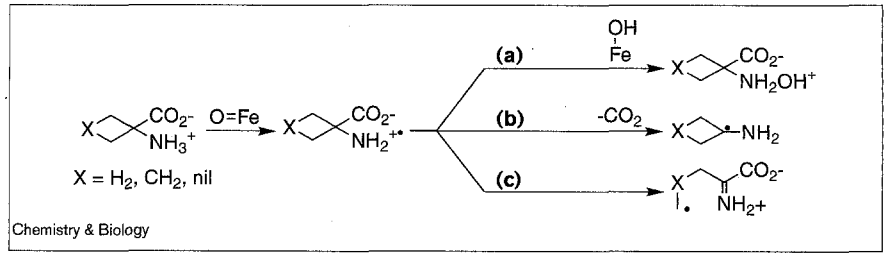
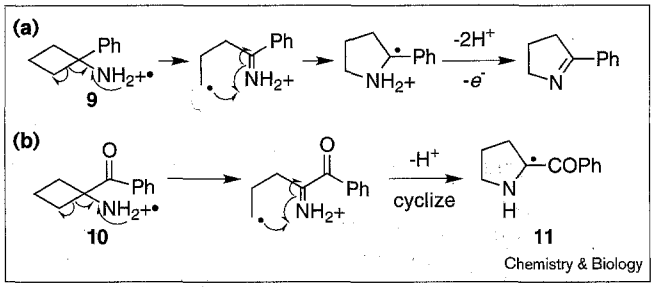


Figure 6



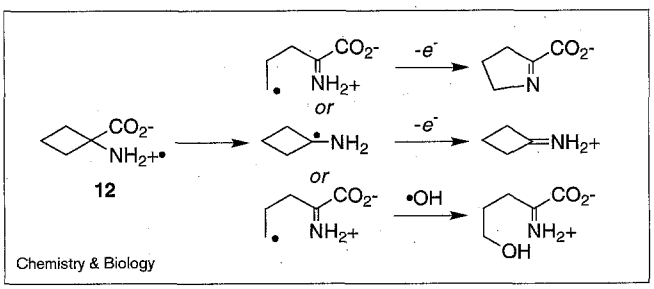
Silverman and co-worker's [31] suggested mechanisms for monoamine oxidase (MAO) processing of cyclobutylamine derivatives. **(a)** The processing of 1-phenylcyclobutylamine. **(b)** The processing of 1-benzoyl cyclobutylamine.

generate *N*-hydroxy-ACC. A Strecker synthesis route using hydroxylamine and cyanide applied to acetone and cyclobutanone (Figure 8) did succeed, however [32]. The yield for the first step is 80–85%, and hydrolysis provides the *N*-hydroxyaminoacids in modest yield after crystallization.

Both *N*-hydroxy AIB and *N*-hydroxy ACBC as well as their corresponding amino acids, AIB and ACBC, were tested as EFE inhibitors against the native apple fruit enzyme at a minimum of five concentrations using a conventional Dixon analysis. The amino acids are relatively weak inhibitors compared to the ~15 μM Michaelis constant for ACC (Figure 9). Both of the hydroxylamines are approximately as effective as substrate (ACC) in binding to the EFE, however.

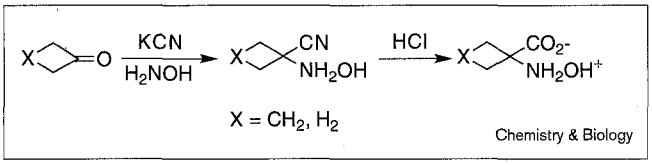
The processing of ACBC by the EFE was investigated with 1-[^{14}C]-labeled ACBC (Figure 10a). EFE was incubated to exhaustion (no more product was produced; the enzyme was cleanly inactivated during this process) and the products analyzed. Only a trace of the radioactivity was converted to carbon dioxide, in contrast to earlier observations with AIB [19]. The nonvolatile labeled product, where most of the radioactivity resided, was identified as Δ^1-pyrroline-2-carboxylic acid (dehydroproline). Because

Figure 7



Reaction pathways for the ACBC radical cation (**12**).

