although improbable, that the gene cloned into *S. sanguis* did not contain the coding region for the 32-residue COOH-terminal part of the streptokinase molecule. It is more likely that the gene cloned in *S. sanguis* is complete, and the protein molecule initially contains the COOH-terminal 32 residue, but this region is lost due to posttranslational processing by a protease, or proteases, present in *S. sanguis*. Support for this possibility comes from the fact that the COOH-terminal residue of cloned SK is leucine, and it is known that Leu-X bonds are rapidly cleaved by chymotrypsin. Further support for a chymotryptic-like posttranslational processing of cloned SK was demonstrated by the fact that when native SK is incubated briefly with α-chymotrypsin, a degradation product is formed that has a mobility equivalent to cloned SK on SDS-polyacrylamide gel electrophoresis. The protease hypothesis is also supported by the presence of a second minor component, cSK_L.

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Registry No. SK, 9002-01-1; plasminogen, 9001-91-6.

REFERENCES

Ambler, R. P. (1972) *Methods Enzymol. 25*, 143-154.
Brauer, A. W., Margolies, M. N., & Haber, E. (1975) *Biochemistry 14*, 3029-3035.
Brockway, W. J., & Castellino, F. J. (1974) *Biochemistry 13*, 2063-2070.
Castellino, F. J. (1979) *Trends Biochem. Sci. (Pers. Ed.) 4*, 1-5.
Castellino, F. J., & Powell, J. R. (1981) *Methods Enzymol. 80*, 365-378.
Gerlach, D., & Kohler, W. (1977) *Zentralbl. Bakteriol., Mikrobiol. Hyg., Abt. 1, Orig. A 238*, 336-349.
Henderson, L. E., Copeland, T. D., & Oroszlan, S. (1980) *Anal. Biochem. 102*, 1-7.
Jackson, K. W., & Tang, J. (1978) *Thromb. Res. 13*, 693-699.
Jackson, K. W., & Tang, J. (1982) *Biochemistry 21*, 6620-6625.
Jackson, K. W., Esmon, N., & Tang, J. (1981) *Methods Enzymol. 80*, 387-394.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Malke, H., & Ferretti, J. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3557-3561.
Malke, H., Gerlach, D., Kohler, W., & Ferretti, J. J. (1984) *MMG, Mol. Gen. Genet. 196*, 360-365.
Malke, H., Roe, B., & Ferretti, J. J. (1985) *Gene 34*, 357-362.
Siefring, G. E., Jr., & Castellino, F. J. (1976) *J. Biol. Chem. 251*, 3913-3920.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190-1206.
Steers, E., Craven, Z. R., Anfinson, C. B., & Bethune, J. L. (1965) *J. Biol. Chem. 240*, 2478-2484.
Summers, M. R., Smythers, G. W., & Oroszlan, S. (1973) *Anal. Biochem. 53*, 624-628.
Tarr, G. E. (1975) *Anal. Biochem. 63*, 361-370.
Violand, B. N., & Castellino, F. J. (1976) *J. Biol. Chem. 251*, 3906-3912.

Mechanism of a Lipoxygenase Model for Ethylene Biosynthesis†

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ABSTRACT: The model system for ethylene biosynthesis developed by Bousquet and Thimann [Bousquet, J.-F., & Thimann, K. V. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1724-1727], consisting of lipoxygenase, linoleic acid, aminocyclopropanecarboxylic acid, Mn^{2+}, and pyridoxal phosphate, has been studied to identify its products and mechanism. As occurs in ethylene biosynthesis in plant tissue, C1-N1 of aminocyclopropanecarboxylic acid are converted to cyanide. Also as in plant tissue, stereospecifically labeled aminocyclopropanecarboxylic acid is converted to ethylene with loss of stereochemistry. The role of pyridoxal phosphate has been shown not to involve Schiff's base formation with aminocyclopropanecarboxylic acid but chelation of the *o*-hydroxybenzaldehyde unit with manganese. A number of other such compounds may replace pyridoxal phosphate as a chelator in the model system. Chelation has the effect of increasing the reduction potential of the metal ion in order to cleave linoleic hydroperoxide more efficiently. The system shows sufficient fundamental differences with the natural system to confidently discount it.

The study of the process by which aminocyclopropanecarboxylic acid is converted to the plant growth hormone ethylene has been frustrated by the lack of an authentic isolated ethylene-forming enzyme. Consequently, a number of cell-free preparations (Konze & Kende, 1979; Mayak et al., 1981; Mattoo et al., 1982; Guy & Kende, 1984; Vinkler & Apelbaum, 1983; Vioque et al., 1981; Konze & Kwiatkowski, 1981; Boller et al., 1979; Bousquet & Thimann, 1984) have been investigated as model systems. These include peroxidases, microsomal membranes, vacuoles, mitochondria, and others. Often, cofactors (pryidoxal, ascorbate), metal ions (Cu^{2+}, Mn^{2+}), and other ingredients are added to these systems to allow them to operate.

