DEAMINATION AND γ-ADDITION REACTIONS OF VINYLGLYCINE BY L-METHIONINE γ-LYASE

Nobuyoshi Esaki, Tetsuya SUZUKI*, Hidehiko TANAKA+, Kenji SODA‡ and Robert R. RANDO**

*Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, *Research Institute for Food Science, Kyoto University, Uji, Kyoto-Fu 611, +Laboratory of Biochemistry, Kyoto College of Pharmacy, Kyoto 607, Japan* and **Department of Pharmacology, Harvard Medical School, Boston, MA 02115, USA*

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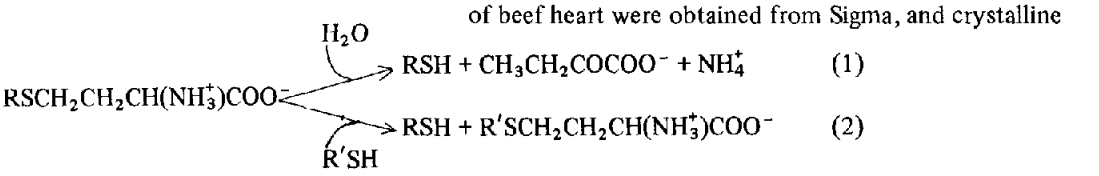
1. Introduction

Recently we have purified L-methionine γ-lyase to homogeneity from *Pseudomonas putida* (*Ps. ovalis*), and showed that the enzyme catalyzes α,γ-elimination and γ-replacement reactions of L-methionine and its derivatives, and also α,β-elimination and β-replacement reactions of *S*-substituted-L-cysteines [1,2]. A Schiff base between pyridoxamine 5′-phosphate and 2-keto-3-butenoic acid is regarded as a key intermediate in the α,γ-elimination (1) and γ-replacement (2) reactions as reviewed by Snell and Di Mari [3], and Davis and Metzler [4].

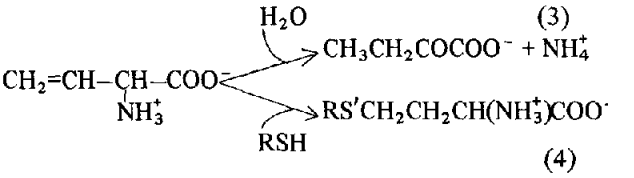


$$RSCH_2CH_2CH(NH_3^+)COO^- \xrightarrow{H_2O} RSH + CH_3CH_2COCOO^- + NH_4^+ \quad (1)$$

$$RSCH_2CH_2CH(NH_3^+)COO^- \xrightarrow{R'SH} RSH + R'SCH_2CH_2CH(NH_3^+)COO^- \quad (2)$$

The conversion of vinylglycine into α-ketobutyrate was demonstrated with serine–threonine dehydratase of sheep liver [5] and tryptophan synthase of *Escherichia coli* [6]. Cooper et al. [7] reported the γ-addition reaction of vinylglycine by L-amino acid oxidase of snake venom.

The present investigation was undertaken to elucidate whether vinylglycine can be a substrate for deamination (3) and γ-addition (4) reactions by L-methionine γ-lyase.



$$CH_2{=}CH{-}CH(NH_3^+){-}COO^- \xrightarrow{H_2O} CH_3CH_2COCOO^- + NH_4^+ \quad (3)$$

$$CH_2{=}CH{-}CH(NH_3^+){-}COO^- \xrightarrow{RSH} RS'CH_2CH_2CH(NH_3^+)COO^- \quad (4)$$

vinylglycine

2. Materials and methods

DL-Vinylglycine [8] and *S*-substituted homocysteines [2] were prepared as described previously. α-Ketobutyric acid and crystalline lactate dehydrogenase of beef heart were obtained from Sigma, and crystalline glutamate dehydrogenase of beef liver was from Boehringer Mannheim Gmbh. L-Methionine γ-lyase was purified to homogeneity from *Pseudomonas putida* (*Ps. ovalis*) [9] IFO 3738 as described [2].