Figure 8

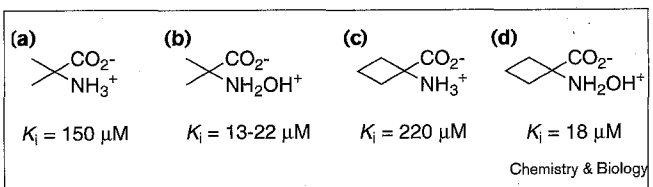


Synthesis of *N*-hydroxyamino acids.

an authentic sample of the ACBC *N*-hydroxy analogue was also available, the possibility of its presence in the reaction could be excluded using radio-thin layer chromatography (TLC). Control experiments in the absence of enzyme showed only traces of dehydroproline. It was also interesting that radioactivity corresponding to ~1.7 molecules of ACBC was retained by each molecule of the enzyme, as determined by scintillation counting after ultrafiltration. This raises the likelihood of a covalent modification whose kinetics and structure bear further investigation.

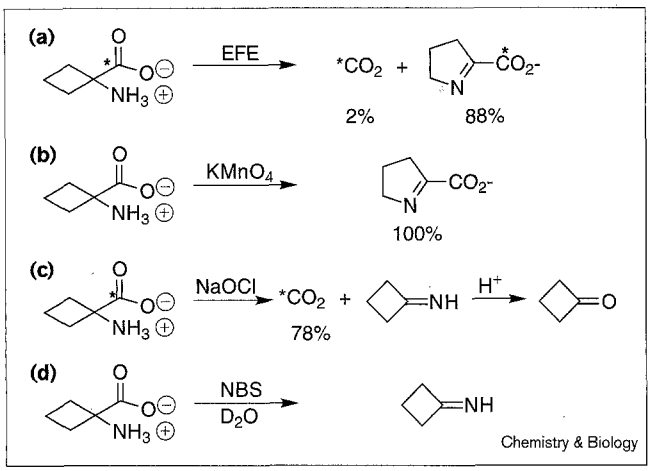
In order to understand ACBC's intrinsic chemical reactivity under various conditions, it was treated with reagents that can convert ACC to ethylene; positive halogen sources are commonly used in assays for ACC [33]. Previous work strongly suggests these reactions proceed via *N*-chlorination and α-elimination to the cyclopropyl nitrene [34,35], which undergoes concerted fragmentation to ethylene. The conversion of stereospecifically dideuterated ACC to ethylene proceeds in a stereospecific manner when promoted by halogen, in contrast to the EFE-catalyzed biosynthetic reaction. Reactions that show loss of stereochemistry in agreement with the biosynthetic reaction involve electrochemical and chemical oxidants [36], which presumably proceed by one-electron mechanisms. We chose to study permanganate, one of the chemical oxidants (Figure 10b). When ACBC is treated with permanganate under the published conditions [36], dehydroproline is produced. Decarboxylation to cyclobutanone or its imine is undetectable, even when using radiolabeled material. In contrast, treatment with sodium hypochlorite initially produces the imine

Figure 9



Structure and inhibition constants for four ACC analogues. **(a)** Aminoisobutyric acid (AIB). **(b)** *N*-Hydroxy aminoisobutyric acid **(c)** Aminocyclobutanecarboxylic acid (ACBC). **(d)** *N*-Hydroxy aminocyclobutanecarboxylic acid.

Figure 10

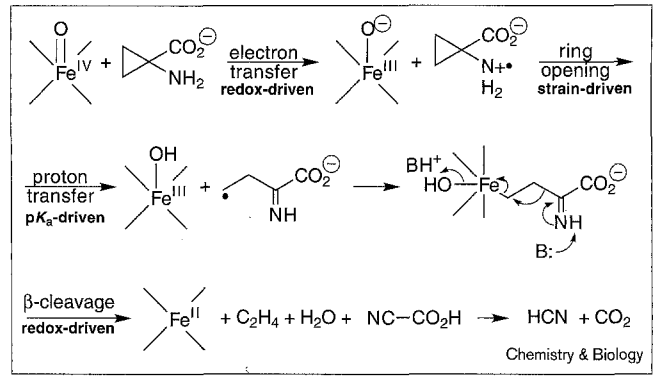


Reactions of ACBC under enzymatic or chemical oxidation by **(a)** the EFE, **(b)** potassium permanganate, **(c)** sodium hypochlorite and **(d)** *N*-bromosuccinimide (NBS).

