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A KINETIC STUDY OF SIMULTANEOUS SUICIDE INACTIVATION AND IRREVERSIBLE INHIBITION OF AN ENZYME. APPLICATION TO 1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) SYNTHASE INACTIVATION BY ITS SUBSTRATE *S*-ADENOSYLMETHIONINE

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This paper deals with the development of an experimental method for the kinetic study of the inactivation of an enzyme by a racemic mixture of an inhibitor, whose isomers operate as suicide substrate and irreversible inhibitor respectively. The ratio between the isomer concentration in the biological or commercial source must be determined, but no separation of them is required. The method involves a kinetic analysis and an experimental design that enables the affinity ($1/K_m$), rate of catalysis (k_{cat}), rate of inactivation (λ_{max}), efficiency of catalysis (k_{cat}/K_m) and efficiency of inactivation (λ_{max}/K_m) to be determined.

The method has been applied to the kinetic characterization of the inactivation of 1-aminocyclopropane-1-carboxylate (ACC) synthase from tomato fruits by its substrate, *S*-adenosylmethionine (AdoMet). The ratio between AdoMet isomers with respect to its sulfonium centre, namely (−)-AdoMet and (+)-AdoMet, present in the commercial sample used, has been determined by ^1H nuclear magnetic resonance.

KEY WORDS: ACC synthase, inactivation, irreversible inhibitor, *Lycopersicon esculentum* Mill., suicide substrate.

INTRODUCTION

Ethylene is a plant hormone which plays a major role in many aspects of plant growth and development, such as seed germination, leaf abscission, senescence of plant tissues and fruit ripening.[1,2]

The pathway by which higher plant tissues synthesize ethylene was revealed when a non-protein amino acid, identified as 1-aminocyclopropane-1-carboxylic acid

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Abbreviations: ACC: 1-aminocyclopropane-1-carboxylic acid; AdoMet: *S*-adenosyl-L-methionine; DTT: dithiothreitol; MTA: methyl-thioadenosine; PLP: pyridoxal phosphate.

(ACC),[3,4] was found to be the precursor of ethylene. ACC is formed from *S*-adenosyl-L-methionine (AdoMet) in a reaction catalyzed by ACC synthase (*S*-adenosyl-*L*-methionine: methylthioadenosine lyase, EC 4.4.1.14), a pyridoxal phosphate-dependent enzyme, first isolated from tomato fruit pericarp.[5] ACC synthase is considered as the key enzyme in ethylene biosynthesis,[6] mainly because of its induction prior to the onset of ethylene production. Likewise, ACC synthase shows a rapid turnover, as deduced from its short half-life of 0.5–2 h.[7,8]

Although the first evidence for a substrate-dependent inactivation of ACC synthase was obtained soon after its discovery in plant tissues,[5,9] it was not until recently that the first work on ACC synthase inactivation appeared.[10] In this work it was shown that the half life of a preparation of ACC synthase extracted from mung bean hypocotyls was reduced from 23.5 to 12 min when AdoMet concentration was increased from 40 to 150 μM. These findings corroborated those, previously communicated by Boller,[11] for a time-dependent inactivation of ACC synthase in ripening tomato pericarp. Lately, Satoh and Yang[12] confirmed that ACC synthase was inactivated by its substrate during catalysis.

Kinetic studies of the inactivation process of ACC synthase involve one main difficulty. It is known that the AdoMet molecule possesses two enantiomeric forms with respect to the sulfonium centre, (+)-AdoMet and (−)-AdoMet. While only the (−)-AdoMet isomer is found in plant tissue,[13] both forms are present in commercial samples of AdoMet in a variable enantiomeric ratio. In addition, (+)-AdoMet and (−)-AdoMet behave differently during catalysis.[14,15] Thus, the naturally occurring isomer, (−)-AdoMet, is the only one capable of acting as substrate and also of causing the suicide inactivation of the enzyme, while the (+)-AdoMet isomer is unable to give ACC but irreversibly inactivates the enzyme.

This high specificity of ACC synthase with respect to the sulfonium centre is also found in other AdoMet-utilizing enzymes, such as catechol *O*-methyltransferase (EC 2.1.1.6), phenylethanolamine *N*-methyltransferase (EC 2.1.1.28), histamine *N*-methyltransferase (EC 2.1.1.8) and hydroxyindole *O*-methyltransferase (EC 2.1.1.4), all of which use only the (−)-AdoMet isomer as a methyl donor.[16]

As regards the molecular mechanism of ACC synthase inactivation, the specific radiolabelling of the enzyme with [3,4-^{14}C]-AdoMet showed that the inhibition mechanism was based on the covalent linkage of a portion of the AdoMet molecule to the active site of ACC synthase,[12] probably through the formation of an intermediate L-vinylglycine-enzyme complex.[14,17] However, nothing is known about the molecular mechanism of the irreversible inactivation of the enzyme.