In some cases, these studies have muddied the waters concerning ethylene biosynthesis in vivo. For example, it is now well established that pyridoxal phosphate is required for ethylene biosynthesis and that pyridoxal inhibitors which form

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stable Schiff's bases inhibit ethylene production (Adams & Yang, 1979; Amrhein & Schneebeck, 1980; Yu et al., 1979a). However, it is also known that in vivo these act by inhibiting the pyridoxal enzyme aminocyclopropanecarboxylic acid synthase (Yu et al., 1979b). They have no effect on the conversion of aminocyclopropanecarboxylic acid to ethylene. It is difficult to ascribe a role for pyridoxal phosphate, then, in the cell-free systems mentioned above.

This report concerns the characterization of the model of Thimann which consists of lipoxygenase, linoleic acid, Mn^{2+}, pyridoxal phosphate, and aminocyclopropanecarboxylic acid in a pH 8 buffer. It is a possible candidate for the ethylene-forming enzyme because of the correlation of peroxide levels and lipid peroxidation with ethylene biosynthesis. It is also attractive because of the presumed intermediacy of linoleic hydroperoxide. Since this is a chiral, optically active hydroperoxide, its reaction with alkylated aminocyclopropanecarboxylic acid analogues (vide infra) might show both enantioselectivity and diastereoselectivity, accounting for the stereospecificity of natural ethylene biosynthesis (Hoffman et al., 1982). This model additionally shows an inhibition profile somewhat similar to ethylene production in plants, including Co^{2+}, propyl gallate, and phenanthroline. In this work, evidence has been obtained for the roles played by both the metal ion and pyridoxal phosphate, and the system has been further studied with probes previously used in vivo. Some correspondence has been found, especially mechanistic, but enough disparities in inhibitor response exist that it is possible to confidently say this does not represent the actual in vivo system.

Materials and Methods

Linoleic acid, lipoxygenase (EC 1.13.11.12), pyridoxal phosphate, and aminocyclopropanecarboxylic acid were obtained from Sigma. Aminoxyacetic acid was obtained from Calbiochem. Salicylaldehyde, 4-methoxy-2-hydroxybenzaldehyde, and *N,N'*-bis(salicylidene)ethylenediamine (SALEN) were from Aldrich. Manganese(II) bis(salicylaldehyde) [$Mn(sal)_2$] was prepared according to the literature (Asmussen & Soling, 1957). *cis*-[2,3-$^2H_2$]Aminocyclopropanecarboxylic acid was available from another study (Pirrung, 1983). Cyclic voltammograms were obtained in dimethyl sulfoxide (Me_2SO)/0.1 M tetrabutylammonium perchlorate solution using Pt wire electrodes. Ethylene was quantified as previously described (Pirrung, 1983). Pentane was identified by coinjection with an authentic sample, using the same gas chromatograph conditions as for ethylene, except for a 150 °C column temperature and 175 °C injector and detector temperatures. IR spectra were also obtained as previously described (Pirrung, 1983). Incubations of aminocyclopropanecarboxylic acid with the lipoxygenase system were conducted as described by Thimann, except still 13-mL polypropylene culture tubes sealed with serum caps were used.

Stereochemistry. In 2 mL of H_2O was dissolved 1.8 mg of *cis*-[2,3-$^2H_2$]aminocyclopropanecarboxylic acid in a 25-mL Erlenmeyer flask with vacuum fittings. Lipoxygenase (4800 units) and the remainder of the reagents were added in 4 mL of solution. After incubation for 11 h, the ethylene concentration was 400 ppm. The liquids were frozen with dry ice/acetone, and the head space was vacuum-transferred to an IR cell. A 1:1 mixture of *cis*- and *trans*-dideuterioethylene was observed. The experiment was repeated, obtaining 260 ppm of scrambled dideuterioethylene. The liquids were adjusted to pH 10 with NaOH and subjected to Dowex 50 (H^+) ion-exchange chromatography. Buffer salts and unreacted substrate were recovered by elution with 1 M NH_4OH and concentration. These were dissolved in 2.5 mL of 10% $EtOH/H_2O$ in an Erlenmeyer flask with vacuum fittings. Mercuric chloride (10 mg) was added, followed by 1 mL of 0.46 M NaOCl/KOH solution. After incubation for 1 h, the solution was frozen, and the head space was vacuum-transferred to an IR cell. Only *cis*-dideuterioethylene was observed. Authentic *cis*-dideuterioethylene was incubated with the Thimann reagent system for 16 h. The solution was frozen and the head space vacuum-transferred to an IR cell. Only *cis*-dideuterioethylene was observed.

