Syntheses of *S*-Substituted L-Homocysteine Derivatives by Cystathionine γ -Lyase of *Streptomyces phaeochromogenes*

Hiroshi KANZAKI, Michihiko KOBAYASHI, Toru NAGASAWA
and Hideaki YAMADA

Department of Agricultural Chemistry, Faculty of Agriculture,
Kyoto University, Sakyo-ku, Kyoto 606, Japan

Received July 2, 1985

Cystathionine γ -lyase from *Streptomyces phaeochromogenes* catalyzes not only the α,γ -elimination reaction of L-cystathionine, but also the γ -replacement reaction of L-homoserine in the presence of thiol compounds. Substrates for the enzyme in the γ -replacement reaction were examined. It was found that D-cysteine, L- and D-homocysteine, and 3- and 2-mercaptopropionate served as preferable substrates in the γ -replacement reaction. D-Allocystathionine,^{*1} L- and meso-homolanthionine, *S*-carboxyethyl-L-homocysteine and *S*-methylcarboxymethyl-L-homocysteine were enzymatically synthesized from L-homoserine and the corresponding thiol compounds. The thus synthesized *S*-substituted L-homocysteine derivatives were isolated from large scale reaction mixtures and identified physicochemically.

Cystathionine γ -lyase (EC 4.4.1.1) is widely distributed in eukaryotic organisms, and plays an important role in the transfer of sulfur between L-homocysteine and L-cysteine. Previous studies^{1,2)} indicated that prokaryotic organisms lack cystathionine γ -lyase. However, we first investigated the distribution of cystathionine cleavage activity in prokaryotic microorganisms in detail, and found the wide distribution of cystathionine γ -lyase in actinomycetes.³⁾ Cystathionine γ -lyase occurs abundantly in *Streptomyces phaeochromogenes* (IFO 3105). We purified and crystallized the enzyme from *S. phaeochromogenes*. The *Streptomyces* enzyme catalyzes not only the α,γ -elimination reaction of L-cystathionine or L-homoserine (Eq. (1)), but also the γ -replacement reaction of L-homoserine to form L-cystathionine in the presence of L-cysteine (Eq. (2)). We optimized the reaction conditions to synthesize L-cystathionine enzymatically through the γ -replacement reaction.⁴⁾

In the present study we examined thiol compounds which can substitute for L-cysteine in the γ -replacement reaction. The enzymatic syntheses of five *S*-substituted L-homocysteine derivatives are described here.

MATERIALS AND METHODS

Materials. D-Homocysteine and L-amino acid oxidase (EC 1.4.3.2) from *Crotalus atrox* venom were obtained from Sigma. Hog kidney D-amino acid oxidase (EC 1.4.3.3) and catalase (EC 1.11.1.6) were purchased from Boehringer Mannheim. Aluminum sheets of silica gel 60 F₂₅₄ were obtained from Merck. All other chemicals were commercially available and of analytical grade.

*1 *S*-(D-2-Amino-2-carboxyethyl)-L-homocysteine.

Microorganism and preparation of cystathionine γ -lyase.

Streptomyces phaeochromogenes (IFO 3105), selected as a source of cystathionine γ -lyase, was cultivated as described previously.³⁾ Purification of the *Streptomyces* cystathionine γ -lyase was carried out according to the previous method.³⁾ In some cases, the partially purified enzyme prepared according to the previous method⁴⁾ was used.

One unit of the enzyme is defined as the amount needed to catalyze the formation of 1 μ mol of α -ketobutyrate per min under the standard reaction conditions.³⁾ The specific activity was defined as units per mg of protein. Protein was determined spectrophotometrically from the absorbance at 280 nm using an absorption coefficient of 0.839 $\text{mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}$, or by the method of Lowry *et al.*⁵⁾ using crystalline bovine serum albumin as a standard.

Analytical methods. The amounts of synthesized *S*-substituted L-homocysteine derivatives were determined with a standard amino acid analyzer K101-AS (Kyowa Seimitsu, Japan) using the respective purified *S*-substituted L-homocysteine derivatives as standards. The amounts of keto acids and cysteine were determined by the methods of Friedemann and Haugen⁶⁾ and Gaitonde,⁷⁾ respectively.

