Phytochemistry, Vol. 24, No. 6, pp. 1141–1145, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

RELATIONSHIP BETWEEN 1-AMINOCYCLOPROPANECARBOXYLATE MALONYLTRANSFERASE AND D-AMINO ACID MALONYLTRANSFERASE

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(*Revised received* 12 *November* 1984)

Key Word Index—*Vigna radiata*; Leguminosae; 1-aminocyclopropane-1-carboxylic acid; D-amino acid; malonyltransferase.

Abstract—An enzyme preparation isolated from mungbean hypocotyls catalyses the malonyl-CoA-dependent *N*-malonylation of 1-aminocyclopropane-1-carboxylic acid (ACC), D-phenylalanine (Phe), D-methionine and 2-aminoisobutyric acid with K_m values of 0.15, 0.8, 3.4 and 5.1 mM, respectively; L-enantiomers of Phe and methionine were, however, not malonylated by the enzyme preparation. When ACC was tested on D-Phe malonyltransferase activity, or when D-Phe was tested on ACC malonyltransferase activity, these compounds exhibited competitive inhibition kinetics with K_i values similar to their respective K_m values. Such a relationship suggests that malonylations of ACC and D-amino acids are catalysed by the same enzyme. This view was further supported by the observations that the ratio ACC–D-Phe malonyltransferase activities remained constant throughout various fractionation steps and both enzyme activities were inhibited similarly by various sulphydryl reagents and 1-aminocycloalkane-1-carboxylic acids.

INTRODUCTION*

Ethylene, an important plant hormone, is biosynthesized in plant tissues via the following sequence: methionine → *S*-adenosylmethionine → ACC → ethylene [1]. Recently, Amrhein *et al.* [2, 3] and Hoffman *et al.* [4] have shown that ACC is effectively conjugated into MACC in higher plants. Since MACC is a poor ethylene precursor and the conjugation of ACC to MACC is essentially irreversible, MACC is thought to be a biologically inactive end-product of ACC [2, 5]. Malonylation of ACC to MACC participates in the regulation of ethylene biosynthesis by reducing the level of free ACC which, in turn, results in reducing the rate of ethylene production [2, 6].

N-Malonyl-D-amino acids have been isolated from many plant tissues following administration of D-amino acids [7–16]. In all cases the conjugates are synthesized only form the D-enantiomers. Since ACC has no asymmetric carbon, it can be recognized as a D- or L-amino acid. *In vivo* studies have established that D-amino acids inhibited the formation of MACC from exogenous ACC and ACC inhibited the formation of *N*-malonyl-D-amino acids from exogenous D-amino acids [2, 12, 16]. These results indicate that malonylations of D-amino acids and of ACC are intimately interrelated.

Recently, a malonyltransferase preparation from mungbean hypocotyls which catalyses the malonylation of ACC has been reported by Kionka and Amrhein [17]. Although they have shown that several non-polar D-amino acids effectively inhibited ACC malonyltransferase, the kinetic properties of D-amino acid malonyltransferase and the relationship between ACC malonyltransferase and D-amino acid malonyltransferase were not examined. In this paper we present experimental data showing that malonylation of ACC and of D-amino acids is mediated by the same enzyme.

*Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AIB, 2-aminoisobutyric acid; Ala, alanine; DTT, dithiothreitol; Gly, glycine; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid; MAIB, 2(-malonylamino)isobutyric acid; Met, methionine; MetSO, methionine sulphoxide; MPhe, *N*-malonyl-D-phenylalanine; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; Phe, phenylalanine.

RESULTS AND DISCUSSION

Distribution and characteristics of malonyltransferase

To study the distribution of ACC malonyltransferase in different parts of mungbean seedlings, seedlings were separated into root, hypocotyl and cotyledon parts and the enzyme activity in each part was individually measured. Since the extract from hypocotyls contained 96% of the enzyme activity, mungbean hypocotyls were routinely employed as the enzyme source.