The enzymatic α,γ-elimination reaction was followed by determining α-ketobutyrate by a method described [2,10] with a quarter scale reaction system (final vol. 0.5 ml). The reaction mixture for the stoichiometric studies contained 4 μmol DL-vinylglycine as a substrate. The γ-replacement and γ-addition reactions were assayed as described [2] except that 10 μmol DL-vinylglycine was used as a substrate. Ammonia was determined with indophenol [11] and with glutamate dehydrogenase [12]. DL-Vinylglycine was determined

‡To whom correspondence should be addressed: Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-FU 611, Japan

with ninhydrin after separation by paper chromatography [13].

Gas chromatography–mass spectrometry was performed with a Shimadzu LKB-9000 gas chromatograph–mass spectrometer. Gas chromatography was run on a column (3 mm i.d. × 2 m) packed with 10% Silar 10 C on Gas Chrom Q 100–120 mesh. Helium was used as carrier gas, flow rate 30 ml/min. Injection port was kept at 200°C. The column was programmed from 70–200°C at a rate of 6°C/min. Mass spectra were taken at 70 eV with 3.5 kV of accelerating voltage.

3. Results

3.1. *Deamination of vinylglycine*

After the reaction mixture containing DL-vinylglycine was incubated at 30°C for 30 min and 60 min, the residual substrate and the products were determined. Ammonia was identified and determined chemically and enzymatically as described above. α-Keto acid derived from vinylglycine reacted with lactate dehydrogenase and NADH to yield NAD. The product was identified as α-ketobutyrate by a comparison of the R_f values of its 2,4-dinitrophenylhydrazone with those of the authentic α-ketobutyrate by paper chromatography, carried out at 25°C using several solvent systems, e.g., 1-butanol/water/ethanol (5:1:1, v/v/v) (R_F 0.6) and methanol/benzene/1-butanol/water (4:2:2:2, v/v/v/v) (R_F 0.8). An attempt also was made to identify the product by gas chromatography–mass spectrometry. After incubation for 2 h, the reaction mixture was deproteinized by addition of 0.1 ml 6 N HCl followed by centrifugation. The supernatant solution was passed through Dowex 50-H^+. The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in a small volume of ethylether, and esterified with diazomethane derived from *p*-tosyl-*N*-methyl-*N*-nitrosoamine [14]. The derivative, which showed a peak at 9.6 min by gas chromatography, was identical with the standard methyl α-ketobutyrate. The mass spectrum of the authentic methyl α-ketobutyrate was identical with that of the product; molecular ion peak at *m/e* 116, fragment peaks at 101 (CH_3CH_2CO–CO–O^+), 59 (CH_3O–$\overset{+}{C}$=O), 57 ($CH_3CH_2C{\equiv}O^+$), 43 ($CH_3C{\equiv}O^+$), and 29 ($CH_3\overset{+}{C}H_2$), among which those at *m/e* 29 and *m/e* 57 were much more abundant than the others. Thus, the product was identified as α-ketobutyrate, which was determined with MBTH. The results in table 1 indicate that the enzyme catalyzes reaction [3], and that the reaction proceeds stoichiometrically.

3.2. *γ-Addition reaction of vinylglycine*

When vinylglycine was incubated with the enzyme in the presence of 1-propanethiol, the formation of a sulfur-containing amino acid was observed by paper chromatography. To isolate and identify the amino acid, incubation was carried out at 30°C for 2 h with a ten-times larger scale reaction mixture. After deproteinization the reaction mixture was chromatographed on Toyo filter paper No. 51 with 1-butanol/acetic acid/water (12:3:5, v/v/v) as a solvent. The area corresponding to the position of the product was cut off and eluted with water. The product was crystallized from the eluate as described [2].

The infrared spectra of the product and the authentic *S-n*-propylhomocysteine were identical. On elemental analysis of the enzymatic product, the following result was obtained. Anal. calcd. for *S-n*-propylhomocysteine ($C_7H_{15}NO_2S$): C, 47.5; H, 8.5; N, 7.9%. Found: C, 47.6; H, 8.3; N, 8.0%. These results reveal that the compound is *S-n*-propylhomocysteine. When 1-propanethiol was replaced by several other thiols (e.g., ethanethiol, 1-butanethiol, toluenethiol, benzene-

Table 1
Stoichiometry of reaction (3)

Incubation time (min)	DL-Vinylglycine disappeared (μmol)	α-Ketobutyrate formed (μmol)	Ammonia formed (μmol)
0	0	0	0
30	1.80	1.75	1.80
60	2.00	2.07	2.03

thiol), the corresponding sulfur amino acids were formed. They were identified paper-chromatographically by a comparison of their R_F values with those of the authentic *S*-substituted homocysteines using two solvent systems, 1-butanol/acetic acid/water (12:3:5, v/v/v) and phenol/water (4:1, w/v).