The ¹H-NMR spectrum was recorded with a JNM-PM $\times$ 60 (JEOL, Japan) in deuterium oxide with 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. The infrared spectrum was obtained with an IR-27G (Shimadzu, Japan). The mass-spectrum was obtained with a Hitachi M-80 mass spectrometer. Optical activity was measured with a Hitachi-Perkin-Elmer 241 polarimeter. Elemental analysis was carried out with a Yanagimoto C.H.N. recorder MT-1. Sulfur was determined by Wagner's method.⁸⁾

Testing of the enzymatic γ -replacement reaction. The reaction mixture (100 μ l) contained 50 mM L-homoserine, 10 or 500 mM various thiol compounds, 84 mM potassium phosphate buffer (pH 8.0), 0.08 mM pyridoxal 5'-phosphate (pyridoxal-*P*) and 0.029 units of the *Streptomyces* cystathionine γ -lyase. An aliquot of a reaction mixture was spotted on a silica gel plate after 1, 2 and 3-hr incubation at 30°C, followed by development with *n*-butanol-acetic acid-water (4:1:1, v/v). Then each plate was sprayed with ninhydrin solution (0.5% in acetone-ethanol (3:7, v/v)) to detect the newly formed amino acid. Furthermore, the formation of ninhydrin-positive compounds was reexamined with an amino acid analyzer.

Purification and isolation of enzymatically synthesized amino acids. When a considerable amount of an *S*-substituted L-homocysteine derivative had been accumulated in the reaction mixture, the reaction was stopped by adding trichloroacetic acid to a final concentration of 5% (w/v), and then the denatured protein was removed by centrifugation at 10,000 $\times g$ for 5 min. For the purification

of D-allocystathionine, the supernatant was applied to a Dowex 50 $\times$ 8 (H^+ form) column (2.4 $\times$ 36 cm). The column was washed with 1 N HCl and then washed fully with distilled water. In this step, L-homoserine, D-cysteine and keto acids were eluted. The synthesized D-allocystathionine was then eluted with 0.5 N ammonia solution. The eluates containing D-allocystathionine were pooled and concentrated *in vacuo*. The concentrated residue was dissolved in a small amount of water and then crystallization was induced by adding ethanol. Recrystallization was carried out in the same manner.

For the purification of L- or *meso*-homolanthionine, the supernatant was incubated with a sufficient amount of dithiothreitol (DTT) for about 4 hr at 30°C to reduce homocystine to homocysteine, and then *N*-ethylmaleimide was added to convert the homocysteine to homocysteinyl-*N*-ethylsuccinimide. The homocysteinyl-*N*-ethylsuccinimide could be easily separated from homolanthionine by Dowex 50 $\times$ 8 (H^+ form) column chromatography. This column chromatography was carried out in the same manner as described above.

In the cases of *S*-carboxyethyl-L-homocysteine and *S*-methylcarboxymethyl-L-homocysteine, the supernatant was applied to a Dowex 1 $\times$ 2 (CH_3COO^- form) column (2.4 $\times$ 36 cm). The column was washed with distilled water and then eluted with 0.1 N acetic acid. The eluates containing the synthesized amino acid were combined and concentrated *in vacuo*. Crystallization was carried out as described above.

Desulfuration and amino acid oxidase treatment of the synthesized S-substituted L-homocysteine derivatives. Raney nickel-W2 was prepared by the method of Mazingo.⁹⁾ Five mg of each synthesized amino acid was suspended in 15 ml of 33% (v/v) ethanol containing about 1 g of Raney nickel-W2, followed by refluxing for 2.5 hr. The nickel precipitate was washed three times with 10% (v/v) ammonia solution and then filtered. The filtrates were combined and then evaporated to dryness *in vacuo*. The residue was dissolved in water and then the resulting light green solution was extracted repeatedly to remove the concomitant nickel with ether containing 2-thenoyltrifluoroacetone. The aqueous phase was evaporated. The concentrated solution was divided into three parts. Each part was treated with L- or D-amino acid oxidase in 1.0 ml of a reaction mixture containing 0.15 μ mol of FAD, 1250 units of catalase, 0.1 mmol of potassium phosphate buffer (pH 8.0) and 1.75 units of L-amino acid oxidase (or 1.13 units of D-amino acid oxidase) at 30°C for 1 hr with shaking. The enzyme was replaced by water in the control. The reaction was stopped by adding trichloroacetic acid to a final concentration of 5% (w/v). After brief centrifugation, α -aminobutyrate and/or alanine in the supernatant was determined with an amino acid analyzer.

RESULTS AND DISCUSSION

Syntheses and isolation of sulfur-containing amino acids

We examined thiol compounds, which can substitute for L-cysteine in the γ -replacement reaction. The testing of synthetic reactions with various thiol compounds was carried out as described under MATERIALS AND METHODS.