The distribution of the enzyme activity in different fractions of ammonium sulphate saturation is shown in Table 1. *Ca* 11%, 69% and 28% of the protein was recovered in the fraction of 0–30%, 30–60% and 60–100% saturation, respectively. However, most (66% of the total activity) of the ACC malonyltransferase and D-Phe malonyltransferase activities were found in the fraction of 30–60% ammonium sulphate saturation. It is to be noted that *ca* 30% of the enzyme activity for both malonyltransferases was lost during the ammonium sulphate fractionation. Although the fraction at 30–60% saturation had the highest sp. act. among the ammonium sulphate fractions, its sp. act. was not higher than that of crude extract. The cause of this loss in enzyme activity is not known. Since the fraction at 30–60% saturation contained most of the enzyme activity and was con-

Table 1. Fractionation of malonyltransferase

Fraction	Protein (mg)	Total activity (nmol/hr) MACC	MPhe	Specific activity (nmol/mg·hr) MACC	MPhe
Supernatant of crude extract	86.7	3000	1543	34.6	17.8
0–30% $(NH_4)_2SO_4$ ppt	9.3	44	29	4.7	3.1
30–60% $(NH_4)_2SO_4$ ppt	59.7	1975	1026	33.1	17.2
60–100% $(NH_4)_2SO_4$ ppt	24.1	27	—*	1.1	—*
Supernatant after 100% $(NH_4)_2SO_4$ precip.	0	0	0	0	0

Mungbean hypocotyls (72.5 g) were homogenized and fractionated as described in the Experimental. The reaction mixture contained 1 mM malonyl-CoA, 0.25 mM [2,3-^{14}C]ACC (1 nCi/nmol) or D-[3-^{14}C]Phe (2.5 nCi/nmol), 0.1 M KCl, 0.1 M potassium phosphate (pH 8) and 15–20 μl enzyme solution in a final volume of 50 μl. Incubation was at 35° for 1 hr.

*Activity is too low to be accurately estimated.

centrated, this fraction was routinely prepared and employed as the enzyme source.

ACC malonyltransferase exhibited maximal activity (31 nmol/mg protein·hr) at pH 8; the enzyme activity at pH 6 was only *ca* 10% of that at pH 8 and at pH 9 it decreased by *ca* 25%. The optimal temperature for the enzyme activity was 35°. The enzyme activity was, therefore, routinely assayed at pH 8 and 35°. Under these conditions, the amount of reaction product formed was linear with incubation time for at least 2 hr, after which the rate declined slightly. Apparent K_ms for ACC and malonyl-CoA were estimated to be 0.15 and 0.5 mM, respectively, from the double-reciprocal plots of MACC formation with variable concentrations of ACC (Fig. 1) and malonyl-CoA. These values were close to the values of 0.17 and 0.25 mM reported by Kionka and Amrhein [17].

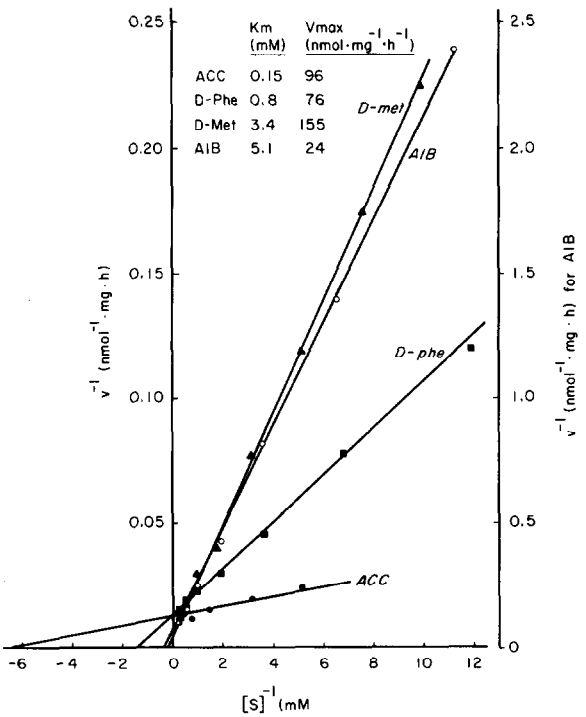

Fig. 1. Double-reciprocal plots of malonyltransferase activity vs concentration of different amino acids as substrates. The reaction mixture and incubation conditions were as in Table 1 except that [2,3-^{14}C]ACC, [1-^{14}C]AIB, D-[3-^{14}C]Phe and D-[3,4-^{14}C]Met (all at 0.25 mM) were used.