The selective behaviour of ACC synthase towards the two AdoMet enantiomers complicates kinetic studies because the variable enantiomeric ratio in the commercial sample of AdoMet might lead to different values in the kinetic constants for the enzyme. One way of avoiding these problems involves separation and purification of both diastereoisomers and then examining their ability to inactivate ACC synthase, which results in a very laborious protocol.[14] These problems in the separation of optical isomers and of contaminants in commercial reagents have led to the derivation of a kinetic analysis which considers single inactivators as well as non-purified mixtures.

The kinetic analysis of enzymatic inactivation induced by suicide substrates[18,19] has been developed for systems with significant[20,21] and negligible[22,23] substrate consumption during the assay time. The suicide inactivation of enzymes measured through coupling reactions[24] and of enzymes involving two substrates[25] has also

been characterized. These kinetic analyses have been applied to the study of the suicide inactivation of tyrosinase by catecholamines[24,26-29] and that of peroxidase by hydrogen peroxide and indoleacetic acid[30-32].

The enzymatic inactivation induced by irreversible inhibitors[33] has been analyzed by means of discontinuous methods.[34,35] Continuous methods have been developed by using one [36-38] or two [39] auxiliary substrates, with control of the substrate consumption,[40-42] as well as in the presence of coupling enzymes.[43,44] Furthermore, the reversible inhibition of enzymes by slow binding inhibitors[45] has also been characterized in the presence of auxiliary substrate.[37,46-50]

This approach has already been employed in the kinetic analysis of enzymes which use a reversible inhibitor and a suicide substrate stoichiometrically,[27] and for enzymes which use a mixture of enantiomers, one of them acting as a slow-binding inhibitor and the other as a classical reversible inhibitor.[51] In a similar way, in this paper we develop a kinetic analysis of the enzymatic inactivation originated by a racemic mixture of isomers acting as suicide substrate and as irreversible inhibitor, respectively. This method has been applied to the experimental inactivation of ACC synthase by racemate AdoMet, whose diasteromeric ratio (R) had been determined in the commercial sample used.

MATERIALS AND METHODS

Plant Tissue Preparation

Tomato (*Lycopersicon esculentum* Mill. nothovar. F1 "Lorena") fruits at the pink stage of ripening[52] were harvested from a local greenhouse (Cooperativa Duran S.A., Mazarron, Murcia). Once collected, the fruits were washed with distilled water and the pericarp was cut into small sections ($0.3 \times 0.2 \times 0.1$ cm). To obtain maximal ACC synthase activity different pre-treatments were assayed on the pericarp sections: wound, LiCl, auxin, ethanol and ethylene (data not shown). The most effective of these treatments was shown to be incubation under an ethylene atmosphere. Thus, for the experiments described here, 10 g of sections were placed on dry paper in Petri dishes and these placed into glass jars (650 ml volume) and ethylene injected through a septum-fitted hole at the top until a concentration of about 5 ppm of ethylene was reached. After an 18 h incubation period at room temperature in the dark, sections were immediately extracted or stored frozen in liquid nitrogen.

Extraction of ACC Synthase

Extraction of ACC-synthase from tomato pricarp was made following the protocol of Tsai *et al.*[53] with slight modifications. Pericarp sections were frozen in liquid nitrogen and ground in a chilled mortar. The triturate was then extracted in a Polytron homogeneizer with 1 M K-phosphate buffer, pH 8.0, containing 4 mM DTT, 5 μM PLP and 0.2% (v/v) Triton X-100 at a ratio of 1 ml/g fresh weight. The homogenate was centrifuged at 25,000 g for 20 min, and the supernatant filtered through glasswool. A 2-ml fraction of the filtrate was passed through a Sephadex G-50 column (1×14 cm, 7 ml bed volume) which was equilibrated with 20 mM K-phosphate buffer, pH 8.0, containing 0.1 mM DTT and 1 μM PLP. A 4-ml protein fraction was collected and assayed for ACC synthase activity and protein content as described below. All steps including gel chromatography were carried out at 4°C.

ACC Synthase and Protein Assays

ACC synthase activity was measured by incubating the enzyme extract at 30°C in 100 mM K-phosphate buffer, pH 8.0, containing 2 μM PLP (assay buffer). In all cases, the reaction was started by the addition of AdoMet. After 1 h incubation with continuous shaking, the reaction was stopped by adding 100 μl of 10 mM $HgCl_2$ and the amount of ACC present determined by its oxidation to ethylene with a highly basic mixture of NaOCl (5%) and saturated NaOH (2:1, v/v).[54] Ethylene formed was then quantified by gas chromatography. In all the experiments, blanks consisted of enzyme incubated under the same conditions but in the absence of AdoMet.

