Bull Soc Chim Fr (1996) 133, 441-455

Review

Chemical and biochemical aspects of the biosynthesis of ethylene, a plant hormone.

Lucien Stella*, Sophie Wouters, Frédéric Baldellon

Laboratoire de chimie moléculaire organique, associé au CNRS, Faculté des sciences Saint-Jérôme, Université d'Aix-Marseille-III, av Escadrille Normandie-Niemen, 13397 Marseille cedex 20, France

(Received 18 December 1995; accepted 12 April 1996)

Summary — Ethylene is a plant hormone which controls numerous aspects of the growth and development of plants. Plants produce ethylene starting from methionine via 1-aminocyclopropanecarboxylic acid (ACC). Two key enzymes take part successively in the course of this transformation: ACC synthase, which is now well known, and ACC oxidase, the mechanism of action and the structure of which are still being studied. In this article we assemble and analyze the chemical and biochemical aspects of the biosynthesis of ethylene and describe studies directed towards the rational design of new classes of regulators of plant growth. These investigations may have commercial importance if they lead to prolonging the viable life of fruit after harvesting or allow the modification of other processes, such as abscission, controlled by ethylene. In humanitarian terms, the impact on agricultural zones lacking refrigerated storage or an effective means of maintaining foodstuffs after harvest may be more important and more worthy of interest. The loss of foodstuffs due to deterioration and mould growth after harvest remains a universal problem both for developed and under-developed countries.

1-aminocyclopropanecarboxylic acid / ethylene / non-heme iron(II)-enzyme / plant hormone

Résumé — Aspects chimiques et biochimiques de la biosynthèse de l'éthylène, hormone végétale. *L'éthylène est une hormone végétale qui contrôle de nombreux aspects de la croissance et du développement des plantes. Les plantes produisent l'éthylène à partir de la méthionine via l'acide 1-aminocyclopropanecarboxylique (ACC). Deux enzymes clef interviennent successivement au cours de cette transformation: l'ACC synthase, maintenant bien connue et l'ACC oxydase dont le mécanisme d'action et la structure sont toujours à l'étude. Dans cet article nous rassemblons et analysons les aspects chimiques et biochimiques de la biosynthèse de l'éthylène qui permettent d'introduire les études actuelles dirigées vers la conception rationnelle de nouvelles classes de régulateurs de croissance des végétaux. Des avantages économiques importants peuvent résulter de ces recherches, si l'on parvient à prolonger la vie des fruits après la récolte ou à modifier d'autres processus contrôlés par l'éthylène, tels que l'abscision. En termes humanitaires, l'impact sur les zones agricoles dépourvues d'installations de stockage réfrigérées, ou de systèmes de manutention efficaces post-récolte, est très sérieux et digne d'intérêt. Les pertes en produits alimentaires résultant d'altération et de pourrissement, après les récoltes, constituent toujours un problème universel à la fois pour les pays développés et non développés.*

acide 1-aminocyclopropanecarboxylique / éthylène / enzyme à fer(II) non-hémique / hormone végétale

Introduction

The different stages of development of plants are controlled by plant hormones which may be divided into five major groups: abscissic acid (a sesquiterpene derivative), auxin (indole-3-acetic acid), the cytokines (aminopurines substituted in the 6-position such as 6-[(4-hydroxy-3-methyl-but-2-enyl)amino]purine, called zeatin), the gibberellins (a large family of tetracyclic diterpenes), and ethylene.

It has long been known that the presence of gaseous compounds can modify the natural growth of plants. In 1864 Girardin [1] made the first scientific observation when he described the premature dropping of leaves from trees situated near a pipe from which gas was escaping. In 1901 Neljubow [2] was the first to show that ethylene, a constituent of gas used for lighting, has effect on the growth of plants. Later Denny [3] showed that ethylene was the active substance in the gas derived from the combustion of hydrocarbons, used for ripening citrus fruits. Finally, in 1934, Gane [4] gave chemical proof of the production of ethylene by ripe apples using techniques of absorption.

Since then, it has been demonstrated that ethylene is produced by practically all parts of plants, notably leaves, shoots, roots, flowers, fruit, and seeds, and it has

* Correspondence and reprints

been shown that this vital hormone is involved in numerous phases of development of plants: germination, embryogenesis, growth of roots, twigs and leaves, abscission and senescence of the leaves, and maturation of the fruits. The synthesis of ethylene is strongly stimulated by exterior damage, be it physical, chemical or environmental, undergone by the plants. Thus ethylene takes part in the reaction to a large number of situations of stress, such as the wounds inflicted by herbivores, insect bites, water or heat damage, the presence of certain chemicals, etc.

Ethylene is of considerable economic interest because it plays a role in several sectors of the agricultural industry, in particular in the control of the maturation of fruits [5], modulation of the growth of shoots and in the control of abscission. A thorough understanding of the means of biosynthesis of ethylene and its regulation is thus indispensable for the control of these processes. A certain number of general works [6–8] and reviews [9–11] deal with the physiological effects of ethylene in the plant world. The object of our review is to assemble and analyze the chemical and biochemical aspects and particularly the mechanisms of the biosynthesis of ethylene. The essence is as follows:

The biosynthesis of ethylene takes place by two major transformations. In the first, now well known, methionine is transformed by the action of ATP to *S*-adenosylmethionine which, under the influence of a key enzyme, ACC synthase, leads to 1-aminocyclopropanecarboxylic acid (ACC). In the second step ACC is transformed into ethylene under the action of a second key enzyme. Until 1991 most of the data concerning the enzyme, then named the 'ethylene-forming enzyme' (EFE), were obtained using tissue material. This method has been well rewarded if one considers the limits of the method employed. The model in vitro systems reported until then were, for the most part, artifacts in the measurements, as they were incapable of recognizing the four stereoisomers of a substituted ACC, 1-amino-2-ethylcyclopropanecarboxylic acid, which they transformed without discrimination (see later: *Stereochemical aspects*). These systems also gave values of K_M for ACC considerably larger than the value estimated for the enzyme from in vivo studies [12]. In a good example of inverse biochemistry, where one begins with the gene to isolate the enzyme, we will describe the discovery of this enzyme, henceforth called ACC oxidase. This marks the beginning of a new era for the in vitro study of the mechanism of transformation of ACC.

Path of biosynthesis of ethylene

Discovering the precursors of ethylene has not been an easy matter. Indeed, vegetable matter such as apple or tomato, which produces elevated quantities of ethylene, is no longer active when it is homogenized and a cellular extract obtained. Progress in this area has thus come from studies in which a labeled potential precursor has been administered to the plant and its conversion to ethylene established. However, because of its very simple structure, ethylene may be evolved starting from a large number of compounds by various chemical reactions. Thus linoleic acid, propanal, β-alanine, acrylic acid, β-hydroxypropionic acid, ethionine, ethanol, ethane, acetic acid, fumaric acid and methionine, among others, have been proposed [13]. It is now firmly established that only methionine is the effective precursor of ethylene in the higher plants.

Methionine as intermediate

The manner by which it has been shown that methionine is the precursor of ethylene is indeed noteworthy. Cellular extracts do not produce ethylene; Lieberman and Mapson [14] thus directed their studies towards model systems. They discovered that linoleic acid peroxide produces, in the presence of cuprous salts and ascorbic acid, ethane, ethylene and other hydrocarbons. For the purpose of testing the participation of radical intermediates they tried to inhibit the reaction by addition of methionine, often used as a radical trap. Contrary to the expected results, the production of ethylene was found to be strongly enhanced. It then became apparent that even in the presence of linoleic acid peroxide, methionine could serve as a precursor of ethylene in the system Cu^+/ascorbate. Shortly after the discovery of Lieberman et al [15], another chemical system also using methionine was developed by Abeles and Rubinstein [16]. These authors observed that ethylene can be formed in a non-enzymatic manner from crude extracts of young pea plants in the presence of flavin mononucleotide. Soon thereafter, Yang and colleagues [17] identified methionine as an active substrate in these extracts, and demonstrated that it is transformed via a radical reaction initiated by light and obeying the following equation where the numbering of the carbon atoms indicates their fate:

$$\overset{5}{C}H_3\text{-}S\text{-}\overset{4}{C}H_2\text{-}\overset{3}{C}H_2\text{-}\overset{2}{C}H(NH_2)\text{-}\overset{1}{C}OOH \rightarrow$$

$$H_3\overset{5}{C}\text{-}S\text{-}\overset{5}{C}H_3 + \overset{4}{C}H_2{=}\overset{3}{C}H_2 + NH_3 + \overset{2}{H}\overset{1}{C}OOH + \overset{1}{C}O_2$$

Direct proof of the role of methionine as the in vivo precursor of ethylene was obtained by administration of labeled methionine to an apple [18]. It was observed that the ability of plant materials to cause this transformation is in line with its ability to produce endogenous ethylene, and that the radioactivity in the recovered ethylene had a specific activity close to that of the methionine administered [13, 19]. These results show that methionine is the major, if not the unique, precursor of ethylene. However, in certain tissues exposed to light, particularly in the presence of Cu^+, non-physiological evolution of ethylene can result from lipid peroxidation [20]. It is thought that this phenomenon may explain the ancient practice of increasing the maturation of figs by the application of olive oil to these fruits [21].