Cyanide Production. Dideuterioaminocyclopropanecarboxylic acid (2.4 mg) was degraded by the Thimann reagent solution over 10.5 h. Scrambled dideuterioethylene (164.9 ppm, 183.9 nmol) was again produced as evidenced by IR studies. The solution was diluted to 10.0 mL with water after the addition of 0.1 mL of 10 M NaOH. Analysis in triplicate by a cyanide ion selective electrode as previously described (Pirrung, 1983; Pirrung & McGeehan, 1983) gave a concentration of 1.82×10^{-5} M.

Results

Comparison of the Thimann Reagent System with Natural Ethylene Biosynthesis. Two groups (Pirrung, 1983; Adlington et al., 1983) have recently provided evidence for the nonconcerted nature of ethylene biosynthesis using deuterated substrates. Submission of *cis*-dideuterioaminocyclopropanecarboxylic acid to the Thimann oxidation conditions leads to the same result as in plants: production of equal amounts of *cis*- and *trans*-dideuterioethylene. Control experiments demonstrate that *cis*-dideuterioethylene is stable to the reaction conditions and that the starting material is not scrambled by them. The latter was demonstrated by isolating unreacted substrate and submitting it to hypochlorite degradation (Lizada & Yang, 1979), which has been shown to proceed with retention of configuration (Adlington et al., 1983). Only *cis*-dideuterioethylene is produced.

It has also recently been established (Peiser et al., 1984; Pirrung, 1985) that ethylene biosynthesis produces cyanide from C1–N1 of aminocyclopropanecarboxylic acid and that this cyanide is further metabolized by known pathways. Since the enzymes required for the metabolism of cyanide are absent from this system, it should be possible to observe it directly. Analysis by a cyanide ion selective electrode of a large-scale incubation with the Thimann reagent system demonstrates that cyanide is produced in a molar amount equivalent to that of ethylene.

An important characteristic of natural ethylene biosynthesis is the discrimination between stereoisomers of alkyl-substituted aminocyclopropanecarboxylic acids (Hoffman et al., 1982). Racemic *cis*- and *trans*-2-methyl-1-aminocyclopropanecarboxylic acids[1] were examined in the Thimann system; a small preference for the trans isomer is observed (Table I). Interestingly, this preference is maintained when *tert*-butyl hydroperoxide or hydrogen peroxide is substituted for linoleic acid and enzyme. The diastereoselection, particularly in the latter two processes, must be purely substrate related and is not easy to explain. The amino acid with the *more* hindered amine reacts preferentially, so steric effects are not responsible [it has previously been shown that reaction at nitrogen of aminocyclopropanecarboxylic acid is critical for ethylene production (Pirrung, 1983)]. Similar levels of diastereose-

[1] The former was provided by Prof. C. T. Walsh. The latter (racemic and optically active) is available in our laboratory (Pirrung & McGeehan, 1985).