D-Cysteine, L- and D-homocysteine, and 3- and 2-mercaptopropionate were found to serve as preferable substrates in the γ -replacement reaction with L-homoserine of *Streptomyces* cystathionine γ -lyase. Methyl, ethyl and *n*-propylmercaptans, and cysteamine were found to be inert as substrates for the γ -replacement reaction of the *Streptomyces* enzyme.

To isolate and identify the newly synthesized amino acids, large-scale syntheses were carried out.

(I) Synthesis of D-allocystathionine.

The reaction was carried out at 30°C in a starting reaction mixture (50 ml) containing 200 mM L-homoserine, 10 mM D-cysteine, 10 mM DTT, 100 mM potassium phosphate

buffer (pH 8.0), 0.1 mM pyridoxal-*P* and 11.2 units of the enzyme (specific activity: 0.06 units/mg). To keep the D-cysteine concentration constant, D-cysteine and DTT were fed to the reaction mixture after 1,2,3,4 and 5-hr incubation at the concentrations of 8, 8, 7, 5 and 5 mM, respectively. As shown in Fig. 1A, the amount of D-allocystathionine formed increased almost linearly, the maximum value of 28.3 mM (6.3 g/liter) being attained after 6.5-hr incubation, and at this time 14% and 66% of the added L-homoserine and D-cysteine had been converted to D-allocystathionine, respectively. After about 4.5-hr incubation, precipitation of the product was observed. Formation of keto acids was very low. Purification and isolation of D-allocystathionine were carried out as described under MATERIALS AND METHODS. Finally 165 mg of pure crystalline D-allocystathionine was obtained.

(II) Syntheses of L-homolanthionine and *meso*-homolanthionine.

L-Homolanthionine (or *meso*-homolanthionine) was synthesized in a reaction mixture (30 ml) containing 100 mM L-homo-

FIG. 1. Syntheses of *S*-Substituted L-Homocysteine Derivatives.

The reactions were carried out as described under MATERIALS AND METHODS. (A) Synthesis of D-allocystathionine. $\circ$, D-allocystathionine; $\bullet$, D-cysteine; $\triangle$, keto acids. (B) Syntheses of L- ($\circ$) and *meso*-homolanthionine ($\bullet$). L- or D-homocysteine (0.6 mmol) was added ($\rightarrow$), and the enzyme (4.8 units) was also fed to the reaction mixture after 3, 6 and 9 hr incubation. (C) Syntheses of *S*-carboxyethyl-L-homocysteine ($\circ$) and *S*-methylcarboxymethyl-L-homocysteine ($\bullet$). The enzyme was added three or four times ($\rightarrow$).

serine, 20 mM L-homocysteine (or D-homocysteine), 100 mM potassium phosphate buffer (pH 8.0), 0.1 mM pyridoxal-*P* and 12.1 units of the enzyme (0.02 units/mg). L- and D-Homocysteine were prepared by incubating L- and D-homocystine with DTT at 30°C for 2~5 hr, respectively. L- or D-Homocysteine was added to the reaction mixture four times (0.6 mmol each). The enzyme was also fed to the reaction mixture after 3, 6 and 9-hr incubation (4.8 units each). As shown in Fig. 1B, after 15-hr incubation, the yields of L-homolanthionine and *meso*-homolanthionine were 32.8 mM (7.78 g/liter) and 30.9 mM (7.31 g/liter), respectively. At this time, the conversion ratios of L-homoserine to L-homolanthionine and *meso*-homolanthionine were 32.8% and 30.9%, respectively. Purification and isolation were carried out as described under MATERIALS AND METHODS.

(III) Syntheses of *S*-carboxyethyl-L-homocysteine and *S*-methylcarboxymethyl-L-homocysteine.

The starting reaction mixture (25 ml) for the synthesis of *S*-carboxyethyl-L-homocysteine contained 200 mM L-homoserine, 2 M 3-mercaptopropionate, 100 mM potassium phosphate buffer (pH 8.0), 0.1 mM pyridoxal-*P* and 6.3 units of the enzyme (0.06 units/mg). For the synthesis of *S*-methylcarboxymethyl-L-homocysteine, 3-mercaptopropionate was replaced by 2-mercaptopropionate. The enzyme (4.8 units each) was fed three and four times to the reaction mixtures for *S*-carboxyethyl-L-homocysteine and *S*-methylcarboxymethyl-L-homocysteine, respectively (Fig. 1C). The amount of *S*-carboxyethyl-L-homocysteine formed reached 153 mM (31.7 g/liter) after 17-day incubation, accordingly 76% of the L-homoserine was converted to the product. Purification and crystallization were carried out as described under MATERIALS AND METHODS, and 600 mg of crystalline *S*-carboxyethyl-L-homocysteine was obtained. In the case of *S*-methylcarboxymethyl-L-homocysteine synthesis, the highest amount of the product was 108 mM (22.4 g/liter) after 23-day incubation, and the conversion of L-

homoserine to the product was 54%. Four hundred and thirty mg of *S*-methylcarboxymethyl-L-homocysteine was obtained as a white crystalline material.