In chemical systems, as in the plant material, carbon atoms 3 and 4 of methionine are the source of the ethylene, while carbon 1 leads to CO_2. From these data it appeared that the mechanisms of production of ethylene in vivo and in the chemical systems were similar. It has turned out that this is not the case and that these

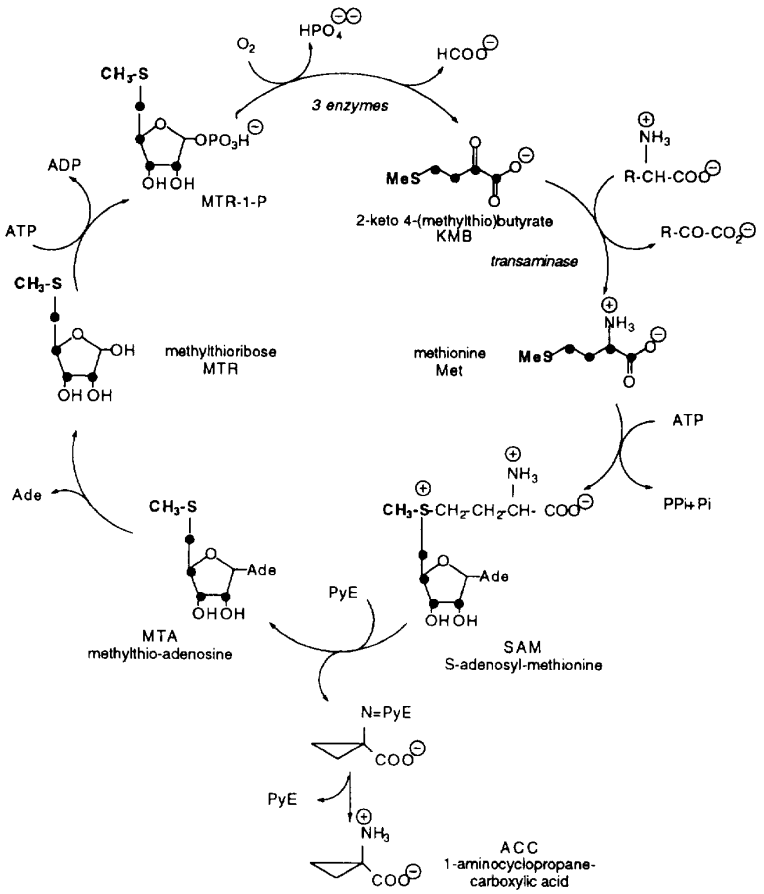

Fig 1. The methionine cycle.

systems are not good models. In fact, in the chemical systems alluded to, ethylene is formed from methionine by way of methional ($CH_3SCH_2CH_2CHO$) and the methylthio group gives rise to volatile dimethyl disulfide (CH_3SSCH_3). Conversely, in apple tissue methionine and not methional is the precursor of ethylene and the methylthio group is not transformed into a volatile compound but remains in the plant material [22].

The methionine cycle

The quantity of methionine in slices of apple is too small to support the normal production of ethylene. Baur and Yang [23] thus assumed that methionine is recycled. This is in agreement with the observation that no volatile sulfur compounds are produced after the administration of labeled (^{35}S)-methionine to apple [22].

The first step in the path of recycling (see fig 1) is the cleavage of methylthioadenosine (MTA). In plants and in many microorganisms MTA is hydrolyzed by MTA nucleosidase to form methylthioribose (MTR) and adenosine [24–29]. In a slice of apple or an extract of tomato the enzyme is so active that MTA is hydrolyzed as soon as it is formed [24, 29]. In animal tissues the phosphorolytic cleavage of MTA leads to the formation of MTR-1-P [30]. MTR-1-P and not MTR is oxidized to 2-keto-4-(methylthio)butyrate (KMB). MTR kinase, which catalyzes the ATP-dependent phosphorylation of MTR to MTR-1-P has been identified in plants [31, 32]. The plant enzyme has a molecular weight of 70 kDa, requires the presence of divalent metal ions (Mg^{2+}, Mn^{2+}) and gives K_M values of 4.3 and 8.3 μM for MTR and ATP respectively. Kushad et al [33] have shown that MTR is transformed to KMB by cellular extracts of avocado solely in the presence of ATP, but that MTR-1-P is metabolized to KMB even in the absence of ATP. This result indicates that MTR is first phosphorylated by MTR kinase before being further transformed. Trackman and Abeles [34] have shown that MTR-1-P is converted to KMB and 2-hydroxy-4-(methylthio)butyrate with stoichiometric consumption of oxygen and formation of formic acid. At least three enzymes are implicated in this transformation which takes place in three successive steps. First MTR-1-P is isomerized to methylthioribulose-1-P in the presence of a protein fraction. Two unidentified intermediates are then formed in the course of a reaction catalyzed by a second protein fraction. The addition of a third protein fraction is accompanied by the consumption of oxygen and the conversion of the two intermediates into KMB. Finally KMB is transaminated to methionine [35].

S-Adenosylmethionine as intermediate

The conversion of methionine to ethylene requires oxygen and is inhibited by 2,4-dinitrophenol, a decoupler of oxidative phosphorylation. On this basis, certain authors [36, 37] have proposed that *S*-adenosylmethionine (SAM), synthesized by methionine adenosyl transferase from methionine (Met) and adenosine triphosphate (ATP), is one of the intermediates in the biosynthesis (fig 2). Experimental proof has been provided by Adams and Yang [24]. These authors have shown that labeled methionine administered to apple tissue gives rise in parallel to ethylene, methylthioadenosine (MTA) and its product of hydrolysis methylthioribose (MTR) (vide supra: fig 1). Since the production of ethylene is inhibited by aminoethoxyvinylglycine (AVG), neither MTA nor MTR are detected in the tissue. Green apples, which do not produce ethylene, are not capable of producing MTA or MTR from introduced methionine.

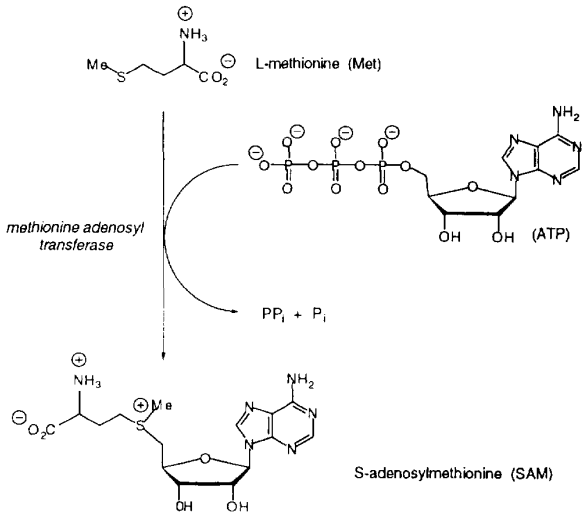

Fig 2. The methionine activation.

1-Aminocyclopropanecarboxylic acid as intermediate

It has long been known that in a nitrogen atmosphere, apples [38] and pears [39] cease production of ethylene, but when one reexposes the tissue to air a burst of ethylene production occurs. The observations can be interpreted as the accumulation of an intermediate during the anaerobic incubation and its subsequent transformation on exposure to oxygen. Thus in the presence of air methionine is transformed efficiently to ethylene, while under nitrogen it is transformed into methylthioribose (MTR) and a substance later identified as 1-aminocyclopropanecarboxylic acid (ACC). In the presence of air, ACC is rapidly transformed into ethylene. This indicates that ACC is an intermediate and that the transformation of ACC into ethylene depends on oxygen. These facts together allow the proposal of the following sequence for the pathway of biosynthesis of ethylene in apple tissue [25]:

methionine → SAM → ACC → ethylene

Although AVG, which is a well-known inhibitor of reactions of enzymes involving pyridoxal phosphate [40], strongly inhibits the conversion of methionine to ACC, it does not block the transformation of methionine to SAM or that of SAM to ethylene. This indicates that AVG inhibits the transformation of SAM to ACC and that it is catalyzed by a pyridoxal phosphate enzyme [25] (fig 3).

Mechanism of formation of ACC

The transformation of SAM to ACC and MTA is a typical α,γ-elimination (1,3-elimination). It is known that a pyridoxal phosphate enzyme facilitates, by nucleophilic activation, the removal of the proton on the α-carbon of an amino acid [41] (fig 3). The previous formation of the sulfonium ion (fig 2) thermodynamically destabilizes the SAM molecule by electrophilic activation. This in turn facilitates the intramolecular nucleophilic substitution reaction leading to ACC and to MTA (fig 3). By using SAM compounds with stereospecific deuterium labeling on carbon atoms 3 and 4, Boller and Arigoni et al [42] have proved that the mechanism of formation of ACC is via direct nucleophilic substitution of the sulfonium group accompanied by a complete inversion of the configuration of carbon atom 4 (fig 4). ACC was isolated for the first time in 1957, simultaneously by Burroughs [43] and by Vätähalo and Virtanen [44].