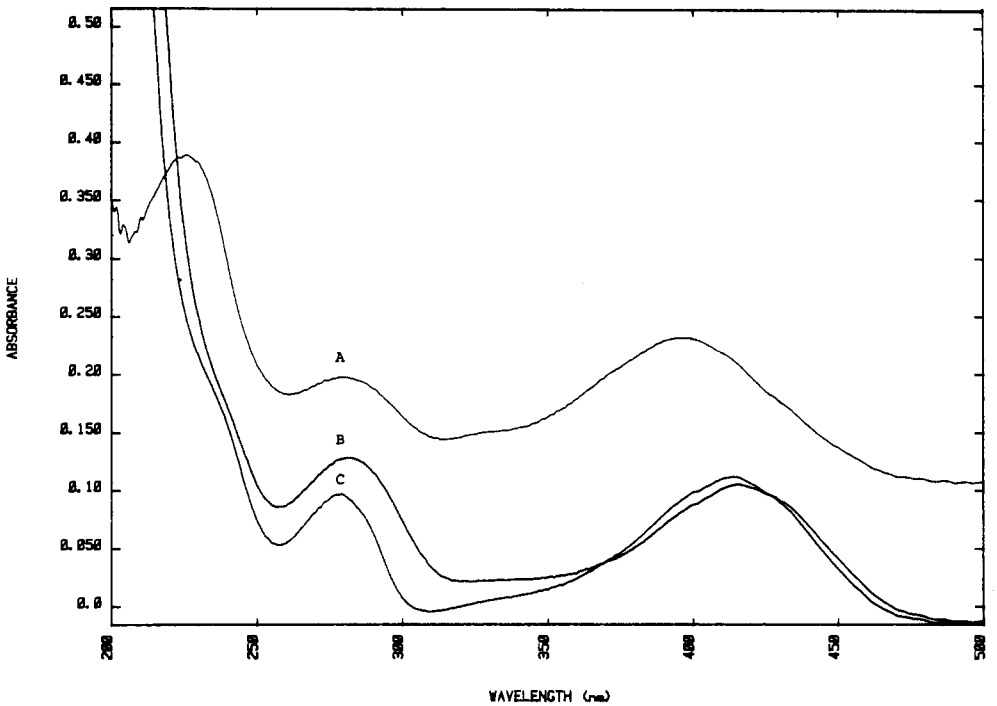


FIGURE 1: UV spectra: (a) 1 mM aminocyclopropanecarboxylic acid (ACC); (b) 0.5 M ACC; (c) 0.5 M glycine. Solution was buffered at pH 8 and contained 0.1 mM pyridoxal phosphate.

Table I: Stereoselection of Processing of 2-Methyl-1-aminocyclopropanecarboxylic Acid (MeACC) to Propene by Various Oxidants[a]

substrate	oxidant	propene (nmol h^{-1})	*trans*:*cis* ratio
cis-MeACC	lipoxygenase/linoleic acid	25.4	
trans-MeACC		46.5	1.8
cis-MeACC	*tert*-butylhydroperoxide	38.0	
trans-MeACC		126.4	3.3
cis-MeACC	hydrogen peroxide	19.7	
trans-MeACC		49.6	2.5
(1*S*,2*R*)-MeACC	lipoxygenase/linoleic acid	42.0	
(1*R*,2*S*)-MeACC		43.2	

[a] Means of two replicates.

Table II: Effect of Ligands on the Ethylene Yield in the Lipoxygenase System[a]

ligand	ethylene (nmol h^{-1})
pyridoxal phosphate	7.9
salicylaldehyde	12.4
SALEN	2.6
pyridoxal phosphate[b]	7.2
none	0.4
$Mn(sal)_2$	11.1
4-methoxy-2-hydroxybenzaldehyde[b]	2.4
pyridoxal phosphate + aminoxyacetic acid	0.0

[a] Means of three to five determinations conducted in duplicate. [b] pH 9.

lection (2–3:1) have been reported in the ethyl-substituted series by studying other cell-free systems (McKeon & Yang, 1984; Venis, 1984). Enantioselection by the Thimann system was also examined by using optically active *trans*-2-methyl-1-aminocyclopropanecarboxylic acid.[1] As Table I also shows, a complete lack of selectivity for the natural[1] 1*R*,2*S* substrate is observed.

In summary, two mechanistic criteria are met by the Thimann system, while it failed the stereochemical test.

Role of Pyridoxal Phosphate. As a multifunctional molecule, there are many ways pyridoxal phosphate might interact with the lipoxygenase/manganese system. Molecules which contain only some of the functionalities of pyridoxal were used to identify which are crucial. Thus, pyridine, benzaldehyde, and 3-hydroxypyridine, singly and in combination, were examined as replacements for pyridoxal phosphate in the Thimann system, all without success. Further, a much larger difference (~20-fold) than reported by Thimann between reagent mixtures which do and do not include pyridoxal phosphate is found. This may have to do with the use of plastic rather than glass containers, which proved important in other studies of peroxidic models for ethylene production.[2] Metallic impurities may be responsible. Importantly, salicylaldehyde is superior to pyridoxal phosphate. This immediately suggested that metal chelation, not Schiff's base formation, is the reason for the utility of pyridoxal phosphate in the system. To confirm this, some other compounds were substituted, with results shown in Table II. While the performed chelate $Mn(sal)_2$ is as effective as Mn and salicylaldehyde allowed to react in situ, it is possible to show by UV spectroscopy (Lemon, 1947) that the primary organic species present in either case is the salicylaldehyde alcoholate. The fact that salicylaldehyde and SALEN give different amounts of ethylene yet would go through the same Schiff's base rules out a rapidly and reversibly formed intermediate. Examination of reaction mixtures by UV spectroscopy (Metzler, 1957) also failed to reveal any Schiff's base intermediate (Figure 1), though one could be generated and identified by a 414-nm band at high (0.5 M) aminocyclopropanecarboxylic acid concentrations.

A stringent test of the validity of the model system with chelating ligands was provided by study of the effects of aminoxyacetic acid, a compound which does not inhibit the aminocyclopropanecarboxylic acid conversion in vivo. In this model system, however, ethylene formation is totally suppressed (Table II). While at first sight this is supportive of Schiff's base chemistry, an equally tenable proposal is that the pyridoxaldoxime/manganese complex has shifted its redox potential outside the useful range. Support for the latter idea is provided below.

[2] M. C. Pirrung and T. J. Kodadek (unpublished results).