One unit of ACC synthase activity was defined as that amount of enzyme which catalyzed the formation of 1 nmol of ACC per hour under the stated conditions of assay, and the specific activity was expressed as units per milligram of protein. Protein was determined according to the method of Bradford[55] with bovine serum albumin as standard.

Assays for Inactivation of ACC Synthase

To determine the decay of the remaining ACC synthase activity, reaction media consisting of 2 ml of enzyme extract, 3 ml of assay buffer and 1 ml of different concentrations of AdoMet were prepared. At different intervals of time, 100-μl aliquots of these media were withdrawn and transferred to a basic reaction medium consisting of 0.4 ml of assay buffer and 0.1 ml of 75 μM AdoMet, and incubated for a subsequent 30 min period after which the ACC produced was quantified as described previously.

To characterize the inactivation process of ACC synthase, time course experiments were performed either varying the enzyme content or AdoMet concentration in 5 ml reaction vessels containing 600 μl of the corresponding reaction mixture. The ACC accumulation was measured at different time intervals, until no further ACC accumulation was detected. This maximal amount of ACC was assumed to be the "infinite product" of the reaction (P_∞). Then, the behaviour of P_∞ was analyzed in relation to substrate and enzyme concentration. In these reactions the following conditions were observed:

$$[S]_o \gg [E]_o \text{ and } P_\infty \ll [S]_o.$$

The data points are means of triplicate assays, and the corresponding standard deviations are shorter than symbols.

Estimation of the (−)-AdoMet/(+)-AdoMet Ratio in the Commercial Sample of AdoMet

AdoMet was purchased as the hydrogen sulphate salt (Boehringer Mannheim, Germany. Lot No. 10041233-40). This salt has been shown to exert no effect on ACC synthase activity even at concentrations up to 2 mM, contrary to other AdoMet salts such as chloride or toluenesulphonate.[56] The ^1H NMR spectrum at 25°C was obtained on a Brucker 200 AC spectrometer (200.13 MHz) by dissolving 2.5 mg AdoMet in 0.5 ml ^2H_2O and using sodium 3-(trimethylsilyl)tetradeuteriopropionate as internal standard. By comparison with the chemical shift values of the *S*-methyl protons of AdoMet obtained by Stolowitz and Minch,[57] two signals corresponding to both (−)-AdoMet and (+)-AdoMet were assigned and their ratio (R) calculated

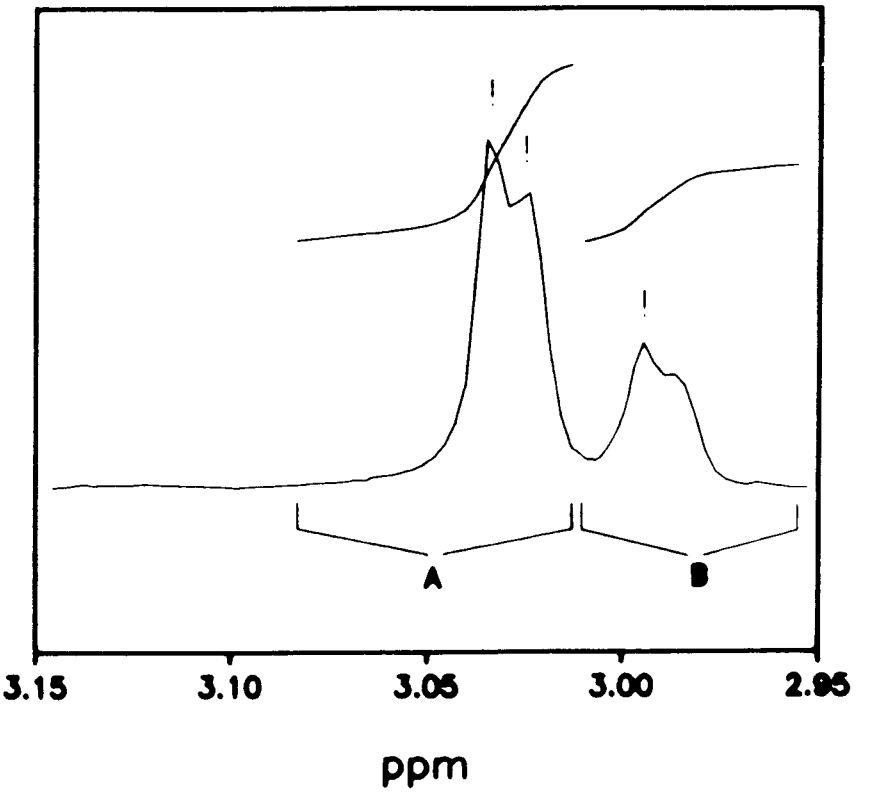


Figure 1 Estimation of the (−)-AdoMet/(+)-AdoMet ratio in the commercial sample used. Three ^1H 200.1-MHz resonance spectra were made on a 85.5 mM solution of AdoMet prepared in ^2H_2O. The *S*-Methyl protons of (−)-AdoMet and (+)-AdoMet gave two peaks with a chemical shift of 2.986 and 2.95 (in ppm relative to the internal standard), respectively. Integrating the area of these two peaks a (−)-AdoMet/(+)-AdoMet ratio (R) of 2.27 ± 0.11 was deduced.

based on the traces of their first derivatives (Figure 1). Under these conditions, a mean value of $R = 2.27 \pm 0.11$ was obtained for three samples of the commercial source of AdoMet.