The effect of different divalent metal ions (0.5 mM) on the ACC malonyltransferase activity is shown in Table 2. The ions Hg^{2+} and Cu^{2+} inhibited the enzyme activity markedly, whereas Mn^{2+} and Mg^{2+} showed no effect. Inhibition of the enzyme activity was *ca* 50–60% with 0.5 mM Co^{2+} and Zn^{2+}. Since heavy metal ions, such as Hg^{2+} and Cu^{2+} can form mercaptides with sulphydryl groups of the protein [18], other sulphydryl reagents, such as PCMB and NEM were, therefore, tested. PCMB was a more potent inhibitor than NEM. These results suggest that sulphydryl groups are involved at the active site of the enzyme.

Table 2. Inhibition of malonyltransferase activity by various divalent metal ions and sulphydryl reagents

Addition	Inhibition of MACC formation (%)	Inhibition of MPhe formation (%)
None	0	0
Co^{2+}	50	—
Zn^{2+}	57	—
Cu^{2+}	88	—
Hg^{2+}	98	98
NEM 0.1 mM	0	0
1 mM	51	68
PCMB 0.1 mM	54	76
1 mM	100	98

The reaction mixture and incubation conditions were as in Table 1 except that 0.5 mM divalent metal ions were added as chloride salts. The enzyme activities without additions were 35.6 and 16.7 nmol/mg·hr, respectively, for MACC and MPhe formation.

Identification of the reaction products of ACC, AIB and D-amino acids

After labelled ACC, AIB, D-Met, or D-Phe was incubated with malonyl-CoA and the enzyme preparation, its reaction products were analysed by PC. Except for D-Met, only two radioactive spots were observed: one of the radioactive spots was the unreacted substrate, ACC, D-Phe or AIB, respectively, whereas the other spot cochromatographed with labelled MACC (R_f 0.57), MPhe (R_f 0.79) or MAIB (R_f 0.72), respectively, which had been synthesized *in vivo* as described in the Experimental. When D-Met was employed as the substrate, the reaction mixture showed the presence of Met, MetSO, *N*-malonyl-Met (R_f 0.72) and *N*-malonyl-MetSO (R_f 0.21). MetSO and *N*-malonyl-MetSO were characterized by the production of Met and *N*-malonyl-Met, respectively, upon reduction with mercaptoethanol as described by Liu *et al.* [12]. The following evidence indicates that the radioactive products observed were indeed *N*-malonyl amino acids: (a) no such product were detected when malonyl-CoA was omitted from the reaction; (b) when these products were subjected to acid hydrolysis, the corresponding free amino acids were recovered, which were verified by paper co-electrophoresis and co-chromatography with the authentic ACC, AIB, D-Met or D-Phe; and (c) the standard conjugates isolated *in vivo* have been identified by various techniques including GC/MS as *N*-malonyl conjugates of ACC, D-Met or D-Phe [3, 4, 12, 15, 16].

The relationship of malonylation of ACC and of D-amino acids

It has been established *in vivo* that only D- but not L-amino acids can form *N*-malonyl conjugates [11–15]. When D-Phe, D-Met and AIB were incubated with the same enzyme preparation used for ACC malonyltransferase, the corresponding *N*-malonyl conjugates of these amino acids were identified. The K_m values for D-Phe, D-Met and AIB were estimated to be 0.8, 3.4 and 5.1 mM, respectively, as compared to the value of 0.15 mM for ACC; the V_{max} values for the corresponding amino acids were 76, 155 and 24 nmol/mg·hr, respectively, as compared to 96 nmol/mg·hr for ACC (Fig. 1). ACC had the lowest K_m value of those amino acids tested.