On addition of the substrates which undergo, α,γ-elimination reaction (e.g., L-methionine and DL-methionine sulfone), the enzyme exhibits a new absorption shoulder around 480 nm, which shows a positive CD band. This shoulder decreases as the substrates are converted into α-ketobutyrate, NH_3 and RSH. Incubation with vinylglycine also gives rise to the same absorption shift. The D- and L-amino acids which are not substrates for α,γ-elimination reaction are ineffective. The 480 nm shoulder is probably attributable to a reaction intermediate derived from a pyridoxal-P Schiff base of vinylglycine.

3.3. *Kinetics*

The kinetic parameters, K_m and V_{max} for DL-vinylglycine and L-methionine in reactions (3), (1) and (4), (2), in which 1-propanethiol was used as a substituent donor, are listed in table 2. The K_m value was calculated on the assumption that only the L-form is active as a substrate since D-methionine and D-cysteine are not substrates [2].

Vinylglycine has been shown to act as a suicide substrate upon L-aspartate aminotransferase [8,15], D-amino acid aminotransferases [16], and L-amino acid oxidase [17]. No inactivation of L-methionine γ-lyase occurred on incubation with 100 mM DL-vinylglycine at 25°C for several hours.

Table 2
Kinetic constants for reaction (1)–(4)

Reactions	K_m (mM)	V_{max} (μmol/mg/min)
Vinylglycine[a]		
Reaction (3)	15	1.81
Reaction (4)	12	1.39
L-Methionine		
Reaction (1)	1.3	1.88
Reaction (2)	11	1.80

[a]DL-Vinylglycine was used as a substrate and the active concentration is assumed to be a half of that of the DL-isomer

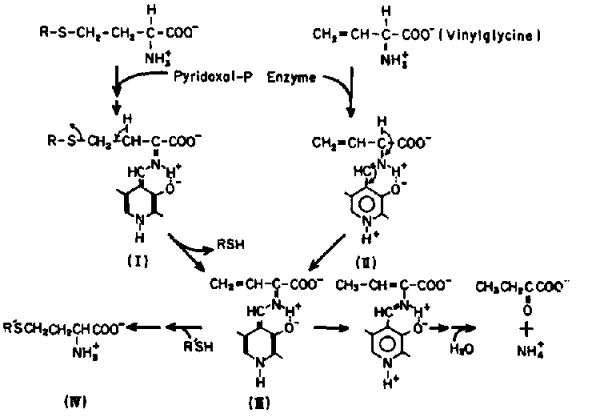
Table 3
Relative activity of alkanethiols

RSH R=	Vinylglycine (%)	Methionine (%)
Ethyl–	100	100
n-Propyl–	82	81
n-Hexyl–	23	17
Benzyl–	72	71

The enzyme shows a high affinity for L-methionine in α,γ-elimination reaction. The K_m and V_{max} values for methionine and vinylglycine in reactions (2), (3), (4) are closely similar. Relative activity of the enzyme for various alkanethiols in γ-addition reaction of vinylglycine is very close to that in γ-replacement reaction of methionine (table 3).

4. Discussion

The results obtained here support the proposed mechanism [3,4,18] for the α,γ-elimination and γ-replacement reactions through a pyridoxal-P Schiff base of vinylglycine as illustrated in Scheme 1. The formation of a ketimine quinoid intermediate of *S*-substituted-homocysteine (I) is followed by loss of a β-hydrogen and γ-substituent. The resulting β,γ-unsaturated compound (III) is derived also from a pyridoxal-P aldimine intermediate of vinylglycine (II). The intermediate III can undergo γ-addition of R'SH



Scheme 1

to form a product IV, or tautomerization followed by hydrolysis to form α-ketobutyrate and NH_3. More detailed investigations on the reaction mechanism are currently in progress in order to elucidate the nature of the intermediate observed spectrophotometrically.

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