ACC synthase, first key enzyme

Shortly after the pathway of biosynthesis of ethylene had been determined, Boller et al [45] showed that cellular extracts prepared from tomato are able to transform SAM to ACC. These authors also showed that this enzyme is soluble and strongly inhibited by AVG as had been foreseen by Adams and Yang [25]. The K_M for SAM was estimated as 13 μM. Using labeled SAM, Yu et al [29] confirmed that it is transformed to ACC and MTR by an enzyme preparation obtained from tomato; MTA is considered to be the primary product of the reaction because the enzyme preparation is rich in very active MTA nucleosidase which rapidly hydrolyzes MTA to MTR. Additionally these authors showed that ACC synthetase is activated by low concentrations of pyridoxal phosphate but that it is strongly inhibited by (aminooxy)acetic acid (AOA), another well known inhibitor of pyridoxal enzymes [29]. These observations support the hypothesis that ACC synthetase is a pyridoxal phosphate enzyme. Its molecular weight has been estimated at 55–58 kDa [46, 47] and its optimum pH is 8.5 [29, 45]. ACC has been found in various plant sources including the apple, in which its activity increases considerably during the course of maturation [48]. The induction of ACC synthase has been shown to occur on wounding, during senescence of the flowers and leaves and also after treatment with lithium chloride or phytohormones such as auxin, the cytokines and ethylene. Following partial purification of the enzyme, a monoclonal antibody against tomato ACC synthetase

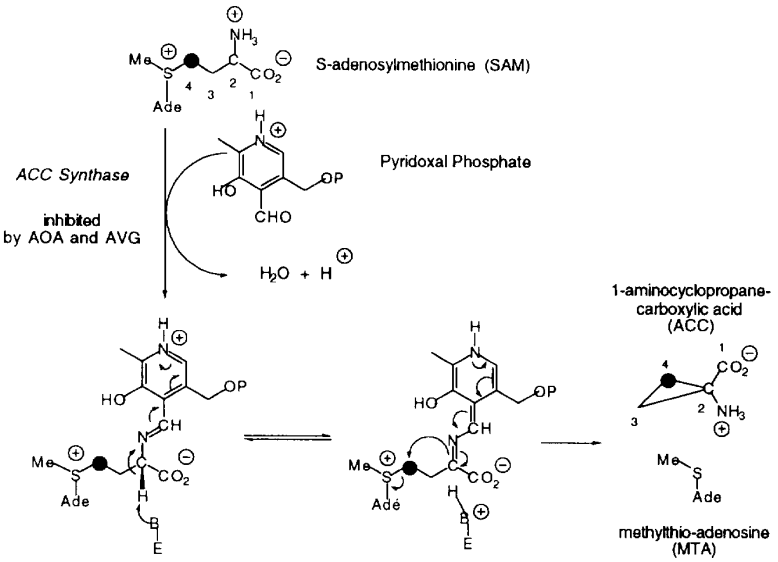

Fig 3. The formation of 1-aminocyclopropanecarboxylic acid (ACC).

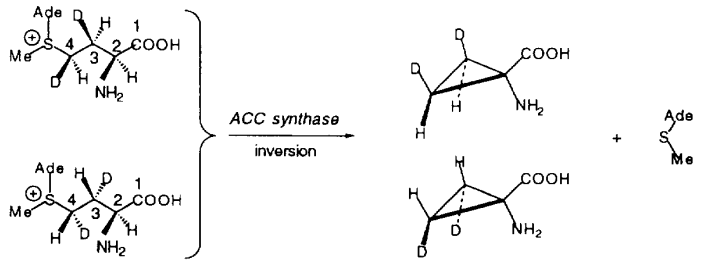

Fig 4. Stereospecific transformation of *S*-adenosylmethionine.

has been obtained [49]. This tool has led to the finding that the protein undergoes a post-translational modification, explaining the difference in molecular weight between the product of in vitro translation (56 kDa) and that of the enzyme isolated in vivo from a plant extract (50 kDa). This proteolysis of the native protein has been confirmed by studies on other plants [50–52]. The existence of several isoforms of ACC synthetase has been considered since it has been shown that the anti-ACC synthetase antibody from wounding does not cross with those obtained against the enzyme induced by auxin [53].

ACC synthetase is inhibited by its substrate following binding between the enzyme site and the 2-aminobutyric acid residue of SAM. The enzyme in fact catalyzes two reactions: the formation of ACC by α,γ-elimination and that of vinylglycine by β,γ-elimination [54]. Vinylglycine binds covalently in the enzyme site, thus irreversibly inhibiting its activity [55]. This property of the enzyme has been exploited to mark the protein with its substrate, then to isolate after partial trypsin digestion a dodecapeptide of sequence SLSKDLGLPGFR corresponding to the active site [56]. It is noteworthy that the first four amino acids of this sequence are characteristic of all enzymes of the known sequence functioning with pyridoxal phosphate as coenzyme.

Conversion of ACC to ethylene

ACC oxidase is the second key enzyme in the biosynthesis of ethylene. Several enzymes and enzyme systems are, however, capable of transforming ACC to ethylene: isolated microsomes [57, 58], peroxidase [59], indoleacetic acid oxidase [60, 61], and lipoxygenase [60–62]. These reactions frequently cause intervention of hydroperoxides or formation of hydrogen peroxide [65, 66]. These enzymes do not always possess the characteristics of ACC oxidase (K_M in the micromolar range with respect to ACC, and stereospecificity with regard to the transformation of ethyl-ACC to 1-butene; see below). Additionally they only partially convert ACC to ethylene, giving rise to other compounds which have not yet been identified [64]. As we have already indicated, the production of ethylene can also result from chemical oxidation of methionine, linoleic acid or ACC [13, 67, 68].

ACC oxidase in vivo

The rapid progress which has been made with ACC synthetase is in contrast to the status quo which has long characterized the situation for ACC oxidase. This latter enzyme, previously called EFE, has not yet been iso-

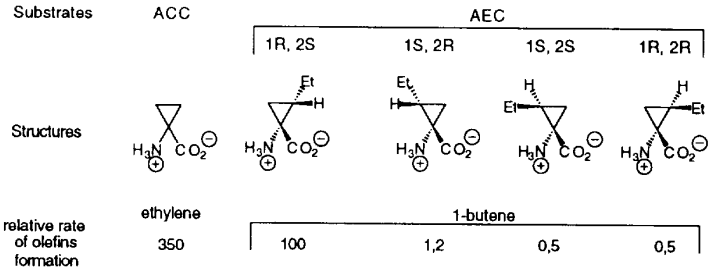

Fig 5. Stereospecificity of ACC oxydase for 2-ethyl-ACC.

lated from cell-free systems. Measurements of its activity have been made on tissue fragments. This method, nevertheless, has allowed the discovery of a number of enzyme effectors.

The apparent K_M of the enzyme with respect to ACC has been determined for numerous plant materials and varies from 8 to 500 μM. To explain the heterogeneity of these results, several authors have considered that it may be due to differential compartmentation of ACC depending on the plant material. The K_M of ACC oxidase with respect to oxygen also varies widely among these studies [69–71]. There is a correlation between the apparent K_M of oxygen and the concentration of ACC in the tissues studied [72], the affinity of ACC oxidase with respect to oxygen being greater when the concentrations of ACC are elevated. A sequential order of binding on ACC oxidase has been suggested, oxygen binding before ACC. Iron is a cofactor or cosubstrate essential for the conversion of ACC to ethylene. Numerous inhibitors of ACC oxidase active in different plant materials are known, and serve to discriminate among the various pathways of production of ethylene. α-Aminoisobutyric acid and inorganic ions such as those of cobalt and nickel inhibit the biosynthesis of ethylene without affecting the activity of ACC synthetase [73]. These cations probably act as competitors for iron. It is considered that free radical traps such as n-propyl gallate inhibit production of ethylene by various plant materials [74–76]; however Dilley et al have recently shown [77] that n-propyl gallate inhibition is due to the chelation of iron, and that it can be countered by addition of iron to the medium. The difficulties of extraction and the role of the modifiers described above have led to the hypothesis that EFE consists of an enzyme complex linked to the membranes and requiring total membrane integrity for its activity.

Stereochemical aspects

ACC is an achiral molecule, but ACC oxidase can also cause olefin formation from 1-amino-2-ethylcyclopropanecarboxylic acid (AEC) which can occur in the form of two couples of diastereoisomers (fig 5). This molecule is of interest because ACC oxidase transforms preferentially the (1R, 2S) diastereoisomer [78]. This is the only active form that causes competitive inhibition of the conversion of ACC to ethylene. This stereospecificity provides one of the surest criteria for differentiation of the ACC oxidase activity from all other transformations by chemical or non-specific enzymic pathways.

This ability of ACC oxidase to discriminate among the four enantiomers of 2-ethyl-ACC has led Yang to propose a model of the active site of ACC oxidase [78]. The first design was subsequently completed following the results obtained by Pirrung et al [79] using 2-methyl-ACC (fig 6). This includes an upper part (roof) which interacts with the alkyl groups in the 2-position, a binding site X which interacts with the carboxylate function, a basic site B well placed to deprotonate the aminium function, and an oxidant situated close to the nitrogen atom. More recently [80], other substituted analogues of ACC have been prepared and studied as inhibitors of ACC oxidase in order to refine the topography of the active site.

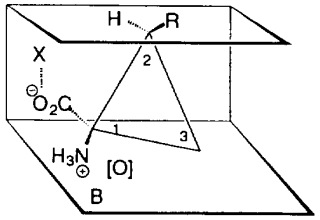

Fig 6. Topography of the ACC oxidase active site.

Mechanism of formation of ethylene

Studies on the products of reaction of ACC oxidase have been decisive in our understanding of the chemical reaction catalyzed by the enzyme. Based on the fact that 1-phenylcyclopropylamine is chemically oxidized by hypochlorite to ethylene and benzonitrile by way of 1-phenyl-N-hydroxycyclopropylamine [81], Yang in 1981 suggested that EFE is an ACC hydroxylase which catalyses the oxidation of ACC to N-hydroxy-ACC [82], a precursor of the nitrenium ion (fig 7). This intermediate would then undergo fragmentation to produce ethylene and cyanoformic acid. The latter would spontaneously decompose to hydrogen cyanide and carbon dioxide. This hypothesis seems to be bolstered by the observations of Peiser et al [83]. These authors have studied the metabolism of labeled radioactive ACC in various plant tissues. They have shown that the carboxyl carbon of ACC is liberated as carbon dioxide, while carbon 1 of ACC is found, not as free hydrogen cyanide, but in the masked form of β-cyanoalanine in a quantity equivalent to the ethylene produced.