The inhibition of ACC malonylation by D-amino acids and the inhibition of D-amino acid malonylation by ACC have been established *in vivo* [2, 12]. The inhibitory effects of various D- or L-amino acids on ACC malonyltransferase activity is shown in Table 3. Although nearly all of the amino acids tested had an inhibitory effect on MACC formation, D-amino acids exerted greater inhibition than their corresponding L-amino acids. Among the amino acids tested, D-Phe exerted the greatest inhibition, whereas L-alanine had little inhibitory effect on MACC formation. Although, L-Met and L-Phe inhibited MACC formation by *ca* 20%, no *N*-malonyl conjugates were detected when L-Met and L-Phe were employed as substrates. These data suggest that these two L-amino acids have some limited ability to compete with ACC for the binding sites, but cannot serve as the substrates of malonyltransferase. These *in vitro* observations are in full agreement with the data obtained *in vivo*. Amrhein *et al.* [2], Liu *et al.* [12, 16] and Kionka and Amrhein [17] have applied various amino acids including Phe, Met, tryptophan, serine, aspartic acid, alanine and AIB to mungbean hypocotyls and oat leaf segments and observed that D-enantiomers were more effective than their respective L-enantiomers in inhibiting MACC formation and that, among those amino acids examined, D-Phe was the most effective followed by D-Met. Reciprocally, D-Phe malonyltransferase was effectively inhibited by ACC. At 0.25 mM ACC inhibited 68% of D-Phe conjugation while, at 10 mM, it almost completely inhibited the malonylation of D-Phe. The mutual inhibitory effect of D-amino acids and ACC on the malonylation reactions *in vivo* has been reported by Liu *et al.* [12]. Thus, an intimate relationship between malonylations of ACC and D-amino acids *in vitro* and *in vivo* is established. If the malonylations of D-amino acids and ACC were catalysed by the same enzyme then, among D-Phe, D-Met and AIB, D-Phe which has the lowest K_m would be the most potent inhibitor of ACC malonyltransferase, whereas AIB which has the highest K_m would be the least effective inhibitor (Fig. 1). The data of Table 3 indicate this is the case. Kinetics of the inhibition of ACC malonyltransferase by D-Phe and, reciprocally, the inhibition of D-Phe malonyltransferase by ACC were, therefore, studied. By Dixon plots (Fig. 2), the K_i value of ACC as a competitive inhibitor on D-Phe malonyltransferase was determined to be 0.15 mM, and the K_i of D-Phe on ACC malonyltransferase to be 1.0 mM. Thus, these experimental K_i values are close to or identical to their respective K_m values of 0.15 mM for ACC and 0.8 mM for D-Phe. An agreement between K_i values and their corresponding K_m values is expected if the malonylations of ACC and D-Phe are carried out by the same enzyme. These data support the view that the malonylations of D-amino acids and ACC are catalysed by the same enzyme.

Table 3. Inhibition of ACC malonyltransferase by various amino acids

Addition	Inhibition (%)
None	0
AIB	24
Gly	9
D-Ala	34
L-Ala	2
D-Met	43
L-Met	20
D-Phe	76
L-Phe	19

The reaction mixture and assay conditions were as in Table 1 except that 5 mM amino acids were added. The enzyme activities in the absence of addition were 35.6 nmol/mg·hr.