RESULTS AND DISCUSSION

Preliminary Experiments

Initial proof of the inactivation process occurring with a sample of tomato ACC synthase was obtained from experiments in which the enzyme was incubated with increasing concentrations of AdoMet. At 15 min intervals, aliquots of these media were withdrawn and reincubated for a subsequent period of 30 min with 75 μM AdoMet. The ACC synthase activity obtained after the latter incubation was found to decay with time, and as a function of the initial AdoMet concentration, following

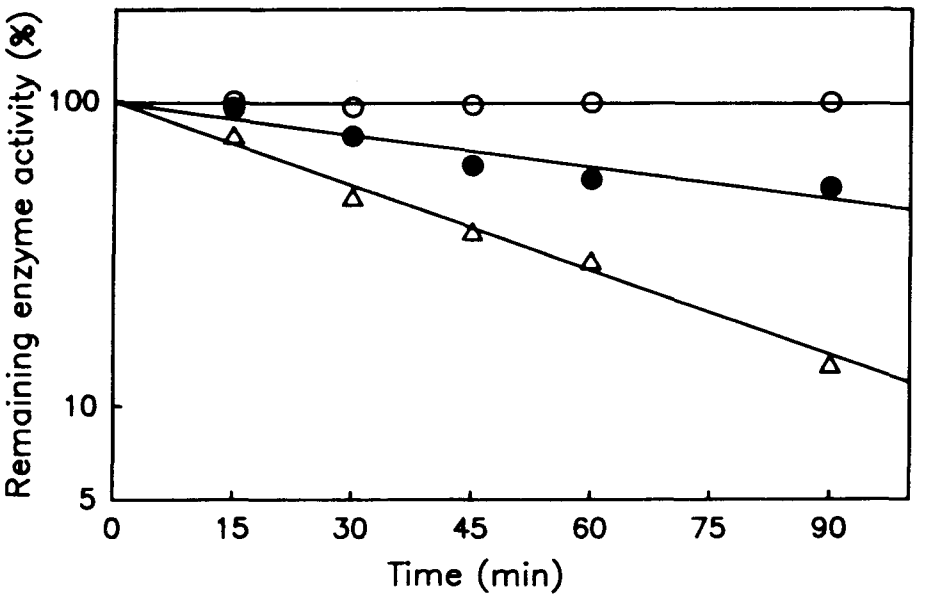


Figure 2 Dependence of the remaining ACC synthase activity on incubation time. A sample (2 ml) of enzyme extract (606.5 μg of protein/ml) was incubated with 1 ml of 0 (○), 50 (●), 125 (△) AdoMet in a final volume of 6 ml under the conditions described in Materials and Methods. At specific time intervals, a 100 μl-aliquot was withdrawn and the ACC synthase activity measured after a subsequent incubation period of 30 min with 75 μM AdoMet.

first order kinetics (Figure 2). The half life of ACC synthase in this preparation was reduced from 78.4 min when incubated with 50 μM AdoMet to 30.7 min with 125 μM AdoMet.

Effects of Enzyme and of Substrate Concentration

In order to analyze the kinetic behaviour of ACC synthase we collected progress curves of ACC production in the presence of several enzyme concentrations, estimated as protein content in the reaction medium. The amount of ACC formed at the end of the reaction (P_∞) was found to vary linearly with enzyme concentration (Figure 3A) and the same was found for the initial rate of the reaction (V_o) (Figure 3B). However, the value of the inactivation constant (λ) was seen to be independent of enzyme concentration (Figure 3C). The dependencies of these kinetic parameters on $[E]_o$ are in accordance with that predicted from equations 9A–11A (see Appendix).

We also tested the effect of the AdoMet concentration on the kinetics of ACC synthase inactivation. P_∞ did not change significantly, within the experimental error (Figure 4A), while V_o increased in a hyperbolic manner (Figure 4B). With regard to λ, a hyperbolic dependence on AdoMet concentration was revealed (Figure 4C). Therefore, the values of these kinetic parameters also changed when varying $[S]_o$ according to the behaviour predicted by equations 9A–11A (see Appendix).