(Figure 10c), which is readily hydrolyzed to cyclobutanone on treatment with acid. The carbon dioxide yield in this decarboxylation, using radiolabeled material, was determined to be 78%. Likewise, monitoring the reaction of ACBC with *N*-bromosuccinimide (Figure 10d) in a nuclear magnetic resonance (NMR) tube shows that (only) the imine is produced; it hydrolyzes to cyclobutanone.

Both *N*-hydroxyaminoacids (Figure 9b, d) are fairly potent inhibitors of the EFE. In principle, this result could either support a mechanism for the biosynthetic reaction involving hydroxylation, based on the fact that hydroxylamines bind tightly to the EFE (hydroxylamines that do not have a strained cyclopropane ring are not substrates, however), or it could refute the hydroxylation hypothesis because hydroxylamines are inhibitors, not substrates of the EFE. That [1-^{14}C]-AIB is converted to radiolabeled carbon dioxide and [1-^{14}C]-ACBC is converted to dehydroproline greatly undermines the hydroxylation theory. If the *N*-hydroxyaminoacids were intermediates toward the formation of these products (carbon dioxide and dehydroproline), they should be substrates, not inhibitors, of the EFE. In fact, of several alternative substrates we have investigated, AIB is the one most likely to be hydroxylated instead of oxidized by electron transfer (Figure 7). The rate of hydroxyl rebound should be approximately constant with ACC, AIB and ACBC, and the favored partitioning would be based on the rate of the C–C bond cleavage step. Because the cycloalkane amino acids are strained, their C–C cleavage rates should be fast (cyclopropanaminium ~10^{11} s^{-1}, cyclobutanaminium ~10^{8} s^{-1}). The C–C bond in a radical or radical cation derived from AIB would not release a methyl radical and so would be likely to undergo hydroxylation, if that were the mechanism.

Figure 11



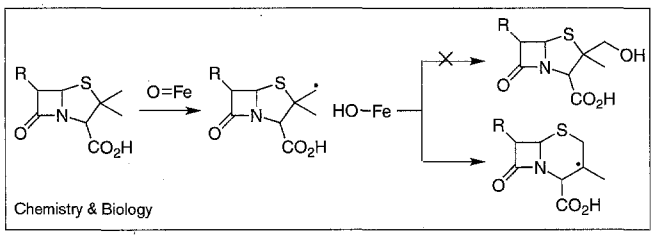
A mechanism for synthesis of ethylene from ACC by the EFE, with observed products, stereochemistry and the iron–oxo intermediate.

Processing of ACBC by the EFE leads to a ring expansion product rather than a hydroxylation or decarboxylation product, which provides strong support for the sequential single-electron transfer mechanism for ethylene biosynthesis. Ring expansion with another free radical, one-electron chemical oxidant, potassium permanganate, gives further support to this idea. As would have been suspected from previous studies, the reactivity of *N*-heteroatom derivatives of these amino acids is divergent from the reactivity of the radical derivatives of these amino acids. Silverman and coworker's [31] previous enzymatic work on MAO, the model reaction with permanganate, and the processing of ACBC by the EFE show that the ring expansion pathway is favored for cyclobutylaminyl radical cations.

Reading the lipoxygenase literature one can find a precedent for the potent inhibition of a non-heme iron enzyme by a hydroxylamine [37]. In this case, the hydroxylamine is believed to inhibit lipoxygenase by reducing the iron from the ferric to the ferrous state. Because the active form of iron in the EFE is expected to be ferrous, a different inhibition mechanism must apply to these *N*-hydroxyaminoacids.

The data presented here, in addition to earlier results, allow us to propose a mechanism for ethylene biosynthesis that exploits purely chemical principles to drive the reaction processes (Figure 11) after the formation of the iron–oxo intermediate. The involvement of reactive radical species may also explain the self-inactivation of the EFE. The overall reaction is quite favorable, with the very high estimated ΔG°_{rxn} of –92 kcal mol^{-1}. Most of the steps have been discussed earlier, but one point is worth reemphasizing. The partitioning of the radical cation between hydroxylation and ring opening is based on the relative rates of oxygen rebound and rearrangement. A similar partitioning exists in the enzymatic mechanism of deacetoxycephalosporin C synthase (DAOCS; Figure 12).