Inhibition by cyclic amino acids

Since ACC is a cyclic amino acid, it is of interest to examine the inhibitory effect of various cyclic amino acids on ACC and D-Phe malonyltransferase. Table 4 shows that all of the cyclic amino acids tested inhibited ACC malonyltransferase, but the inhibition became smaller as the ring size became larger. Moreover, the relative effectiveness of these cyclic amino acids on the inhibitions of

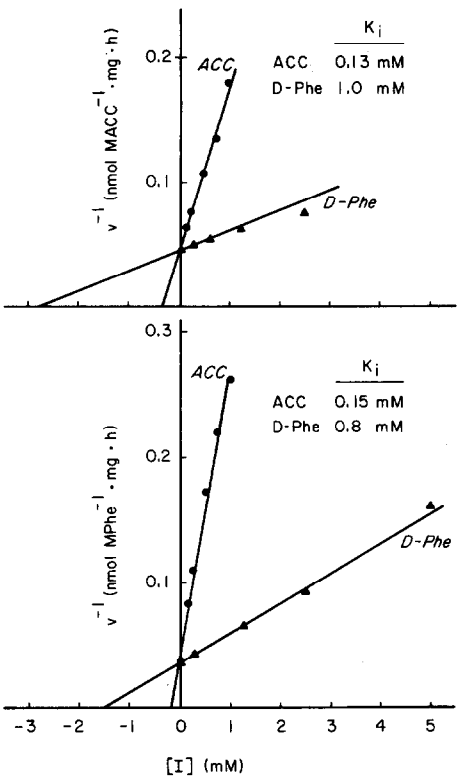

Fig. 2. Dixon plots for reciprocal of malonyltransferase activity vs concentration of different amino acids as inhibitors. The reaction mixture and incubation conditions were similar to those described in Table 1, except that 0.25 mM [2,3-^{14}C]ACC was used as the substrate and unlabelled ACC or D-Phe was employed as the inhibitor (upper panel) or 0.7 mM D-[3-^{14}C]Phe was used as the substrate and unlabelled ACC or D-Phe was employed as the inhibitor (lower panel).

Table 4. Inhibition of ACC and D-Phe malonyltransferase activities by various 1-aminocycloalkane-1-carboxylic acids

Addition	Inhibition (%) MACC formation	MPhe formation
None	0	0
	83	95
	42	76
	16	54
	13	40

The reaction mixture and incubation conditions were as in Table 1 except that 10 mM cyclic amino acids were added. The enzyme activities in the absence of cyclic amino acids were 22.5 nmol MACC/mg · hr and 11.4 nmol MPhe/mg · hr, respectively.

D-Phe malonyltransferase followed the same order as those on ACC malonyltransferase. These observations are in agreement with the view that malonylations of ACC and D-amino acids are catalysed by the same enzyme.

The view that malonylations of ACC and D-amino acids are catalysed by the same enzyme is further supported by the observation that the ratio of ACC malonyltransferase activity to D-Phe malonyltransferase activity remains roughly constant throughout the enzyme fractionation steps (Table 1) and both enzyme activities are inhibited similarly by various sulphydryl reagents (Table 2). Comparing the stereoselectivity of ACC malonyltransferase toward stereoisomers of 1-amino-2-ethylcyclopropane-1-carboxylic acid, Liu *et al.* [19] have observed that those two stereoisomers, which have an *R*-configuration as a D-amino acid, have lower K_m and K_i values than their enantiomers, which have an *S*-configuration as an L-amino acid. These results suggest that ACC is recognized by the enzyme as a D-amino acid and support the view that ACC malonyltransferase and D-amino acid malonyltransferase are the same enzyme. However, the confirmation that a single enzyme carries out both malonylation reactions awaits purification of the enzyme. If there is indeed only one enzyme, it is pertinent to ask whether the primary evolutionary role of the enzyme in plants is to regulate ethylene production by decreasing the ACC level or to detoxify D-amino acids.