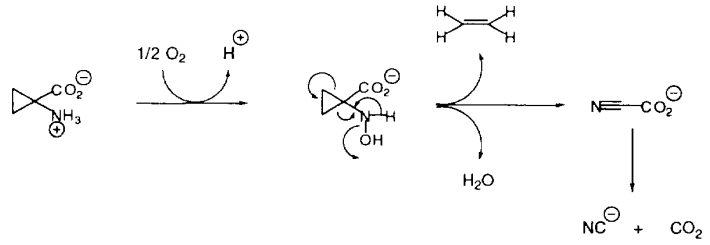

Fig 7. The mechanism of ACC oxidation suggested by Yang [82].

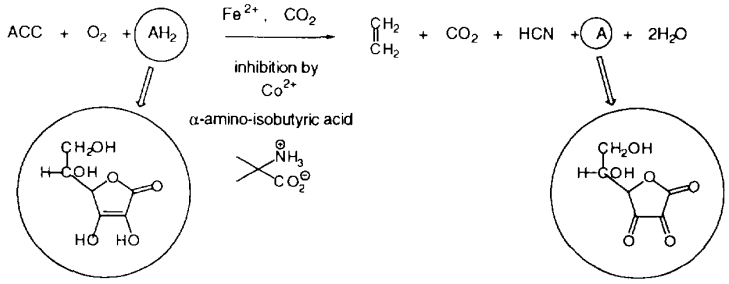

Fig 8. The stoichiometry of ACC oxidation.

Given that hydrogen cyanide is rapidly metabolized to β-cyanoalanine by the same plant tissues, these results are interpreted as proof that hydrogen cyanide is the primary product from carbon 1 of ACC in the course of the biosynthesis of ethylene. Using purified enzyme, Yang et al [84] have shown that for each mole of ACC consumed, one mole of dioxygen is utilized and that equimolar quantities of dehydroascorbate, ethylene, hydrogen cyanide and carbon dioxide are formed. The stoichiometry of the oxidation of ACC to ethylene, catalyzed by ACC oxidase, can thus be represented as shown in figure 8.

Baldwin and colleagues [85] have shown that 2,2,3,3-tetradeuterio ACC is transformed into tetradeuterioethylene by apple tissues. This proves that the oxidation step in the biosynthesis of ethylene proceeds without affecting the carbon–hydrogen bonds of the cyclopropane ring of ACC. Transformation by apple, be it of an equimolecular mixture of the two isomers of *cis*-2,3-dideuterio-ACC, or of (±)-*trans*-2,3-dideuterio-ACC, produces in each case a 1:1 mixture of *cis* and *trans* 1,2-dideuterioethylene [86]. In contrast, the chemical oxidation by sodium hypochlorite of these substances proceeds with total retention of configuration (fig 9). These results indicate that the biological reaction does not proceed by a concerted mechanism but via a mechanism including steps which lead to a loss of configuration of the carbon atoms 3 and 4.

The stereochemical results of the biosynthetic reaction can be reproduced exactly [87] by oxidizing ACC or AEC by various transition metal ions such as Cu^{2+}, MnO_4^- and FeO_4^{2-}. In these reactions the site of oxidation is the amino group. This is indicated by the absence of reaction in acid medium, and by the fact that cyclopropylamine gives ethylene with cupric sulfate while neither *N*-ethoxycarbonyl-ACC nor cyclopropanecarboxylic acid produce ethylene when oxidized under the same conditions.

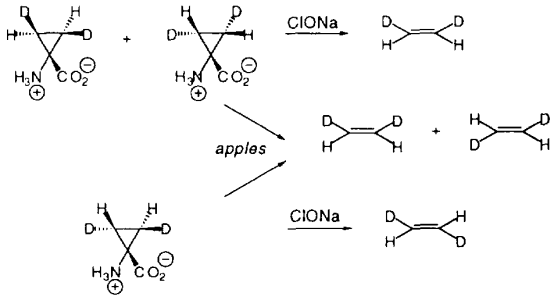

Fig 9. Chemical oxidation, but not biochemical oxidation, is stereospecific.

Radical-cation aminium, nitrene or nitrenium cation?

The clear distinction between, on the one hand, oxidation by hypochlorite and, on the other hand, biological oxidation and oxidations by metal ions, can be explained on the basis of two different mechanisms which operate respectively in the two cases (fig 10). The first oxidation implies a nitrene A and simultaneous cleavage of the two carbon–carbon bonds accompanied by retention of stereochemistry, while the latter oxidations imply a radical cation B which leads to a rapid opening of the ring and rotation of the acyclic intermediate.

While the primary *N*-chloroamine, the presumed precursor of nitrene A in the oxidation by hypochlorite, has never been characterized, similar species have been seen. Thus, peracid oxidation of the imine represented in figure 11 leads to an oxaziridine which decomposes at −40 °C to give ethylene, *para*-methoxybenzaldehyde and methyl cyanoformate. Starting from *cis*-2,3-dideuterio-ACC, only *cis*-1,2-dideuterioethylene is obtained. These results are explained as a decomposition of the oxaziridine to nitrene followed by stereospecific

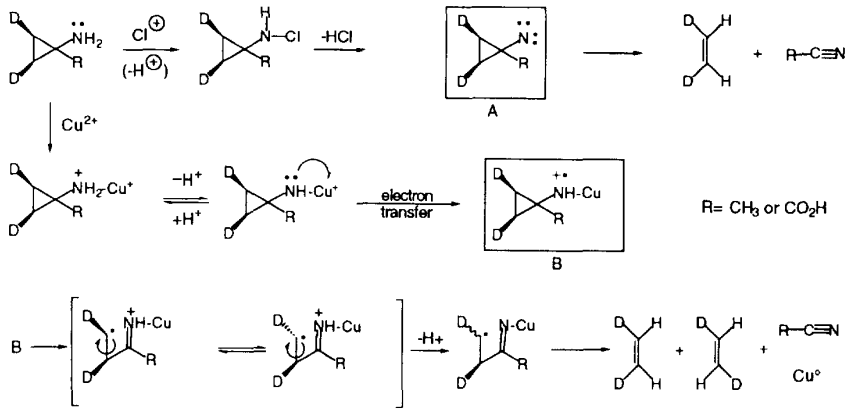

Fig 10. Two different mechanisms can operate.

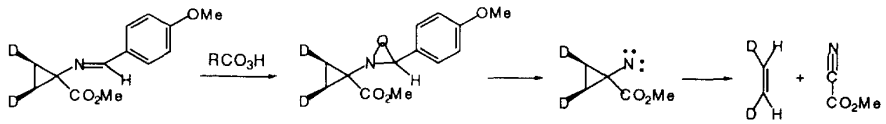

Fig 11. Nitrene rearranges stereospecifically.

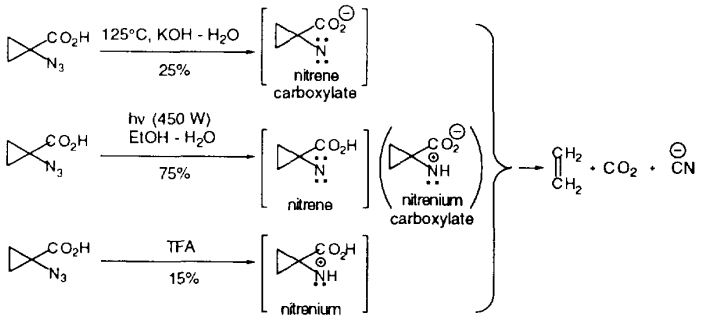

Fig 12. Nitrene or nitrenium intermediates.

rearrangement of the latter to ethylene with retention of stereochemistry [87].

In order to evaluate the merits of the mechanism proposed by Yang (fig 7), Pirrung [88] examined the possibilities of intervention of nitrene and nitrenium ions; the latter was envisaged even though the state of protonation of the nitrene intermediate is not known at the pH optimum for the biosynthesis of ethylene. The decomposition of 1-azidocyclopropanecarboxylic acid (fig 12), both thermally and by photochemical means, proceeds by way of the nitrene intermediate and leads to ethylene, carbon dioxide and the cyanide ion.

Kinetic analysis of the photochemical reaction shows that these three products are formed at the same rate. First order kinetics are observed with a rate constant of 2.2×10^{-4} s^{-1}. The same rate is observed whether it be in neutral or basic solution, which suggests that the reactive species in both cases is the nitrene and not the zwitterionic form of the nitreniumcarboxylate. This suggests that the pK_a of the nitrenium ion is less than that of the cyclopropanecarboxylic acid ($\sim$ 5). Acid-catalyzed decompositions going by way of the nitrenium ion either as a singlet (TFA, AcOH) or a triplet (TFA, $CHBr_3$), occur at a much greater rate ($\sim 1.5 \times 10^{-2}$ s^{-1}). These fragmentation reactions cannot be classified among the reactions labeled 'chelotropic' [89], but among those recently named 'coarctate' [90, 91].