A proposal for the mechanism of ACC synthase inactivation can be described by Scheme I.[18,19] In this mechanism, the reaction with isomer A of AdoMet [(−)-AdoMet] gives the product of the reaction, ACC, but also determines the suicide

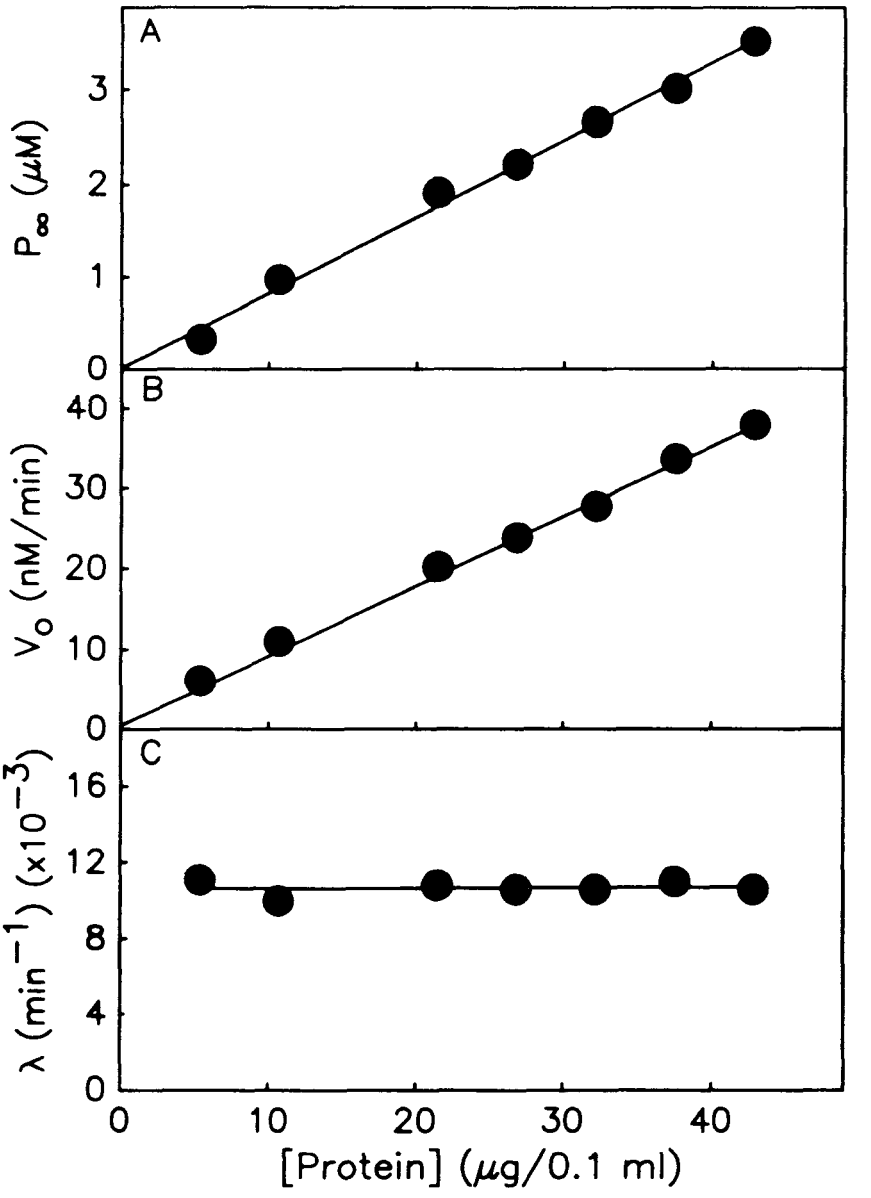


Figure 3 Relationship between inactivation parameters of tomato ACC synthase and protein content. (A) ACC formed at the end of the reaction (P_∞). Data were taken from time courses each with specific enzyme samples incubated with 100 μM AdoMet under the conditions described in Materials and Methods. (B) Dependence of the initial rate of reaction (V_o) on protein content. Data were taken from the same time courses as for Figure 3A by calculating the slopes of the curves during their linear phase. (C) Apparent inactivation constant (λ) of ACC synthase as a function of enzyme concentration, expressed as protein content in the extract. Data were obtained from remaining ACC synthase activity decay curves similar to those shown in Figure 2, with different enzyme concentrations. λ was calculated, according to the relationship: $\ln [X]/[E]_o = \ln a_r/a_o = -\lambda t$, (where a_r is the residual activity and a_o the initial activity of the enzyme) obtained from equation 4A.