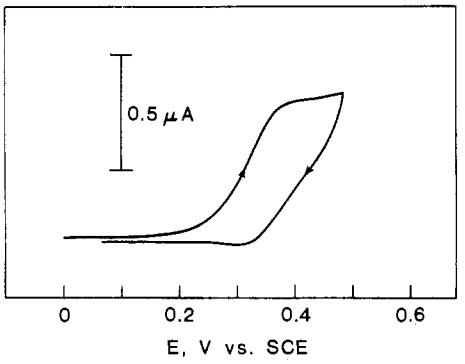


FIGURE 2: Cyclic voltammogram of 1 mM $Mn(sal)_2$ in Me_2SO. Scan rate 5 mV s^{-1}.

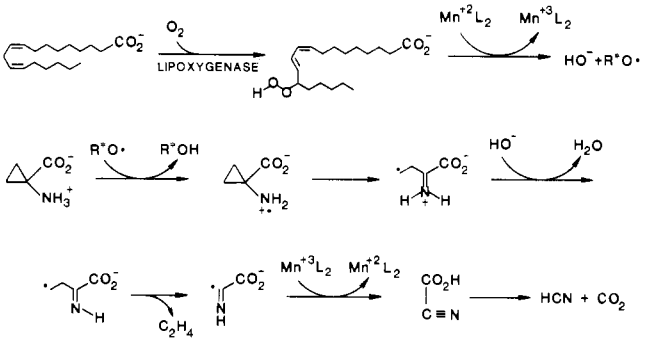
Redox Considerations. Since it is demonstrated that pyridoxal phosphate is a required component of the model system, and the evidence indicates it acts as a metal chelator, the properties of the metal chelate must be connected with its ability to enter into a reaction leading to ethylene. Mn^{2+} has a one-electron oxidation potential of +1.25 V vs. a saturated calomel electrode (SCE). Data are available for the highly air-sensitive $Mn(SALEN)_2$ complex which is presumably formed here in situ (Coleman et al., 1981). Its oxidation potential is −0.22 V vs. SCE in Me_2SO, and it is a quasi-reversible redox couple. The more stable $Mn(sal)_2$ complex is less easily oxidized (ca. +0.34 V vs. SCE in Me_2SO) in an even less reversible manner than $Mn(SALEN)_2$ (Figure 2). Attempts to prepare an analogous manganese/pyridoxal complex by the literature methods resulted in the $Mn(pyridoxal)_2(NH_3)_2$ complex, which is reversibly oxidized at +0.36 V. Interestingly, exchanging salicylaldoxime for salicylaldehyde (Rani et al., 1981) considerably stabilizes the complex. No oxidation is observed in the potential range of interest. This may explain the inertness of the pyridoxal phosphate/aminoxyacetic acid adduct in the Thimann system as described above. Generally, one can see the effect of chelation in making manganese a good reductant. Combined with linoleic hydroperoxide, this constitutes a radical generating system as described below.

Involvement of Linoleic Hydroperoxide. It was hypothesized that linoleic hydroperoxide is first produced by the action of lipoxygenase on linoleic acid and it is then cleaved to hydroxide and an alkoxy radical (Gardner & Kleiman, 1981). This species would then attack substrate to yield ethylene. Strong evidence for the presence of the linoleyl alkoxy radical was obtained by examining the head space of the reaction mixture by gas chromatography. The identification of pentane in addition to ethylene is confirmatory for this species (Evans et al., 1967; Gardner & Plattner, 1984).

Discussion

The mechanistic probes which were applied to the lipoxygenase/manganese model reaction, stereochemistry and cyanide production, produced results similar to the natural system. That chelators such as salicylaldehyde may replace pyridoxal in these model reactions should lay to rest the idea that Schiff's base chemistry is involved in the conversion of aminocyclopropanecarboxylic acid to ethylene. This is expected since, for two reasons, aminocyclopropanecarboxylic acid should be reluctant to enter into pyridoxal/Schiff's base chemistry. One is its reduced electron density at nitrogen, caused by conjugation with the Walsh orbitals of the cyclopropane ring. This is evidenced by the lower pK_a of aminocyclopropanecarboxylic acid derivatives (by 1 pK_a unit; Kimura & Stammer, 1983) and may manifest itself by a reduced rate

Scheme I: Proposed Mechanism of Ethylene Production in the Lipoxygenase/Linoleic Acid/Manganese/Pyridoxal System



of nucleophilic attack on the carbonyl of pyridoxal phosphate. The second is the steric hindrance brought about by α,α-disubstitution. Metzler showed (Metzler, 1957) that such amino acids have the smallest equilibrium constants for Schiff's base formation in solution. However, the involvement of pyridoxal phosphate in one *enzyme*-mediated transformation of aminocyclopropanecarboxylic acid is unambiguous (Walsh et al., 1981).