Coarctate transition state

The term 'cheletropic' is actually incorrect for this reaction, which is not even pericyclic since there is no cyclic rearrangement of bonds (which is Hoffmann's original definition). In pericyclic reactions (see fig 13), only one bond is made and one bond broken at each atom involved in the reaction, or, as in chelotropic reactions, two bonds are made or broken respectively at the expense or in favor of forming a lone pair [89]. In coarctate reactions there is at least one atom at which two bonds are made and broken simultaneously [90].

With the qualitative topological analysis of Herges [90], the stereochemistry of this fragmentation is easily understood without any calculation. The elimination of

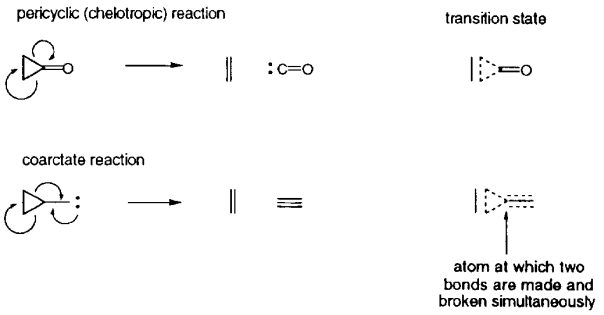

Fig 13. Cheletropic vs coarctate reactions.

ethylene from ACC nitrene is isoelectronic with cyclopropylcarbene fragmentation, which can be viewed as the prototype of these elimination reactions, and which has been well investigated both experimentally and theoretically. Following the coarctate transition state description, we can use a similar drawing (fig 14) to that used for the cyclopropylcarbene fragmentation [92]. There are six delocalized electrons in a Hückel coarctate transition state: two electrons in the π-system of the two carbons which will form ethylene, two at the central cyclopropyl carbon (at which two bonds are made and broken simultaneously) in two orthogonal p-orbitals, and two in the nitrene lone pair. The delocalized system of electrons in the transition state is thus topologically equivalent to or isoconjugate with the π-system of the aromatic cyclopentadienyl anion. The reaction is therefore thermochemically favored. In contrast to coarctate chemical oxidations of ACC, the biological ethylene formation from ACC is certainly not a concerted process.

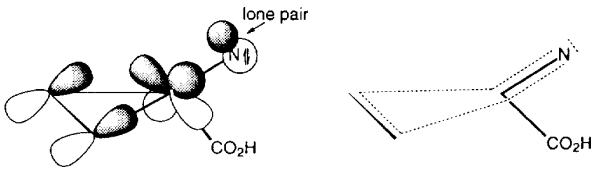

Fig 14. The coarctate transition state.

ACC oxidase functions by successive transfers of one electron

The stereochemical results clearly indicate that the biological oxidation of ACC does not take place in a concerted manner, but by successive steps as indicated in figure 10 under the action of metallic ions. In support of this hypothesis and paralleling the works of Baldwin et al, Pirrung examined the behavior of ACC and related compounds when they are submitted to electrochemical oxidation [93]. Thus, cyclopropylamine and cyclopropanecarboxylate anion are oxidized in an irreversible manner at very similar potentials (+1.1 V with respect to a calomel electrode). The carboxylate anion of ACC is oxidized at a lower potential (+0.7 V). In these conditions ACC carboxylate forms ethylene as does, with lesser yield, cyclopropylamine. Under the same electrochemical conditions, the oxidation of *cis*-2,3-dideuterio-ACC leads, as in the biological case, to a mixture of *cis* and *trans* stereoisomers of 1,2-dideuterioethylene (fig 15).

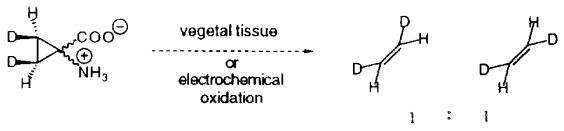

Fig 15. Biochemical oxidation, like electrochemical oxidation, is not stereospecific.

The mechanism which is suggested by these results, common to the natural system and the electrochemical model, is represented in figure 16. The first oxidation is by transfer of one electron leading to the aminium radical cation which is analogous to the cyclopropylaminyl radicals studied by Ingold [94, 95]. These species undergo a very rapid ring opening. It was therefore supposed that, after deprotonation of the aminium cation, a second single-electron oxidation could lead to a diradical cation which gives ethylene and cyanoformic acid by β-fragmentation. Decarboxylation of the latter compound gives, as in the natural system, carbon dioxide and the cyanide ion. Incubation of apple tissue with *trans*-cyclopropyl-ACC (fig 17) leads to a slow release of 1,4-pentadiene [96]. Treatment of the same substrate with sodium hypochlorite gives only the vinylcyclopropane. From the point of view of the mechanism, this study provides additional proof of the sequential one-electron transfer processes in the path of the biosynthesis of ethylene. Indeed the opening of the aminium cation radical resulting from the first single-electron transfer leads to a carbon radical of the cyclopropylmethylene type often used as a 'rapid radical clock'. If the second step of oxidation by single-electron transfer was more rapid than the rearrangement reaction which leads to a second ring opening, one would thus obtain a carbocation which, as in the case of oxidation by hypochlorite, would evolve towards the formation of vinylcyclopropane. The result obtained in this study shows that the second step of single-electron transfer has a rate constant less than that of opening of the methylene cyclopropyl radical (10^8 s^{-1}). Other radical clocks should be used if one wishes to determine more precisely the lifetime of the open intermediate.

Structural and theoretical studies have been carried out by Pirrung [97] by means of semi-empirical methods of molecular orbital calculation. The qualitative results which have been obtained can be summarized as in figure 18, which completes the two previous figures. For the electrochemical model, as apparently for the biosynthetic reaction, the formation of the aminium radical cation constitutes the rate determining step. The presence of the intermediate **3**, essential for the proposed mechanism, is deduced from the previous study. It is also deduced from the studies using isotope labels [93]. The second single-electron transfer, which has been postulated as starting from the nitrogen atom, appears in the light of the calculations to be more probable for the transformation of the carbon radical to the carbocation. The enzyme oxidation step of radical **4** presents a problem of displacement of about 3 Å of

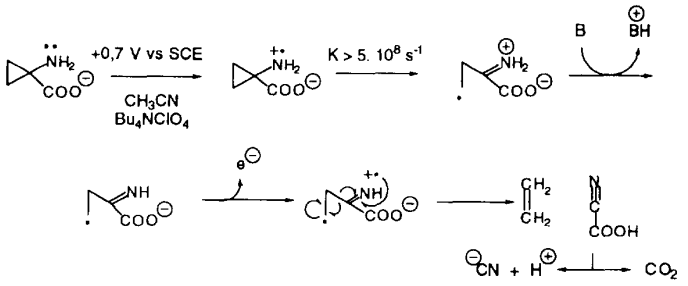

Fig 16. Mechanism for electrochemical and biochemical oxidations.

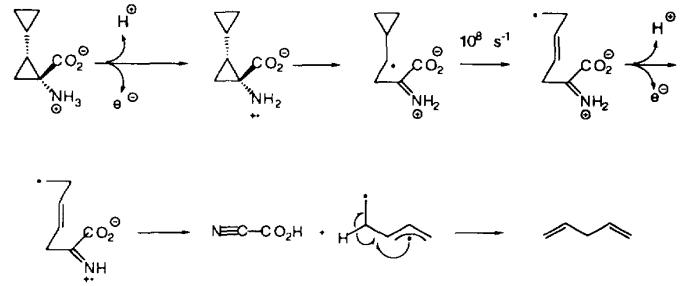

Fig 17. Mechanism from a 'rapid radical clock' study.

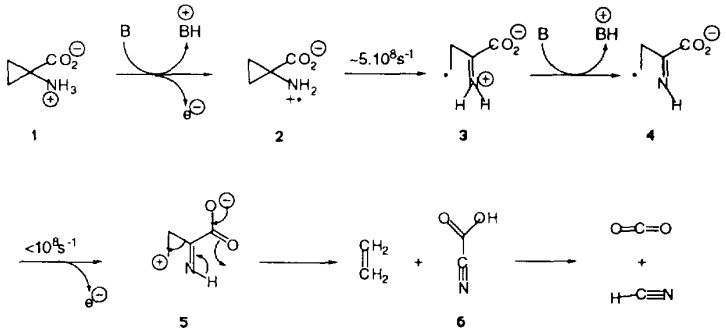

Fig 18. Mechanism from structural and theoretical studies.

the first site of oxidation with respect to the second, but presents the advantage of a thermodynamic gain already entirely paid. Cyanoformic acid **6** has never been described, except in error. In the biosynthesis of ethylene it would provide an attractive intermediate as source of carbon dioxide and of hydrogen cyanide. Its calculated heat of formation (−55 kcal/mol) suggests an exothermic reaction greater than 5 kcal/mol for its transformation to these compounds.

ACC oxidase in vitro

The discovery of the nature and properties of ACC oxidase has been the result of important advances realized in the domain of molecular biology of fruit maturation. Among the climacteric fruit such as the tomato, the process of maturation is preceded by a spectacular increase in respiration and in the synthesis of ethylene. This phenomenon, called climacteric crisis, is accompanied by changes in the expression of the genes which appear to be at the origin of the process of maturation [98, 99]. Several cDNA clones corresponding to mDNA which increase during maturation have been isolated [100], but only those coding for polygalacturonase have been identified [101]. With the aim of isolating the genes implicated in the biosynthesis and response to ethylene, clones expressed concurrently during maturation and wounding have been obtained in the knowledge that the two phenomena strongly induce ethylene synthesis [102]. This strategy has led to the isolation of a clone named pTOM13, which satisfies these criteria and which is found, in addition, to be strongly expressed in senescent leaves [103]. The sequencing of clone pTOM13 [104] and corresponding genomic clones [105] has not provided any information on the function of the protein coded by this family of genes. The role of clone pTOM13 has more recently been studied using the strategy of *anti*-sense RNAs.