Physicochemical properties and identification of products

The identification of the five products was carried out by infrared, ¹H-NMR and mass spectroscopy, elemental analysis and melting point measurement. The synthesized products showed only one ninhydrin-positive peak on amino acid analysis. The physicochemical properties of these *S*-substituted L-homocysteine derivatives are summarized in Table I. The infrared spectrum of *S*-carboxyethyl-L-homocysteine well agreed with the data of Kodama.¹⁰ The chemical shift of each proton in the ¹H-NMR spectra coincided with the structure of each compound. The presence of a molecular ion peak was definitely observed in the mass spectra of these amino acids, and the fragmentation pattern of *S*-carboxyethyl-L-homocysteine coincided with the spectrum reported by Posner.¹¹

The configurations of these products were determined by optical rotation measurement. The $[\alpha]_D^{25}$ of D-allocystathionine, L-homolanthionine, *meso*-homolanthionine and *S*-carboxyethyl-L-homocysteine determined were almost the same as the reported values.^{10,12,13} Furthermore, to confirm their configurations, Raney nickel desulfuration, followed by amino acid oxidation, was carried out as described under MATERIALS AND METHODS. It was determined from the sensitivity to L- or D-amino acid oxidase whether the formed amino acids were L- or D-isomers. In the case of the synthesized cystathionine, almost the same amounts of alanine and α -aminobutyrate were formed on desulfuration treatment, and the alanine and α -aminobutyrate formed were degraded by D- and L-amino acid oxidase, respectively (Table II). These results suggest that the C₃ unit of cystathionine is the D-form and the C₄ unit is the L-form; that is, the synthesized cystathionine is D-allocystathionine. The enzymatically synthe-

TABLE I. SUMMARY OF PHYSICOCHEMICAL PROPERTIES OF S-SUBSTITUTED L-HOMOCYSTEINE DERIVATIVES SYNTHESIZED BY *Streptomyces* CYSTATHIONINE γ -LYASE

For the ^1H -NMR spectra, the notations d, t and q refer to doublets, triplets and quartets, respectively. The notations Ha, Hb, Hc, Hd and He correspond to the protons indicated in the molecular formula for each compound.

Compound and molecular formula	Molecular ion peak in MS	Elemental analysis (%)				[α] _D ²⁵ in 1 N HCl	Melting point	¹ H-NMR spectra (ppm)	
		C	H	N	S				
D-Allocystathionine									
<div><div><div>HOOCCHCH₂CH₂CH₂CHCOOH</div><div><div>NH₂</div><div>NH₂</div></div><div><div>a</div><div>b</div><div>c</div><div>d</div><div>e</div></div></div></div>	223 ^a	calcd: found:	37.83 37.29	6.35 6.38	12.60 12.53	14.42 14.30	+24.5° (c = 1.0)	> 300°C	Ha: 4.33 (d) Hb: 3.20 (d) Hc: 2.82 (t) Hd: 2.31 (t) He: 4.22 (t)
L-Homolanthionine									
<div><div><div>HOOCCHCH₂CH₂CH₂SCH₂CH₂CHCOOH</div><div><div>NH₂</div><div>NH₂</div></div><div><div>a</div><div>b</div><div>c</div><div>c</div><div>b</div><div>a</div></div></div></div>	237 ^b	calcd: found:	40.67 40.56	6.82 6.65	11.86 11.93	13.57 13.65	+42.4° (c = 1.0)	264°C (dec.)	Ha: 4.28 (t) Hb: 2.35 (t) Hc: 2.78 (t)
<i>meso</i> -Homolanthionine									
<div><div><div>HOOCCHCH₂CH₂CH₂SCH₂CH₂CHCOOH</div><div><div>NH₂</div><div>NH₂</div></div><div><div>a</div><div>b</div><div>c</div><div>c</div><div>b</div><div>a</div></div></div></div>	237 ^b	calcd: found:	40.67 39.92	6.82 6.85	11.86 11.44	13.57 13.30	0 (c = 1.0)	266°C (dec.)	Ha: 4.24 (t) Hb: 2.32 (t) Hc: 2.78 (t)
S-Carboxyethyl-L-homocysteine									
<div><div><div>HOOCCH₂CH₂SCH₂CH₂CHCOOH</div><div><div>NH₂</div></div><div><div>a</div><div>b</div><div>c</div><div>d</div><div>e</div></div></div></div>	207 ^c	calcd: found:	40.57 40.34	6.32 6.51	6.76 6.75	15.47 15.19	+19.2° (c = 2.0)	252°C	Ha,Hb: 2.78 (t) Hc: 2.86 (t) Hd: 2.33 (t) He: 4.23 (t)
S-Methylcarboxymethyl-L-homocysteine									
<div><div><div>HOOCCHSCH₂CH₂CHCOOH</div><div><div>CH₃</div><div>NH₂</div></div><div><div>b</div><div>c</div><div>d</div><div>e</div></div></div><div>a</div></div>	207 ^c	calcd: found:	40.57 40.58	6.32 6.50	6.76 6.88	15.47 15.31	+95.8° (c = 2.0)	216°C	Ha: 1.43 (d) Hb: 3.63 (q) Hc: 2.86 (t) Hd: 2.33 (t) He: 4.23 (t)