The different amounts of ethylene produced with different ligands are likely a reflection of their differing redox properties. The salicylaldehyde complex, which produces the highest amount, has an intermediate oxidation potential. This is important so it may rapidly reduce the hydroperoxide without being such a good reductant that it reacts with water or the air which is required for the lipoxygenation. Thermodynamics may not be the whole story, however; matching the rate of electron transfer to the rate of hydroperoxide production may be crucial and account for the unique qualities of manganese in cell-free ethylene formation. The studies of Gardner (Gardner & Jursinic, 1981; Gardner & Kleiman, 1981) give further indication of the importance of redox properties in the metal-catalyzed reduction of fatty acid peroxides. This also explains the inhibition by ethylenediaminetetraacetic acid (EDTA) when none is seen in vivo.

Another aspect of manganese coordination chemistry which may be important is its affinity for ligands. In the divalent late transition series, manganese has the smallest equilibrium constants for complex formation (Cotton & Wilkinson, 1972). Cobalt, conversely, is the most favorable transition metal ion for forming tetrahedral complexes [e.g., $Co(sal)_2$] and would compete strongly with manganese for ligand ($K_{s,Co} = 10K_{s,Mn}$). Since cobalt is much less stable in high oxidation states than manganese, such complexes would be poor reductants for peroxides. This readily accounts for the inhibition of ethylene production by Co^{2+} in this model as reported by Thimann.

The chemical mechanism which can be written for the processes involved in this model system is summarized in Scheme I. Linoleic hydroperoxide is generated by the normal enzymatic mechanism and cleaved reductively to an alkoxy radical. This proposal is similar to previous mechanisms for the cooxidation of substrates in the presence of linoleic acid and lipoxygenase (Streckert & Stan, 1975; Sanders et al., 1975; Gibian & Galaway, 1977) or prostaglandin synthetase (Marnett et al., 1979). Reaction of the alkoxy radical with aminocyclopropanecarboxylic acid ensues via hydrogen abstraction. This generates the amine radical cation intermediate previously proposed (Pirrung, 1983) as an intermediate in the electrochemical oxidation of aminocyclopropanecarboxylate. Ring opening to a radical which undergoes free rotation is also as previously proposed. From this point, the exact sequence which leads to loss of ethylene is difficult to describe without

more information. It certainly seems likely that cyanoformate is the penultimate precursor to CO_2 and HCN, so a sequence leading to it is important. Since there is no evidence that aminocyclopropanecarboxylic acid radical cation fragments to ethylene [at least in the gas phase (Coulter & Fenselau, 1972)], some other step must precede ethylene loss. Deprotonation should be a rapid reaction at this pH, and then loss of ethylene might occur. Back electron transfer from the radical anion would regenerate the manganous complex and yield cyanoformate. There is no evidence concerning the final state of the metal, and previous workers have shown that it is quite difficult to assign structures to such oxygenated manganese complexes using physical methods (Boreham & Chiswell, 1977). The metal is oxidized in solution at a rate somewhat slower than that of the reaction to produce ethylene, so it is unclear if the metallic products have any significance for the reaction overall [however, substantial quantities of salicylaldehyde are recovered from large-scale reactions of $Mn(sal)_2$, *tert*-butyl hydroperoxide, and aminocyclopropanecarboxylic acid]. The fact that substochiometric quantities of Mn^{2+} and pyridoxal phosphate are required suggests the complex operates catalytically as described above or as a free-radical initiator.

This mechanistic picture also explains previous reports that fatty acids are precursors to ethylene (Lieberman & Mapson, 1964; Galliard et al., 1968). When cell-free extracts of apple tissue are combined with linolenic acid and ascorbate, they yield ethylene. However, no evidence was found for radioactivity in the ethylene when [^{14}C]linolenic acid was used (Mapson et al., 1970). Aminocyclopropanecarboxylic acid is naturally expected in apple homogenates (Burroughs, 1957), and if lipoxygenase is also present, the complete system of Thimann is reproduced: hydroperoxide, reducing agent, and substrate.

Conclusions

This study establishes that the model system for ethylene biosynthesis developed by Thimann has mechanistic features outwardly similar to those in plants. However, many of its characteristics concerning inhibitors and the function of individual components do not agree. On the basis of these results, caution should be exercised concerning any cell-free ethylene-forming system requiring pyridoxal phosphate or manganese.

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Registry No. *cis*-MeACC, 93621-77-3; *trans*-MeACC, 93621-76-2; (1*S*,2*R*)-MeACC, 98244-45-2; (1*R*,2*S*)-MeACC, 98302-67-1; SALEN, 94-93-9; $Mn(sal)_2$, 15246-70-5; ACC, 22059-21-8; Mn, 7439-96-5; cyanide, 57-12-5; linoleic hydroperoxide, 7324-21-2; propene, 115-07-1; lipoxygenase, 9029-60-1; linoleic acid, 60-33-3; *tert*-butyl hydroperoxide, 75-91-2; hydrogen peroxide, 7722-84-1; pyridoxal phosphate, 54-47-7; salicylaldehyde, 90-02-8; 4-methoxy-2-hydroxybenzaldehyde, 673-22-3; aminoxyacetic acid, 645-88-5; ethylene, 74-85-1.