Some tomato plants have been transformed by a Ti-modified plasmid of *Agrobacterium tumefasciens* carrying an anti-sense cDNA construction pTOM13 which

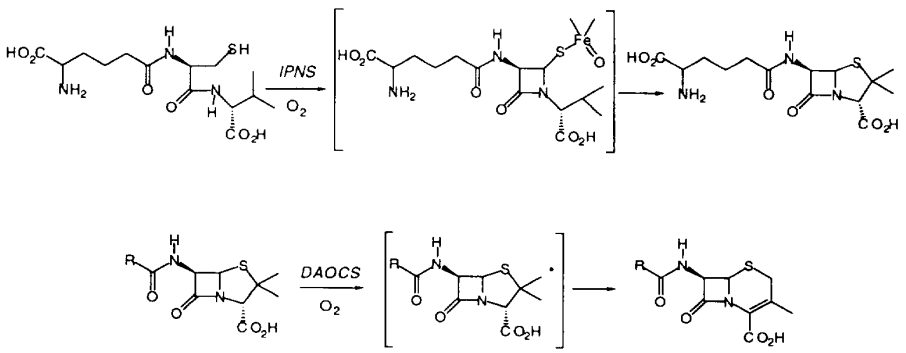

Fig 19. Isopenicillin-*N*-synthase and diacetoxycephalosporin-*C*-synthase.

depends on the promoter CaMV 35S of the cauliflower mosaic virus [106]. In the transgenic plants so obtained, the synthesis of ethylene as well as the accumulation of mRNA corresponding to the clone pTOM13 are strongly reduced, thus implicating the clone pTOM13 in the production of ethylene. Since there is a lack of homology of this sequence with the known genes of ACC synthetase, the authors have proposed the hypothesis that this clone codes for ACC oxidase. The function of the protein has definitively been determined by heterologous expression of cDNA pTOM13 in organisms which are normally incapable of producing ethylene from ACC. The two cellular models used in the course of these experiments were the yeast *Saccaromyces cerevisiae* [107] and oocytes of the frog *Xenopus* [108]. In contrast to the wild types, the transformed yeast was capable of converting ACC to ethylene in the same biochemical manner as the plant, and notably of discriminating among the different stereoisomers of AEC a substituted analog of ACC. These results, obtained in very different expression systems, constitute direct proof that a polypeptide of 35 kDa coded for by a unique gene is capable by itself of converting ACC to ethylene. This discovery is inconsistent with the idea of a multi-enzyme complex associated with the cell membrane, which had previously held favor.

An important observation was made in 1990 by Hamilton [106] who commented that the sequence of amino acids deduced from cDNA pTOM13 [107, 108] showed homology with that of flavone-3β-hydroxylase [109], considered to be a non-heme iron protein [110], as well as with other known hydroxylases [106, 111]. The known instability of such enzymes suggested a reasonable explanation for the difficulties which had been encountered up until then in obtaining appreciable ACC oxidase activity. Ververidis and John [112] had the idea of using conditions of extraction and added constituents (the presence of Fe^{2+} and ascorbate), permitting the solubilization of flavone-3β-hydroxylase and measurement of its activity. They were thus able to obtain soluble active ACC oxidase from melon. Authentic ACC oxidase activity has also been obtained using apple [113, 114] and avocado [115]. Soon thereafter, total purification of the enzyme was achieved in the apple by Pech [117], Yang [84], and then by Pirrung [118], and the biochemical properties have been determined.

In contrast to the accepted notion [9], ACC oxidase appears to be a relatively abundant enzyme. Its molecular weight is 35 kDa. Its optimum temperature has been estimated to be 26 °C and its optimum pH 7.4. The K_M for ACC is about 12 ± 4 μM, and for iron about 1 μM [118]. The purified enzyme demonstrates an absolute requirement for Fe^{2+} and for ascorbate. Concentrations of Fe^{2+} as low as 10 μM are sufficient to maintain maximum enzyme activity, and ascorbate concentrations of 1 mM are necessary. In the presence of ascorbate, dithiothreitol increases the enzymatic activity by 10–15%, but in itself it is not effective in replacing ascorbate as an electron donor. Carbon dioxide is an essential activator of ACC oxidase activity [84]. In the absence of CO_2, enzyme activity is completely suppressed. A maximum increase in activity is obtained with 4% CO_2. At this concentration, the activity is at least ten times greater than at the normal concentration of CO_2 in ambient air (0.03%). For comparison it has been shown that CO_2 activates ribulose-1,5-diphosphate carboxylase by carbamate formation on an ε-amino residue of lysine in the enzyme, while decarbamation causes inactivation of the enzyme [119]. In the case of ACC oxidase, activation by CO_2 is also reversible.

The history of the discovery of this enzyme, presently called ACC oxidase, is an example of 'inverse biochemistry' in that the enzyme has been isolated by means of the gene. Since then, several clones coding for ACC oxidase have been isolated from diverse types of fruit [115, 116, 120, 121], from flowers [122] and from other plants [123].

Work has been done on the homology of the proteins produced by transcription of the cDNA of ACC oxidase of tomato (pTOM13) [104], of apple (pAE12) [116] and of avocado (AVOe3) [124]. Some enzymes possessing strongly homologous regions have been detected. In these regions several residues are perfectly conserved, as for example the histidines which are supposed to complex with an essential Fe^{2+} cation. This small family of non-heme iron enzymes consists of an oxidase and a dioxygenase implicated in the biosynthesis of the antibiotics penicillin and cephalosporin, and of dioxygenases implicated in two pathways of biosynthesis in plants. Isopenicillin-*N*-synthase (IPNS) is the most studied member of this family. It removes four hydrogen atoms from the tripeptide Asp-Cys-Val to produce directly isopenicillin *N* and two molecules of water [125] (fig 19). The mechanism of activation of oxygen by this enzyme is not clear. The ligands on Fe(II) have been studied spectroscopically in detail using ESR and NMR

[126–128]. It is thought that, in the catalytically-active complex, iron is surrounded by three histidine residues and perhaps an aspartate residue, and the thiol function of the tripeptide is bonded to iron [127].

Diacetoxycephalosporin-*C*-synthase (DAOCS) is not strictly an oxygenase [129]. No oxygen atom is introduced into the substrate, but the ring expansion is accompanied by unsaturation such that the product is two levels of oxidation higher than that of the starting material. Several experiments have suggested that the enzyme transforms the methyl group of the substrate to a primary alkyl radical [130, 131] by a reaction of hydrogen abstraction caused by an oxo–iron species. Flavone-3β-hydroxylase (F3H) [109] and hyoscyamine-6β-hydroxylase (H6H) [111] (fig 20) both add two atoms of oxygen to their substrates, probably via an oxo–iron intermediate such as is generally invoked for enzymatic hydroxylations.

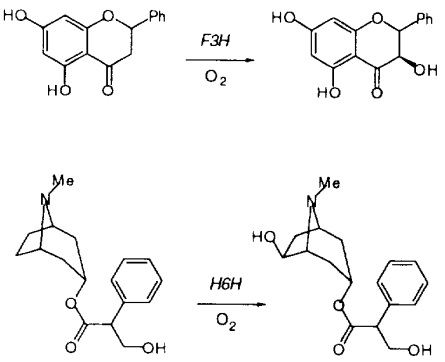

Fig 20. Flavanone-3β-hydroxylase and hyoscyamine-6β-hydroxylase.

Present hypothesis and outlook

The availability of the purified enzyme [84, 117, 118] will now allow unambiguous studies to be carried out on the mechanism by which ethylene is formed and offers the possibility of finding, by screening or by design, molecules which block such activity. We are presently carrying out studies following two complementary approaches. The first deals with the mechanism of action of ACC oxidase: the tailoring of modified radical clocks based, for example, on the principle of a radical cyclization which occurs on a slower timescale than opening of the cyclopropyl methylene radical (fig 21). This should allow confirmation that an acyclic radical intervenes, and precise measurement of its lifetime [132]. The second approach involves the use of suicide substrates which are capable of specifically labeling the enzyme [133]. This approach should allow (i) deduction, from the labeling, of information about the active site of the enzyme at the molecular level, a necessary requirement for all works of mutagenesis directed to the active site, and (ii) study of the subcellular localization of the protein.