EXPERIMENTAL

Chemicals. Malonyl-CoA was purchased from Sigma. [2,3-^{14}C]ACC was prepared from *S*-adenosyl-L-[3,4-^{14}C]methionine with ACC synthase prepared from aged tomato fruit slices [4]. *S*-Adenosyl-L-[3,4-^{14}C]methionine, D-[3,4-^{14}C]Met and DL-[3-^{14}C]Phe were products of Research Products International Corp. [1-^{14}C]AIB was from Amersham International. D-[3-^{14}C]Phe was prepared from DL-[3-^{14}C]Phe, which had been reacted with L-amino acid oxidase to oxidize L-Phe to phenylpyruvic acid as described in ref. [20]. When the reaction was complete, the reaction mixture was passed through a Dowex 50 (H^+ form) column and D-Phe was eluted from the column with 2 M NH_4OH. When DL-[3-^{14}C]Phe was employed as the substrate, two anionic reaction products were obtained; one was MPhe and the other was tentatively identified as cinnamic acid probably by the action of L-Phe ammonia-lyase in the enzyme preparation. This product was not observed when D-[3-^{14}C]Phe was employed as the substrate. 1-Aminocyclobutane-1-carboxylic acid and 1-amino(3-cyclopentene)-1-carboxylic acid were generously provided by Professor C. T. Walsh and Professor J. E. Baldwin, respectively. 1-Aminocyclopentane-1-carboxylic acid (cycloleucine) was from Sigma and 1-aminocyclohexane-1-carboxylic acid was obtained from Aldrich Chemical Co.

Enzyme extraction. Etiolated mungbean (*Vigna radiata* L.) hypocotyls grown and prepared as previously described [12, 16] were homogenized with an equal wt of an extraction medium containing 0.1 M KCl, 0.1 M KPi (pH 7.2) and 0.4 mM DTT at 0°. The homogenate was centrifuged at 12 000 *g* for 30 min and the supernatant was passed through a layer of glass wool. The filtrate (crude extract) was fractionated by pptn with $(NH_4)_2SO_4$. The fraction ppting at 30–60% satn was collected by centrifugation at 12 000 *g* for 30 min. The pptant was re-dissolved in a vol. of the extraction medium *ca* 1/12 of plant material wt and was dialysed overnight against a buffer soln containing 0.01 M KCl, 0.01 M KPi (pH 7.2) and 0.2 mM DTT at 0°. Unless noted otherwise, this dialysed preparation was employed as the enzyme source. Protein concn was determined by the method of ref. [21].

The protein concn was *ca* 6 mg/ml. The enzyme can be stored at −20° for 1 month with a loss of less than 30% in enzyme activity.

Identification of reaction products. The formation of *N*-malonyl conjugates of ACC, D-Phe, D-Met and AIB from their respective amino acids in mungbean hypocotyls and other plant tissues has been established [2, 9, 12–16]. For chromatographic comparison, these radioactive conjugates were prepared from radioactive ACC, D-Phe, D-Met or AIB with mungbean hypocotyls and purified as described previously [12, 16]. The identification of radioactive enzymatic products was performed by PC using *n*-BuOH–HOAc–H_2O (4:1:1.5) as the solvent system. In addition, the enzymatic products were hydrolysed with 2 M HCl at 100° for 3 hr, and the resulting radioactive hydrolysates were identified by PC and electrophoresis as described previously [5, 12, 16].

Enzyme assay. A standard reaction mixture consisted of 0.1 M KPi (pH 8), 0.1 M KCl, 1 mM malonyl-CoA, 0.25 mM radioactive ACC or D-Phe, enzyme soln containing *ca* 0.1 mg protein and various test chemicals with concns as indicated, in a total vol. of 50 μl. Incubation was at 35° for 1 hr. At the end of the incubation, the reaction was stopped by passing the reaction mixture through a small column (bed vol = 0.3 ml) of Dowex 50 (H^+ form) ion-exchange resin which adsorbs the unreacted free amino acids. The radioactivity in the effluent containing *N*-malonyl conjugates was determined by a Beckman liquid scintillation counter.

Acknowledgements—We thank Dr. N. E. Hoffman for preparing the radioactive ACC. We are also grateful to Professor N. Amrhein for helpful discussion and for providing us with the manuscript of ref. [17] prior to publication. This work was supported by Grant PCM-8414971 from the National Science Foundation.

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