References

Adams, D. O., & Yang, S. F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 170–174.

Adlington, R., Baldwin, J., & Rawlings, B. (1983) *J. Chem. Soc., Chem. Commun.*, 290–291.

Amrhein, N., & Schneebeck, D. (1980) *Physiol. Plant. 49*, 62.

Asmussen, R., & Soling, H. (1957) *Acta Chem. Scand. 11*, 1331–1339.

Boller, P., Herner, R., & Kende, H. (1979) *Planta 145*, 293.

Boreham, C., & Chiswell, B. (1977) *Inorg. Chim. Acta 24*, 77–83.

Bousquet, J.-F., & Thimann, K. V. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1724–1727.

Burroughs, L. (1957) *Nature (London) 179*, 360–361.

Coleman, W., Boggess, R., Hughes, J., & Taylor, L. (1981) *Inorg. Chem. 20*, 700–726.

Cotton, F. A., & Wilkinson, G. (1972) in *Advanced Inorganic Chemistry*, pp 596, 846–849, 874–878, Wiley Interscience, New York.

Coulter, A., & Fenselau, C. (1972) *Org. Mass Spectrom. 6*, 105–111.

Evans, C., List, G., Dolev, A., McConnel, D., & Hoffman, R. (1967) *Lipids 2*, 432–434.

Galliard, T., Hulme, A., Rhodes, M., & Wooltorton, L. (1968) *FEBS Lett. 1*, 283–285.

Gardner, H. W., & Jursinic, P. A. (1981) *Biochim. Biophys. Acta 665*, 100–112.

Gardner, H. W., & Kleiman, R. (1981) *Biochim. Biophys. Acta 665*, 113–125.

Gardner, H. W., & Plattner, R. D. (1984) *Lipids 19*, 294–299.

Gibian, M., & Galaway, R. (1977) in *Bioorganic Chemistry* (van Tamelen, E. E., Ed.) Vol. I, pp 117–135, Academic Press, New York.

Guy, M., & Kende, H. (1984) *Planta 160*, 276–281.

Hoffmann, N., Yang, S. F., Ichihara, A., & Sakamura, S. (1982) *Plant Physiol. 70*, 195–199.

Kimura, H., & Stammer, C. H. (1983) *J. Org. Chem. 48*, 2440–2441.

Konze, J., & Kende, H. (1979) *Planta 146*, 293–301.

Konze, J., & Kwiatkowski, G. (1981) *Planta 151*, 320–326.

Lemon, H. W. (1947) *J. Am. Chem. Soc. 69*, 2998–3000.

Lieberman, M., & Mapson, L. (1964) *Nature (London) 204*, 343–345.

Lizada, M., & Yang, S. F. (1979) *Anal. Biochem. 100*, 140.

Mapson, L., March, J., Rhodes, M., & Wooltorton, L. (1970) *Biochem. J. 117*, 473–479.

Marnett, L., Bienkowski, M., & Pagels, W. (1979) *J. Biol. Chem. 254*, 5077–5081.

Mattoo, A., Achilea, O., Fuchs, Y., & Chalutz, E. (1982) *Biochem. Biophys. Res. Commun. 105*, 271–278.

Mayak, S., Legge, R., & Thompson, J. (1981) *Planta 153*, 49.

McKeon, T., & Yang, S. F. (1984) *Planta 160*, 84–87.

Metzler, D. (1957) *J. Am. Chem. Soc. 77*, 485–490.

Peiser, G., Wang, T.-T., Hoffman, N., Yang, S. F., Liu, H.-W., & Walsh, C. T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3059–3063.

Pirrung, M. C. (1983) *J. Am. Chem. Soc. 105*, 7207–7209.

Pirrung, M. C. (1985) *Bioorg. Chem. 13*, 219–226.

Pirrung, M. C., & McGeehan, G. M. (1983) *J. Org. Chem. 48*, 5143–5144.

Pirrung, M. C., & McGeehan, G. M. (1985) *J. Am. Chem. Soc.* (submitted for publication).

Rani, L., Dandeya, K. B. & Singh, R. P. (1981) *J. Indian Chem. Soc. 58*, 73–74.

Sanders, T., Pattee, H., & Singleton, J. (1975) *Lipids 10*, 568.

Streckert, G., & Stan, H. (1975) *Lipids 10*, 847.