At present, all the data in the literature converge to the hypothesis that ACC oxidase is a non-heme Fe(II) enzyme [134, 135]. Numerous reagents reacting on sulfhydryl functions of proteins [136, 137] strongly

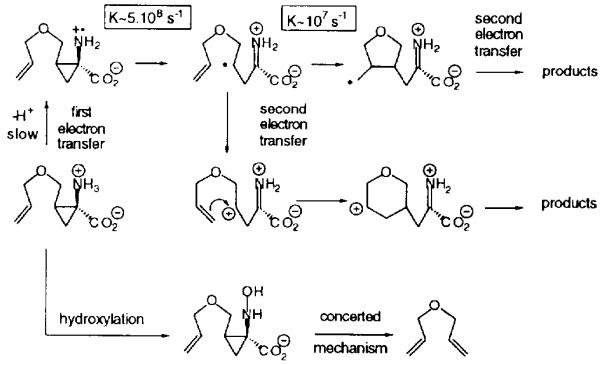

Fig 21. Tailoring of a modified radical clock.

inhibit the production of ethylene, suggesting the presence of a cysteine thiol group within the active site of the enzyme [138]. Christoffersen has utilized specific chemical modifications of the histidine residue (reaction with diethyl pyrocarbonate) to inactivate ACC oxidase [124]. He has shown that the enzyme is protected from this chemical inactivation only when it is carried out in the presence simultaneously of Fe(II) and ACC. Additionally, the study of fluorescence spectra of ACC oxidase under diverse conditions [139] suggests the presence of a tryptophan residue in the immediate vicinity of Fe(II). Zhang et al reached similar conclusions by a comprehensive study of expression, purification and characterization of ACC oxidase from tomato in *Escherichia coli* [141]. A reasonable working hypothesis, based mainly on the strong analogy between ACC oxidase and isopenicillin *N* synthase (IPNS) [124, 127], postulates a hexacoordinated metal center with three histidine residue ligands, one site for cysteine thiol, one site for oxygen, apparently able to be occupied by a carboxylate group of an Asp residue, and the presence near the active site of a tryptophan residue, which affects the reactivity of the enzyme but is not essential for catalysis, as well as an ε-amino group of lysine which reacts reversibly with CO_2. Additional studies are necessary to determine the validity of such a model (fig 22, cf fig 6) using chemical probes [140], spectroscopic techniques (Mössbauer, EPR, NMR, EXAFS) and active-site-directed mutagenesis. The recently-obtained crystal structure of IPNS complexed with manganese reveals that the active site is, unusually, buried within a 'jelly-

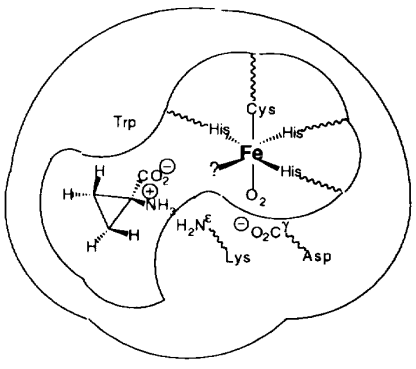

Fig 22. ACC oxidase active site: the working hypothesis.

roll' motif and lined by hydrophobic residues [142]. Sequence analysis indicates that IPNS, ACC oxidase and many of the 2-oxo-acid-dependent oxygenases contain a conserved jelly-roll motif, forming a new structural family of enzymes.

Acknowledgments

We express our gratitude to all those who have prompted, facilitated and encouraged our participation in this area of research: Professor Pierre Potier who has drawn us to the chemistry–biology interface by his persuasive dynamism, Jean-Claude Pech, Mondher Bouzayen and Alain Latché of the École nationale supérieure d'agronomie de Toulouse, who have allowed us to share their knowledge with enthusiasm, Professor Rainer Herges, University of Erlangen, for his expert theoretical opinion, the CNRS for its financial aid and Professor James C Orr, Memorial University of Newfoundland, Canada, who gave us invaluable help with the translation of this article.

References

1 Girardin JPL, *Jahresb Agr* (1864) 7, 199
2 Neljubow D, *Bot Centralbl Beih* (1901) 10, 128-139
3 Denny FE, *J Agr Res* (1924) 27, 757-769
4 Gane R, *Nature* (1934) 134, 1008
5 Pech JC, Balagué C, Latché A, Bouzayen M, *Sci Alim* (1994) 14, 3-15
6 *The Plant Hormone Ethylene*, Mattoo AK Suttle JC, Boca Raton, Fl, CRC Press Pub, 1991, 337pp
7 *Ethylene in Plant Biology*, Abeles FB, Morgan PW, Saltveit ME Jr, San Diego Academic, 2nd ed, 1992, 414pp
8 *Cellular and Molecular Aspects of the Plant Hormone Ethylene*, Pech JC, Latché A, Balagué C, Kluwer, Dordrecht, The Netherlands, 1992, 385pp
9 Yang SF, Hoffman NE, *Ann Rev Plant Physiol* (1984) 35, 155-89
10 Theologis A, *Current Biology* (1993) 3, 369-371
11 Kende H, *Ann Rev Plant Physiol Plant Mol Biol* (1993) 44, 283-307
12 McKeon TA, Yang SF, *Planta* (1984) 160, 84-87
13 For review, see: Yang SF, *Recent Adv Phytochem* (1974) 7, 131-64
14 Lieberman M, Mapson LW, *Nature* (1964) 204, 343-345
15 Lieberman M, Kunishi AT, Mapson LW, Wardale DA, *Biochem J* (1965) 97, 449-459
16 Abeles FB, Rubinstein B, *Biochim Biophys Acta* (1964) 93, 675-677
17 Yang SF, Ku HS, Pratt HK, *Biochem Biophys Res Commun* (1966) 24, 729-743
18 Lieberman M, Kunishi AT, Mapson LW, Wardale DA, *Plant Physiol* (1966) 41, 376-382
19 for review, see: Lieberman M, *Ann Rev Plant Physiol* (1979) 30, 533-591
20 Sandmann G, Boger P, *Plant Physiol* (1960) 66, 797-800
21 Saad FA, Crane JC, Maxie EC, *J Am Soc Hortic Sci* (1969) 94, 335-337
22 Burg SP, Clagett CO, *Biochem Biophys Res Commun* (1967) 27, 125-130
23 Baur AH, Yang SF, *Phytochemistry* (1972) 11, 3207-3214
24 Adams DO, Yang SF, *Plant Physiol* (1977) 60, 892-896
25 Adams DO, Yang SF, *Proc Natl Acad Sci USA* (1979) 76, 170-174
26 Baxter C, Coscia CJ, *Biochem Biophys Res Commun* (1973) 54, 147-154
27 Ferro AJ, Barrett A, Shapiro SK, *Biochim Biophys Acta* (1976) 438, 487-494
28 Guranowski AB, Chiang PK, Cantoni GL, *Eur J Biochem* (1981) 114, 293-299
29 Yu YB, Adams DO, Yang SF, *Arch Biochem Biophys* (1979) 198, 280-286
30 Ferro AJ, Vandenbark AA, Marchito K, *Biochim Biophys Acta* (1979) 588, 294-301
31 Guranowski A, *Plant Physiol* (1983) 71, 932-935
32 Kushad MM, Richardson DG, Ferro AJ, *Biochem Biophys Res Commun* (1982) 108, 167-173
33 Kushad MM, Richardson DG, Ferro AJ, *Plant Physiol* (1983) 73, 257-261
34 Trackman PC, Abeles RH, *Biochem Biophys Res Commun* (1981) 103, 1238-1244
35 Backlund PS Jr, Chang CP, Smith RA, *J Biol Chem* (1982) 257, 4196-4202
36 Burg SP, *Proc Natl Acad Sci USA* (1973) 70, 591-597
37 Murr DP, Yang SF, *Plant Physiol* (1975) 55, 79-82
38 Hansen E, *Bot Gaz* (1942) 103, 543-558
39 Burg SP, Thimann KV, *Proc Natl Acad Sci USA* (1959) 45, 335-344
40 Rando RR, *Science* (1974) 185, 320-324
41 Davis L, Metzler DE, *Enzymes* (1972) 7, 33-74
42 a) Wiesendanger R, Martinoni B, Boller T, Arigoni D, *Experientia* (1986) 42, 207-209
b) see also: Ramalingam K, Lee KM, Woodard RW, Bleecker AB, Kende H, *Proc Natl Acad Sci USA* (1985) 82, 7820-7824
43 Burroughs LF, *Nature* (1957) 179, 160-161
44 Vätähalo ML, Virtanen AI, *Acta Chem Scand* (1957) 11, 741-743
45 Boller T, Herner RC, Kende H, *Planta* (1979) 145, 293-303
46 Acaster MA, Kende H, *Plant Physiol* (1983) 72, 139-145
47 Yang SF, *Hort Science* (1980) 15, 238-243
48 Mansour R, Latché A, Vaillant V, Pech JC, Reid MS, *Physiol Plant* (1986) 66, 495-502
49 Bleecker AB, Robinson G, Kende H, *Planta* (1988) 173, 385-390
50 Nakajima N, Imaseki H, *Plant Cell Physiol* (1986) 27, 969-980
51 Nakagawa J, Mori H, Yamazaki K, Imaseki H, *Plant Cell Physiol* (1990) 32, 1153-1163
52 Sato T, Oeller PW, Theologis A, *J Biol Chem* (1991) 266, 3752-3759
53 Nakagawa J, Nakajima N, Imesaki H, *Plant Cell Physiol* (1988) 27, 1021-1029
54 Satoh S, Yang SF, *Plant Physiol* (1989) 91, 1036-1039
55 Satoh S, Yang SF, *Arch Biochem Biophys* (1989) 271, 107-112
56 Yip WK, Dong JG, Kenny JW, Thompson GA, Yang SF, *Proc Natl Acad Sci USA* (1990) 87, 7930-7934
57 Mayak S, Legge RL, Thompson JE, *Planta* (1981) 153, 49-55
58 Mc Rae DG, Baker JE, Thompson JE, *Plant Cell Physiol* (1982) 23, 375-383
59 Rohwer F, Mader M, *Z Pflanzenphysiol* (1981) 104, 363-372
60 Vioque A, Albi MA, Vioque B, *Phytochemistry* (1981) 20, 1473-1475