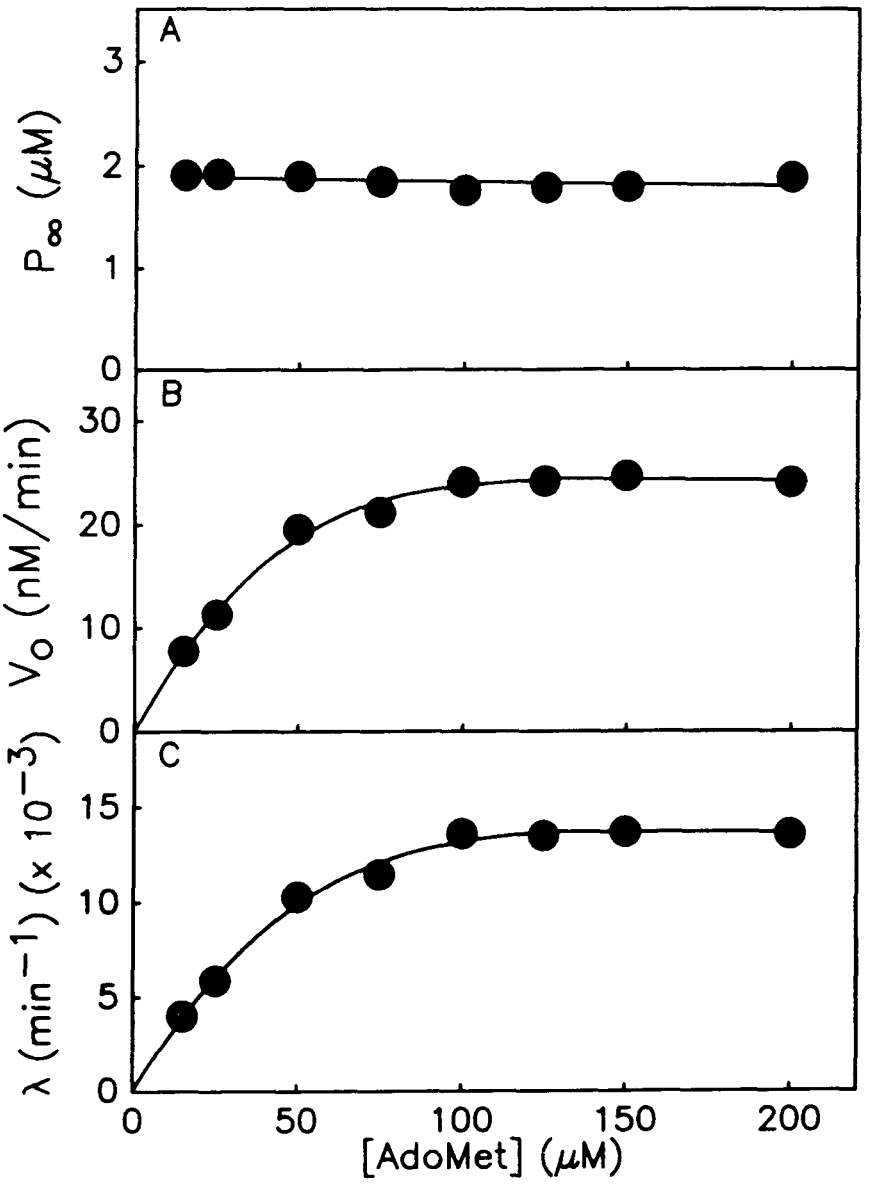
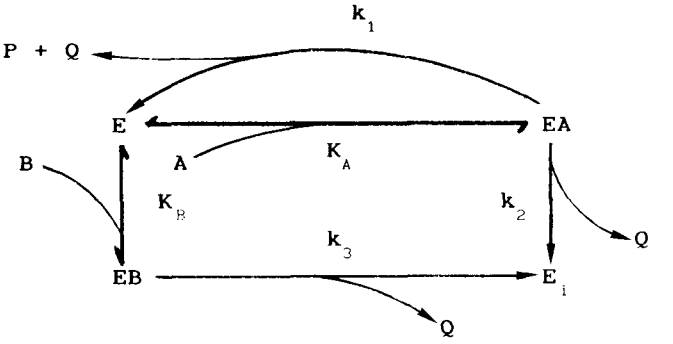


Figure 4 Relationship between inactivation parameters of tomato ACC synthase and substrate concentration. (A) ACC formed at the end of the reaction (P_∞). Data were taken from time courses consisting in 200 μl of enzyme extract (600 μg protein/ml), 100 μl of different AdoMet concentrations, and 300 μl of assay buffer incubated under the conditions described in Materials and Methods. (B) Dependence of the initial rate of reaction (V_o) on AdoMet concentration. Data were taken from the same time courses as in Figure 4A. (C) Apparent inactivation constant (λ) as a function of AdoMet concentration. Data were obtained from ACC synthase decay curves with different substrate concentrations, as explained for Figure 3C.