The *a*, *b* and *c* values were obtained on field desorption MS (FDMS), secondary ion MS (SIMS) and in beam electron impact MS (EIMS), respectively. *a* and *b* were (M+H)⁺, and *c* was M⁺.

The syntheses of D-alloctathionine and homolanthionine by mammalian liver cystathionine γ -lyases were reported.^{14,15)} However, the products were not isolated or identified. We have shown for the first time that cystathionine γ -lyase catalyzes the γ -replacement reaction of L-homoserine in the presence of 3- or 2-mercaptopropionate, mercaptans having no-amino groups, to form S-carboxyethyl-L-homocysteine and S-methylcarboxymethyl-L-homocysteine, respectively, although S-carboxyethyl-L-homocysteine was synthesized from O-succinyl-L-homoserine and 3-mercaptopropionate by the cystathionine γ -synthase of *Salmonella typhimurium*.¹¹⁾

L-Homolanthionine and S-carboxyethyl-L-homocysteine occur in relatively large amounts in the urine of homocystinuric patients. Our present results suggest that cystathionine γ -lyase possibly participates in the formation of these amino acids in organisms.

Acknowledgments. The authors wish to thank Dr. T. Ueno and Dr. T. Nakashima for their technical assistance in the measurement of the mass spectra and Mr. Y. Koizumi for the ¹H-NMR spectra.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- 1) C. Delavier-Klutchko and M. Flavin, *J. Biol. Chem.*, **240**, 2537 (1965).
- 2) M. Flavin, "Metabolism of Sulfur Compounds," ed. by D. M. Greenberg, Academic Press Inc., New York, 1975, pp. 457~503.
- 3) T. Nagasawa, H. Kanzaki and H. Yamada, *J. Biol. Chem.*, **259**, 10393 (1984).
- 4) H. Yamada, H. Kanzaki and T. Nagasawa, *J. Biotechnol.*, **1**, 205 (1984).
- 5) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 6) T. E. Friedemann and G. E. Haugen, *J. Biol. Chem.*, **147**, 415 (1943).
- 7) M. K. Gaitonde, *Biochem. J.*, **104**, 627 (1967).
- 8) H. Wagner, *Mikrochim. Acta*, 19 (1957).
- 9) R. Mozingo, "Organic Syntheses," Coll. Vol. III, ed. by E. C. Horning *et al.*, John Wiley & Sons Inc., New York, 1955, pp. 181~183.
- 10) H. Kodama, *Physiol. Chem. Phys.*, **3**, 159 (1971).
- 11) B. I. Posner, *Biochim. Biophys. Acta*, **276**, 277 (1972).
- 12) W. P. Anslow, Jr., S. Simmonds and V. du Vigneaud, *J. Biol. Chem.*, **166**, 35 (1946).
- 13) S. Weiss and J. A. Stekol, *J. Am. Chem. Soc.*, **73**, 2497 (1951).
- 14) D. Deme and F. Chatagner, *Biochim. Biophys. Acta*, **258**, 643 (1972).
- 15) H. H. Tallan, T. A. Pascal, K. Schneideman, B. M. Gillam and G. E. Gaull, *Biochem. Biophys. Res. Commun.*, **43**, 303 (1971).
- 16) G. E. Gaull, Y. Wada, K. Schneideman, D. K. Rassin, H. H. Tallan and J. A. Sturman, *Pediat. Res.*, **5**, 265 (1971).