61 Shimokawa K, *Phytochemistry* (1983) 22, 1903-1908
62 Bousquet JF, Thimann KV, *Proc Natl Acad Sci USA* (1984) 81, 1724-1727
63 Kacperska A, Kubacka-Zebalska M, *Physiol Plant* (1985) 64, 333-338
64 Wang TT, Yang SF, *Planta* (1987) 170, 190-196
65 Steginк SJ, Siedow JN, *Physiol Plant* (1986) 66, 625-631
66 Acosta M, Casa JL, Arnao MB, Sabater F, *Biochim Biophys Acta* (1991) 1077, 273-280
67 Lieberman M, Kunishi AT, *Science* (1967) 19, 938
68 Mapson LW, Wardale JE, *Phytochemistry* (1971) 10, 29-30
69 Burg SP, *Proc Natl Acad Sci USA* (1973) 70, 591-597
70 Konze JR, Jones JF, Boller T, Kende H, *Plant Physiol* (1980) 66, 566-571
71 Banks NH, in *Ethylene and Plant Development*, Roberts JA, Tucker GA Eds, Butterworths, London, 1985, 29-36
72 Yip WK, Jiao XZ, Yang SF, *Plant Physiol* (1988) 88, 553-558
73 Bradford KJ, Hsiao TC, Yang SF, *Plant Physiol* (1982) 70, 1503-1507
74 Baker JE, Lieberman M, Anderson JD, *Plant Physiol* (1978) 61, 886-888
75 Boller T, Kende H, *Nature* (1980) 286, 259-260
76 Apelbaum A, Wang SY, Burgoon AC, Baker JE, Lieberman M, *Plant Physiol* (1981) 67, 74-79
77 Dilley DR, Kuai J, Poneleit L, Zhu Y, Pekker Y, Wilson ID, Burmeister DM, Gran C, Bowers A, in [8], pp 46-52
78 Hoffman NE, Yang SF, Achihara A, Sakamura S, *Plant Physiol* (1982) 70, 195-199
79 Pirrung MC, McGeehan GM, *J Org Chem* (1986) 51, 2106-2110
80 Pirrung MC, Cao J, Chen J, *J Org Chem* (1995) 60, 5790-5794
81 Hiyama T, Koide H, Nozaki H, *Bull Chem Soc Jpn* (1975) 48, 2918-2921
82 Yang SF in *Recent Advances in the Biochemistry of Fruits and Vegetables*, Friend J, Rhodes MJ Eds, Academic, London, 1981, pp 89-106
83 Peiser GD, Wang TT, Hofmann NE, Yang SF, Liu HW, Walsh CT, *Proc Natl Acad Sci USA* (1984) 81, 3059-3063
84 Dong JG, Fernandez-Maculet JC, Yang SF, *Proc Natl Acad Sci USA* (1992) 89, 9789-9793
85 Adlington RM, Aplin RT, Baldwin JE, Rawlings BJ, Osborne D, *J Chem Soc, Chem Commun* (1982) 1086-1087
86 Adlington RM, Baldwin JE, Rawlings BJ, *J Chem Soc, Chem Commun* (1983) 290-292
87 Baldwin JE, Jackson DA, Adlington RM, Rawlings BJ, *J Chem Soc, Chem Commun* (1985) 206-207
88 Pirrung MC, McGeehan GM, *J Org Chem* (1983) 48, 5143-5144
89 Woodward RB, Hoffmann R, *The Conservation of Orbital Symmetry*, Verlag Chemie, Weinheim and Academic, New York, 1970, pp 152-163
90 Herges R, *Angew Chem, Int Ed Engl* (1994) 33, 255-276
91 Herges R, *J Chem Inf Comput Sci* (1994) 34, 91-102
92 in [90], fig 7, p 259
93 Pirrung MC, *J Am Chem Soc* (1983) 105, 7207-7209
94 Ingold KU, Griller D, *Acc Chem Res* (1980) 13, 317-323
95 Sutcliffe R, Ingold KU, *J Am Chem Soc* (1982) 104, 6071-6075
96 Pirrung MC, McGeehan GM, *Angew Chem, Int Ed Engl* (1985) 24, 1044-1045
97 Pirrung MC, *J Org Chem* (1987) 53, 4179-4184
98 Maunders MJ, Holdsworth MJ, Slater A, Knapp JE, Bird CR, Schuch W, Grierson D, *Plant Cell Envir* (1987) 10, 177-184
99 Lincoln JE, Cordes S, Read E, Fischer RL, *Proc Natl Acad Sci USA* (1987) 84, 2793-2797
100 Slater A, Maunders MJ, Edwards K, Schuch W, Grierson D, *Plant Mol Biol* (1985) 5, 137-147
101 Grierson D, Tucker GA, Keen J, Ray J, Bird CR, Schuch W, *Nucleic Acids Res* (1986) 14, 8595-8603
102 Smith CJS, Slater A, Grierson D, *Planta* (1986) 168, 94-100
103 Davies KM, Grierson D, *Planta* (1989) 79, 73-80
104 Holdsworth MJ, Schuch W, Grierson D, *Nucleic Acids Res* (1987) 15, 10600
105 Holdsworth MJ, Schuch W, Grierson D, *Plant Mol Biol* (1988) 11, 81-88
106 Hamilton AJ, Lycet GW, Grierson D, *Nature* (1990) 346, 284-287
107 Hamilton AJ, Bouzayen M, Grierson D, *Proc Natl Acad Sci USA* (1991) 88, 7434-7437
108 Spanu P, Reinhardt D, Boller T, *EMBO J* (1991) 10, 2007-2013
109 Britsch L, *Arch Biochem Biophys* (1990) 276, 348-354
110 Hedden P, *Biochem Soc Trans* (1992) 20, 373-376
111 Matsuda J, Okabe S, Hashimoto T, Yamada Y, *J Biol Chem* (1991) 266, 9460-9464
112 Ververidis P, John P, *Phytochemistry* (1991) 30, 725-727
113 Kuai J, Dilley DR, *Postharvest Biol Technol* (1992) 1, 203-211
114 Fernandez-Maculet JC, Yang SF, *Plant Physiol* (1992) 99, 751-754
115 McGarvey J, Christoffersen RE, *J Biol Chem* (1992) 267, 5964-5967
116 Dong JG, Olson D, Silverstone A, Yang SF, *Plant Physiol* (1992) 98, 1530-1531
117 Dupille E, Latché A, Roques C, Pech JC, *CR Acad Sci Paris* (1992) 315 (Ser III) 77-84
118 Pirrung MC, Kaiser LM, Chen J, *Biochemistry* (1993) 32, 7445-7450
119 Lorimer GH, Miziorko HM, *Biochemistry* (1980) 19, 5328-5334
120 Ross GS, Knighton ML, Lee ML, *Plant Mol Biol* (1992) 19, 2131-2138
121 Balagué C, Watson CF, Turner AJ, Rougé P, Picton S, Pech JC, Grierson D, *Eur J Biochem* (1993) 212, 27-34
122 Wang H, Woodson WR, *Plant Physiol* (1991) 96, 1000-1001
123 Kende H, *Ann Rev Plant Physiol Plant Mol Biol* (1993) 44, 283-307
124 Christoffersen RE, McGarvey DJ, Savarese P, in [8] pp 65-70
125 Abraham EP, Huddleston JA, Jayatilake BS, O'Sullivan J, White RL, *Spec Publ Chem Soc* (1981) 38, 125-134
126 Chen VJ, Orville AM, Harpel MR, Frolic CA, Suresnes KK, Munck E, Lipscomb JD, *J Biol Chem* (1989) 264, 21677-21681
127 Ming L-J, Que L Jr, Kriauciunas A, Frolik CA, Chen VJ, *Inorg Chem* (1990) 29, 1111-1112
128 Scott RA, Wang S, Eidness MK, Kriauciunas A, Frolic CA, Chen VJ, *Biochemistry* (1992), 31, 4596-4601
129 Baldwin JE, Adlington RM, Cates JB, Crabbe MJC, Crouch NP, Keeping JW, Knight GC, Schofield CJ, Ting H-H, Vallejo CA, Abraham EP, *Biochem J* (1987) 245, 831-841

130 Baldwin JE, Adlington RM, Kang TW, Lee E, Schofield CJ, *J Chem Soc, Chem Commun* (1987) 104-106

131 Baldwin JE, Adlington RM, Crouch NP, Keepling JW, Leppard JW, Pitlik J, Schofield CJ, Sobey WJ, Wood ME, *J Chem Soc, Chem Commun* (1991) 768-769

132 Baldellon F, Thesis, Marseille, in progress

133 Wouters S, Synthèse de substrats suicides de l'ACC oxydase par réactions de cyclopropanation des didehydroaminoacides. PhD thesis, Marseille, 1996

134 Prescott AG, *J Expt Biol* (1993) 94, 849-851

135 Feig AL, Lippard SJ, *Chem Rev* (1994) 94, 759-805

136 Yu YB, Yang SF, *Plant Physiol* (1979) 64, 1074-1077

137 Forney CJ, Arteca RN, Wallner SJ, *Physiol Plant* (1982) 54, 329-332

138 Dupille E, *Purification et caractérisation de l'enzyme formant l'éthylène chez la pomme*, Thesis, Toulouse, 1992

139 Christoffersen RE, unpublished results

140 See for examples, the mechanistic studies of IPNS: Baldwin JE, Bradley M, *Chem Rev* (1990) 90, 1079-1086

141 Zhang Z, Schofield CJ, Baldwin JE, Thomas P, John P, *Biochem J* (1995) 307, 77-85

142 Roach PL, Clifton IJ, Fülöp V, Harlos K, Barton GJ, Hajdu J, Anderson I, Schofield CJ, Baldwin JE, *Nature* (1995) 375, 700-704