Figure 12



Putative mechanism for deacetoxycephalosporin C synthetase. The molecular rearrangement proceeds faster than non-heme Fe–OH rebound.

Significant evidence suggests this enzyme generates a radical, which must undergo a rearrangement from the penicillin to the cephalosporin skeleton faster than rebound, at the methyl group of the substrate by hydrogen-atom abstraction with an iron–oxo intermediate [38]. The fact that this β-thiyl radical rearrangement, which probably involves less strain relief than the cyclopropane cleavage under consideration here, competes successfully with the rebound step suggests that rebound of Fe^{III}-OH in the non-heme iron coordination environment is slower than in cytochrome P-450. The conversion of a carbon radical to an alkene by Fe^{III}-OH is common to proposed mechanisms for both ethylene biosynthesis and cephalosporin production. It has been suggested earlier that in the prostaglandin (PGH_2–TxA_2) biosynthetic pathway Fe^{III}-OH can convert a radical to a cation. The iron atom in this latter system is thiol-ligated [39], however, which is not believed to be the case in either the EFE or DAOCS. Our revised mechanism for ethylene production uses an iron–alkyl intermediate to avoid the formation of a high-energy, primary carbocation.

The conversion of the Fe^{II} species (presumably the resting state of the enzyme) to the iron–oxo intermediate is key to the enzyme-catalyzed part of the cycle, and involves at the least oxygen and a reductant, produces another molecule of water, and will surely be the focus of future investigations of all the enzymes of this class. Generation of an iron–oxo intermediate in mammalian non-heme iron enzymes such as prolyl hydroxylase has been proposed in the past and may be relevant [40], although these systems are dependent on α-ketoglutarate and ethylene formation is not. The non-heme iron proteins and their ligands are likely to be most important in gathering together the substrate and multiple cofactors/reactants, overcoming entropy and generating the oxidizing species. For this reason, the lessons learned about the activation of oxygen in the EFE may be directly applicable to other enzymes in the family. Furthermore, as Benner and colleagues [41,42] have pointed out, maximizing an enzyme's catalytic rate is not the only solution to the problem of biological throughput in the conversion of a substrate to product. Simply having large amounts of enzyme may be more expedient, and it seems that this is the solution used in plants and microorganisms that use these non-heme iron enzymes in their biosynthetic pathways. Thus, clavaminate synthase comprises a significant fraction of total protein in *Streptomyces clavuligerus*, with the purification factor required to reach homogeneity being 50–90-fold [43], and the purification factor required to obtain homogeneous EFE from apples is only ~30-fold, suggesting that it is also an abundant protein. Consistent with this, Woodson *et al.* [44] have shown that EFE mRNA is highly expressed in generative (reproductive) tissue, as measured by a high proportion (up to 25%) of EFE-homologous cDNAs. The enzyme also 'commits suicide' at a significant rate, as inactivation occurs after relatively few (25–50) substrate turnovers. It is unlikely that inhibiting EFE, even by a mechanism-based inactivator with low or no turnover, would have much effect on the physiology of fruit-ripening. Our studies of 1-aminocyclopropenecarboxylic acid [45], which is a good enzyme inhibitor but has only weak anti-senescence properties, support this idea.

Significance

Ethylene gas is the simplest of the plant hormones, and its dramatic effects on plant senescence have been well documented. A sharp increase in ethylene production is associated with the ripening process in climacteric fruits (such as bananas, pears and tomatoes). A thorough understanding of the mechanisms involved in the production of (and autocatalytic regulation of) ethylene should help to identify potential sites of control of this process, which could provide the opportunity to prevent or delay fruit ripening in a reversible manner.

The final step in ethylene biosynthesis is the conversion of 1-aminocyclopropanecarboxylic acid (ACC) to ethylene by ACC oxidase (also known as the ethylene-forming enzyme, EFE). We studied the processing of an ACC analog, 1-aminocyclobutanecarboxylic acid (ACBC), which is also a substrate of the EFE. We observed that processing of ACBC by the EFE proceeds via ring expansion to produce dehydroproline. This result is consistent with a one-electron oxidation mechanism and inconsistent with a two-electron oxidation mechanism; these facts, in addition to the absence of hydroxylated intermediates, refute the proposed *N*-hydroxylation mechanism previously suggested for ethylene biosynthesis. Instead, our results provide support for an alternative mechanism that involves a radical. This, in turn, may explain why the enzyme is readily inactivated during turnover. It is possible that the short lifespan of the EFE results in plants producing large quantities of the enzyme in ripening fruit. Our results suggest that development of an anti-ripening agent that targets the EFE will be problematic.