Venis, M. (1984) *Planta 162*, 85–88.
Vinkler, C., & Apelbaum, A. (1983) *FEBS Lett. 162*, 252–256.
Vioque, A., Albi, M., & Vioque, B. (1981) *Phytochemistry 20*, 1473–1475.
Walsh, C., Pascal, R. A., Johnston, M., Raines, R., Dikshit, D., Krantz, A., & Honma, M. (1981) *Biochemistry 20*, 7509–7519.
Yu, Y., Adams, D. O., & Yang, S. F. (1979a) *Plant Physiol. 63*, 589.
Yu, Y., Adams, D. O., & Yang, S. F. (1979b) *Arch. Biochem. Biophys. 198*, 280–286.

Conversion of Bovine Cardiac Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase to a Heterodimer by Removal of 45 Residues at the N-Terminus of the Regulatory Subunit†

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ABSTRACT: The type II adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinase from bovine heart, consisting of a dimeric regulatory subunit and two catalytic subunits, was converted to a heterodimer by limited tryptic digestion. Loss of the tetrameric structure was accompanied by proteolysis of the regulatory subunit to a form with an apparent molecular weight of 45 000 vs. 52 000 for the native subunit. The proteolyzed subunit behaved as a monomer, in contrast to the dimeric native subunit. Amino acid sequence analysis established that proteolysis removed 45 residues at the N-terminus, indicating that these 45 residues constitute the dimerizing domain of this protein. The kinetic properties of this heterodimer were indistinguishable from those of the native tetramer: half-maximal kinase activation occurred at 48 nM cAMP with a Hill coefficient of 1.45, the regulatory subunit bound 1.5 equiv of cAMP with half-maximal binding occurring at 33 nM, and kinetics for dissociation of bound cAMP were biphasic, indicating the presence of two different binding sites. These observations suggest that residues 1–45 function only in the formation of dimers and that dimerization has little influence on other functional properties of the regulatory subunit. More extensive proteolysis cleaved the monomeric fragment at Lys-311. The fragments resulting from this second cleavage did not dissociate, and the complex inhibited the catalytic subunit in a cAMP-dependent manner.

The adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinases play an important role in mediating the effects of hormones that increase intracellular levels of cAMP (Krebs & Beavo, 1979; Flockhart & Corbin, 1982). These kinases exist in the form of a tetramer composed of a regulatory subunit, which is a homodimer, and two catalytic subunits. Addition of cAMP to the inactive holoenzyme results in dissociation of the complex into two catalytic subunits and a regulatory subunit dimer that binds 4 equiv of cAMP (Flockhart & Corbin, 1982). There are two major forms of the holoenzyme: type I and type II. These two forms have different regulatory subunits but the same catalytic subunits. Recently the amino acid sequences have been elucidated for the catalytic subunit from bovine heart (Shoji et al., 1983), for the type I regulatory subunit from bovine skeletal muscle (Titani et al., 1984), and for the type II regulatory subunit from bovine heart (Takio et al., 1984a). Comparison of the amino acid sequences (Takio et al., 1984b) has shown that the two types of regulatory subunit are structurally homologous as predicted from their functional similarity. The type II regulatory subunit has 400 residues, with residues 135–256 and 257–400 constituting the cAMP binding domains (Takio et al., 1984a).

The functional domains of both types of R[1] must include the following: two binding sites for cAMP, a domain that interacts with and inhibits C, and a domain responsible for dimer formation. The dimerizing domain is within the N-terminal 90 residues, since limited proteolysis of the regulatory subunit generates a monomeric C-terminal fragment of about 37 000 daltons (Corbin et al., 1978; Zoller et al., 1979) and a dimeric N-terminal fragment (Potter & Taylor, 1980). Additional evidence regarding the dimerizing domain comes from purified heterodimer forms, which can arise from proteolysis during purification of the cAMP-dependent protein kinase (Reimann & Rapino, 1974; Sugden & Corbin, 1976; Taylor & Stafford, 1978; Vogel & Heinz, 1980; Rannels et al., 1985). The heterodimer forms are cAMP-dependent and retain both binding sites for cAMP (Vogel & Heinz, 1980; Rannels et al., 1985). However, the size of the fragment

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[1] Abbreviations: AMP, adenosine 5′-monophosphate; cAMP, adenosine cyclic 3′,5′-phosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; R, regulatory subunit of the cAMP-dependent protein kinase; C, catalytic subunit of the cAMP-dependent protein kinase; R^T, the 45 000-dalton form of the regulatory subunit obtained by proteolysis of the holoenzyme with trypsin; R^S, the 36 000-dalton form of the regulatory subunit obtained by proteolysis of the holoenzyme with trypsin; SDS, sodium dodecyl sulfate; CAP, catabolite gene activator protein of *Escherichia coli*; Tris, tris(hydroxymethyl)-aminomethane; HPLC, high-performance liquid chromatography; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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