Scheme 1 Proposed catalytic and inactivation pathways of ACC synthase [14]. Keys for this Scheme are: A, (−)-AdoMet; B, (+)-AdoMet; P, ACC and Q, MTA.

inactivation of the enzyme, while the reaction with isomer B [(+)-AdoMet] does not give ACC and leads to irreversible inhibition of the enzyme.

The kinetic analysis of this mechanism is shown in the Appendix of this work and is based on the (−)-AdoMet/(+)-AdoMet ratio (R). This ratio may vary according to the commercial sample of AdoMet used.

Kinetic Data Analysis

The above experiments with different $[S]_o$ provide sets of V_o vs. $[S]_o$ (Figure 4B) and of λ vs. $[S]_o$ (Figure 4C) which can be fitted to equations 11A and 9A, respectively. Thus, values for K_m, V_{max} and λ_{max} have been determined (Table 1). The value obtained for K_m (20 μM) is similar to that reported by other authors.[5,9,12,58,59]

The catalytic constant under the conditions of our kinetic analysis is defined in equation 11A as follows: $k_{cat} = k_1 R/(1+R)$. k_1 has been given as 300 min^{-1} in the literature[60] and R has been calculated by us using NMR as 2.27 ± 0.11. Therefore, k_{cat} can be obtained from equation 11A (Table 1).

From equation 10A, it is also possible to calculate $r = k_{cat}/\lambda_{max}$, the number of catalytic turnovers that a mole of enzyme gives before being inactivated.[20–23] The value obtained for r (Table 1) involves the joint contribution of the suicide substrate and the irreversible inhibitor and, therefore, the enzyme must have been inactivated earlier than it was in the presence of the suicide substrate alone.[14]

Other kinetic constants that can be calculated from these data are the catalytic (k_{cat}/K_A) and inactivation (λ_{max}/K_A) efficiencies. The catalytic efficiency was found to be about four orders of magnitude higher than the inactivation efficiency (Table 1). This indicates that (±)AdoMet is a much better substrate than it is inactivator, even taking into consideration its actions as suicide substrate and as irreversible inhibitor.

An estimation of the enzyme concentration can be made using the obtained values for V_{max} and the catalytic constant (k_{cat}) (Table 1). The molecular weight for this enzyme in tomato has been calculated as 50000 Da.[12,59,61,62] Thus, our enzyme concentration calculation accounts for 0.00098% of the total protein content of the tissue, a value very similar to the 0.001% estimated by Bleecker *et al.*[60] and slightly lower than that given by Van der Straeten *et al.*[63]

Table 1 Kinetic constants of the inactivation of ACC-synthase from tomato fruits originated by (±)-AdoMet. Values determined and calculated from the experimental data of Figure 4

Constant	Value
$K_A \approx K_B = {}^{K}{}_{m}$ (μM)	20.1 ± 1.7
V_{max} (nM min^{-1})	24.7 ± 1.2
k_{cat} [(*)] (min^{-1})	$(20.8 \pm 2.0) \times 10$
λ_{max} (min^{-1})	$(13.9 \pm 0.6) \times 10^{-3}$
	$(15.0 \pm 2.1) \times 10^{3}$ r. [(**)]
k_{cat}/K_A (μM^{-1} min^{-1})	10.4 ± 1.9
λ_{max}/K_A (μM^{-1} min^{-1})	$(69.3 \pm 8.9) \times 10^{-5}$

[(*)]Value calculated by using the expression: $k_{cat} = k_i R/(1+R)$ (equation 11A), with[60] $k_i = 300$ min^{-1} and $R = 2.27 \pm 0.11$ (^{1}H NMR determination, see Materials and Methods section). [(**)]Value calculated by using the expression: $r = k_{cat}/\lambda_{max}$ (equation 10A).

In summary, the kinetic approach developed here allows the characterization of the affinity ($1/K_A$), rate of catalysis ($k_{cat} = \lambda_{max} r$), rate of inactivation (λ_{max}), efficiency of catalysis (k_{cat}/K_A) and efficiency of inactivation (λ_{max}/K_A), for enzymes inactivated by a racemic compound whose isomers operate as suicide substrate and as irreversible inhibitor, respectively. The further separation of the isomers, cumbersome when possible, enables the corresponding individual rate constants to be determined.[22-32] The applicability of the method is illustrated with the experimental study of ACC synthase (Table 1).

Although several features point to inactivation of ACC synthase as a process of physiological relevance, further studies are required in order to establish its actual involvement *in vivo*. For instance, the inactivation mechanism of ACC synthase based on the covalent linkage of a portion of AdoMet molecule allowed the sequencing of the active site of the enzyme and suggested the existence of several isoenzymes of ACC synthase.[64] However, whether ACC synthase inactivation participates in the enzyme turnover and, thus, in the control of the enzyme level *in vivo*, or in restoring the enzyme to its normal level after a stress situation still needs elucidation.