Materials and methods

General

[1-^{14}C]-1-Aminocyclobutane-1-carboxylic acid with a specific radioactivity of 53.9 mCi/mmol was obtained from NEN. Cyclobutanone was obtained from Aldrich.

Synthesis of N-*hydroxy-1-aminocyclobutanecarboxylic acid*

Cyclobutanone (5.09 g; 73 mmol) was added to a solution of hydroxylamine hydrochloride (5.55 g, 80 mmol) in 25 ml of water. Over a period of 30 min a solution of sodium cyanide (3.73 g, 76 mmol) in 12.5 ml of water was added with vigorous stirring, and stirring was continued for 72 h. The solvent was then removed *in vacuo* and an unstable light brown oil was obtained (6.45 g, 80%). Electrospray ionization mass spectrometry (ESI–MS); *m/z* 113 (M^++1). A solution prepared from this crude product (6.45 g) and concentrated hydrochloric acid (40 ml) was cooled in an ice bath, allowed to stand for 24 h and warmed to room temperature for another 48 h with stirring. The solution was diluted with 20 ml of water and heated at 80°C for 8 h. The reaction mixture was concentrated by rotary evaporation and then cooled in an ice bath for 3 h. A white solid was collected by filtration, dissolved in 1 N HCl, and run through a Dowex 50W-X8 column. The crude product obtained from the column was recrystallized from water/ethanol (1/3) yielding white crystals (0.9 g, 15%). mp 190–190.5°C (dec.). R_f 0.30 (silica gel, water:*n*-butanol:acetic acid = 5:4:1, silver nitrate and ninhydrin dips). Gas chromatography mass spectrometry (GC/MS); *m/z* 276 (M^++1, TMS derivative). ^{1}H NMR (D_2O): δ 1.84–1.93 (m, 2H), 2.26 (t, J = 8.3 Hz, 4H). ^{13}C NMR (D_2O): δ 15.15, 26.83, 67.53, 176.57. Anal. Calc'd for $C_5H_9NO_3$: C, 45.80; H, 6.92; N, 10.68. Found: C, 45.93; H, 6.98; N, 10.62.

Synthesis of N-*hydroxy-aminoisobutyric acid*

To 17.5 g of acetone (0.3 mol) was added 23.0 g of hydroxylamine hydrochloride in 50 ml of water. To the stirring mixture was added 15.2 g (0.3 mol) of NaCN over a period of 30 min. Stirring was continued for 48 h. The solution was concentrated under high vacuum to ~30 ml and cooled to 10°C. The precipitate was collected by vacuum filtration, washed with 10 ml of ice water, and recrystallized from ether/hexanes to give 14.9 g (55%) of the known hydroxyaminonitrile. ^{1}H NMR (DMSO): δ 7.33 (s, 1H), 5.88 (s, 1H) 1.39 (s, 6H). ^{13}C NMR (DMSO): δ 23.64, 55.8, 122.6. IR (KBr): 3183, 2995, 2235, 1419, 1211, 1058, 987, 761, 572 cm^{-1}. A solution of 5 g (55 mmol) of the hydroxyaminonitrile in 10 ml of conc. HCl and 40 ml of water was heated at 60°C for 6 h. The solvent was removed under high vacuum and the resulting wax was triturated with ethanol. The mother liquor was decanted and kept at −10°C for 18 h. The precipitate was collected by filtration to give the hydroxylamine hydrochloride (3.6 g) mp 170–171°C. ^{1}H NMR (DMSO): δ 1.42 (s, 6H). ^{13}C NMR (DMSO): δ 176.45, 57.89, 23.92. IR: 3350, 2955, 1778, 1559, 1373, 1260, 1185, 871, 769, 564 cm^{-1}. This material was identical to that in the literature [46].

Transformation of [1-^{14}C]-1-aminocyclobutane-1-carboxylate by the EFE

Products. In a scintillation vial was placed 1 ml of 40 mM Tris buffer, pH 7.2 containing 20 μM $FeSO_4$, 10 mM sodium ascorbate, 10 mM sodium bicarbonate and 100 μl [1-^{14}C]-1-aminocyclobutane-1-carboxylate (0.25 mCi in 0.5 ml, 100 μl = 0.927 μmol, final concentration = 0.618 μM). A center tube containing 0.5 ml of aminoethanol and methoxyethanol (1:1) was placed inside the vial [47]. A solution of EFE (0.5 ml, 25 units) purified using our previously described procedure through the Butyl Toyopearl column [3] was added to initiate the enzymatic reaction, which was allowed to proceed for 3 h. The CO_2-absorbing mixture of aminoethanol/methoxyethanol was withdrawn via syringe and injected into a scintillation vial containing 20 ml of scintillation cocktail. After the assay mixture was purged by air for about 1 h, 20 μl was placed in a scintillation vial containing 20 ml of scintillation cocktail. The radioactivity in each vial was counted. The buffer solution was lyophilized overnight. The final residue contained mainly glycerol, about 10% of the original volume. TLC analysis and autoradiography (10 day exposure) in comparison to authentic samples developed using the same solvent system (*n*-butanol:glacial acetic acid:water, 4:1:1; ACBC – R_f = 0.43; *N*-hydroxy-1-aminocyclobutane-1-carboxylic acid – R_f = 0.58; Δ^1-pyrroline-2-carboxylic acid – R_f = 0.23) and stained by ninhydrin identified the product. The only hot spot on the developed film was at the R_f of Δ^1-pyrroline-2-carboxylic acid, indicating that the reaction had gone to completion. A control was run under the same conditions as above, omitting the EFE. Without enzyme, ACBC is stable in assay buffer, as evidenced by 500-fold greater counts in the experiment as compared to control.

CO_2 release. The above experiment was repeated using 2.5 U of EFE. Radioactive 1-aminocyclobutane-1-carboxylic acid solution (100 μl) was divided into two parts, half for use as control and half to which was added 80 μl of EFE solution (about 2.5 U). After incubation overnight, each aminoethanol/ethoxyethanol mixture was withdrawn, injected into a scintillation vial containing 20 ml scintillation cocktail, and counted. A fresh mixture of aminoethanol/ethoxyethanol was introduced into the inside center tube and the enzymatic assay solution was acidified adding 100 μl of concentrated HCl. The resulting solution was incubated for 3 h and the aminoethanol/ethoxyethanol counted. Any CO_2 remaining in the assay solution was trapped in a liquid nitrogen trap and re-absorbed into another fresh aminoethanol/ethoxyethanol mixture and counted. The radioactivities of all three vials from the enzymatic reaction were counted separately and added together to obtain the total CO_2 released from the ACBC reaction catalyzed by the EFE. The radioactivity remaining in the assay solution, which represented any unreacted ACBC and transformed product(s) which did not go through decarboxylation, was also counted. Only 2% of the total radioactivity was released as CO_2, and 88% of the starting radioactivity was recovered in the nonvolatile fraction in which TLC showed only Δ^1-pyrroline-2-carboxylic acid. About 90% of the radioactivity was recovered (total released radioactive CO_2 plus remaining radioactivity in the solution).

Reaction of 1-aminocyclobutane-1-carboxylate with $KMnO_4$

Concentrated NH_4OH (0.050 ml; 29.8%) and 9.5 mg of $KMnO_4$ were added to an aqueous solution (0.8 ml) of 1-aminocyclobutane-1-carboxylate (4.4 mg). The reaction was monitored by TLC using *n*-butanol:glacial acetic acid:water (4:1:1) as a solvent system and ninhydrin as a staining reagent. TLC displayed a brown spot below the starting material ACBC, which was a pink spot. The brown spot corresponded to Δ^1-pyrroline-2-carboxylic acid when compared with authentic TLC data. The reaction mixture was passed through a layer of Celite in a pipette and dried *in vacuo*. ^{1}H NMR data (D_2O) showed the three sets of peaks which were identical to Δ^1-pyrroline-2-carboxylic acid. Neither cyclobutanone itself nor its imine (see below) were produced (based on the ^{1}H NMR data).