APPENDIX

KINETIC ANALYSIS

The postulated mechanism in Scheme I can be schematically written as follows:

$$E_i + Q < \underset{k_2 f_{EA} + k_3 f_{EB}}{\longrightarrow} X \underset{k_1 f_{EA}}{\longrightarrow} > P + Q$$

where X indicates the species included in the restricted steady state ($X = E + EA + EB$) and f_{EA}, f_{EB} represent the fractions of EA and EB existing in X as a function of K_A, K_B and concentration of A and B:[65-67]

$$f_{EA} = [A]_o K_B/(K_A K_B + [B]_o K_A + [A]_o K_B)$$

$$f_{EB} = [B]_o K_A/(K_A K_B + [B]_o K_A + [A]_o K_B) \qquad (1A)$$

The conditions under which the kinetic analysis is carried out are: $[A]_o, [B]_o \gg [E]_o$; $k_2, k_3 \ll k_1$ and $[S]_o = [A]_o + [B]_o$ with $R = [A]_o/[B]_o$. The system will progress with time according to the following equations:

$$[\dot{X}] = -(k_2 f_{EA} + k_3 f_{EB})[X] \tag{2A}$$

$$[\dot{P}] = k_1 f_{EA}[X] \tag{3A}$$

For $t = 0$, $[X] = [E]_o$, $[P] = 0$ and $[E_i] = 0$, and thus the resolution of these equations results in the following expressions:

$$[X] = [E]_o e^{-\lambda t} \tag{4A}$$

$$[P] = P_\infty(1 - e^{-\lambda t}) = \frac{V_o}{\lambda}(1 - e^{-\lambda t}) \tag{5A}$$

According to equation 5A, the parameters which define the inactivation process, the apparent inactivation constant (λ), the concentration of product at final time of reaction (P_∞), and the initial rate of the restricted steady-state (V_o) are given by the following expressions:

$$\lambda = k_2 f_{EA} + k_3 f_{EB} = \frac{k_2 K_B [A]_o + k_3 K_A [B]_o}{K_A K_B + [B]_o K_A + [A]_o K_B} \tag{6A}$$

$$P_\infty = \frac{V_o}{\lambda} = \frac{k_1 f_{EA}[E]_o}{k_2 f_{EA} + k_3 f_{EB}} = \frac{k_1 K_B [A]_o [E]_o}{k_2 K_B [A]_o + k_3 K_A [B]_o} \tag{7A}$$

$$V_o = k_1 f_{EA}[E]_o = \frac{k_1 K_B [A]_o [E]_o}{K_A K_B + [B]_o K_A + [A]_o K_B} \tag{8A}$$

In our case, the concentrations of the two enantiomeric forms of the substrate, (−)-AdoMet $[A]_o$ and (+)-AdoMet $[B]_o$ are related by means of the expressions: $[A]_o = R \times [B]_o$ and $[S]_o = [A]_o + [B]_o$. Furthermore,[15,68] $K_A \approx K_B = K_m$, and therefore the above equations can be written as:

$$\lambda = \frac{\lambda_{max}[S]_o}{K_m + [S]_o} = \frac{\frac{k_2 R + k_3}{R + 1}[S]_o}{K_B + [S]_o} \tag{9A}$$

$$P_\infty = \frac{V_o}{\lambda} = \frac{V_{max}}{\lambda_{max}} = \frac{k_1 R}{k_2 R + k_3}[E]_o = r[E]_o \tag{10A}$$

$$V_o = \frac{V_{max}[S]_o}{K_m + [S]_o} = \frac{\frac{k_1 R}{1 + R}[S]_o [E]_o}{K_B + [S]_o} \tag{11A}$$

From equation 10A, it is evident that $r = k_{cat}/\lambda_{max}$.

Experimental Design

When $[E]_o$ is known, for instance from active-site titration, a series of measurements with variation of $[E]_o$ yield values of P_∞ vs. $[E]_o$, which lead to determination of r from equation 10A. Then, further experiments at different $[S]_o$ provide sets of λ vs. $[S]_o$ values, enabling K_m and λ_{max} to be determined from equation 9A. Since $k_{cat} = \lambda_{max} r$ (equation 10A), all the kinetic constants detailed in Table 1 can therefore be determined.

For many enzymes, it is difficult to determine $[E]_o$, but the corresponding turnover number (k_{cat}) has been obtained by complex techniques and reported in the literature.[60] In this case, assays with the variation of $[S]_o$ supply values of λ vs. $[S]_o$ and V_o vs. $[S]_o$, which can be fitted to equations 9A and 11A, respectively, thus leading to the evaluation of K_m, V_{max} and λ_{max}. By using the value of k_{cat} from the literature, $r = k_{cat}/\lambda_{max}$ can be calculated (equation 10A), as well as the corresponding efficiencies of catalysis and inactivation (Table 1).

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