Reaction of 1-aminocyclobutane-1-carboxylate with NBS/D_2O

ACBC (4.4 mg) was dissolved in 0.7 ml of D_2O. The ^{1}H NMR spectrum was recorded and 8.8 mg NBS was added. After 10 min, the ^{1}H NMR spectrum was recorded again. These data show that cyclobutanone is produced in this reaction, whose ^{1}H NMR spectrum was readily distinguished from Δ^1-pyrroline-2-carboxylic acid: ^{1}H NMR (D_2O): δ 1.852 (m, 2H); 2.941 (m, 4H); the reaction mixture when spotted and run out on TLC did not give a positive test upon staining with ninhydrin, showing that the product was not the imine.

Reaction of 1-aminocyclobutane-1-carboxylate with NaOCl/NaOH/$HgCl_2$

$HgCl_2$ (10 μmole in 0.1 ml) and 0.1 ml of a cold mixture of 5% NaOCl and saturated NaOH (2:1)were added to an aqueous solution (0.9 ml) of 1-aminocyclobutane-1-carboxylate (3.45 mg, 30 μmole). The reaction was monitored by TLC, which showed a yellowish brown spot (R_f = 0.22) for cyclobutanone imine after staining with ninhydrin. The product evidently hydrolyses to ammonia on the TLC plate, to give a positive color. The spot develops much more quickly than Δ^1-pyrroline-2-carboxylic acid. This spot disappeared if the reaction mixture was acidified before TLC development, because the imine is hydrolysed to the volatile cyclobutanone when the reaction mixture is acidified and ammonia does not move on TLC under these conditions. Under basic reaction conditions, it remains as cyclobutanone imine.

Reaction of 1-aminocyclobutane-1-carboxylate with NaOCl/NaOH

To an aqueous solution (0.9 ml) of 1-aminocyclobutane-1-carboxylate (3.45 mg, 30 μmole) was added 0.2 ml of a cold mixture of 5% NaOCl and saturated aqueous NaOH (2:1). The reaction was monitored by TLC and was complete in 1 min. Two spots of the reaction mixture were spotted on a TLC plate and one of them was acidified by a drop of 20% HCl. The TLC plate was allowed to dry under an air stream. After development and ninhydrin staining, only the spot without acidification displayed a brown color. This indicates that the product from reaction with NaOCl/NaOH is the imine of cyclobutanone.

Reaction of [1-^{14}C]-1-aminocyclobutane-1-carboxylate with NaOCl/NaOH

Stock solution (25 μl; 53.9 mCi/mmol; 0.25 mCi in 0.5 ml) of ACBC was diluted to 250 μl by addition of 225 μl of water. As a control, diluted stock solution (50 μl) was added to 20 ml of scintillation cocktail and 1 ml of a 1:1 mixture of aminoethanol:methoxyethanol and counted. In the experiment, diluted stock solution (100 μl) was chilled in an ice-water bath and 100 μl of a cold mixture of 5% NaOCl and saturated NaOH (2:1) was added. After 3 h, the aqueous layer was acidified by addition of 100 μl of concentrated HCl. The mixture was allowed to stand overnight, the mixture of aminoethanol/methoxyethanol in the center tube was withdrawn, and it was injected into a 20 ml solution of scintillation cocktail in a scintillation vial with a rubber-sealed cap. The radioactivity was counted, showing that 78% of the radioactivity was released as CO_2.

Reaction of [1-^{14}C]-1-aminocyclobutane-1-carboxylate with $KMnO_4$

A diluted stock solution of [1-^{14}C] ACBC (75 μl) was mixed with 25 μl of concentrated NH_4OH (final concentration 3.5%) and 100 μl of aqueous $KMnO_4$ (4.1 mg in 4 ml). The reaction was kept at room temperature overnight. The reaction solutions were acidified to release CO_2, which was trapped in an aminoethanol:ethoxyethanol mixture (1:1) for radioactivity counting, which was not detectable above background. Most of ACBC reacted with NaOCl/NaOH, releasing carbon dioxide. The $KMnO_4$ reaction did not release carbon dioxide. These observations were consistent with the results obtained in the previous experiments monitored by ^{1}H NMR and TLC/ninhydrin.

Supplementary material

Supplementary material available with the on-line version of this paper describes the preparation of an authentic sample of dehydroproline.

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