L-α-Amino-β-thio-ε-caprolactam, a new sulfur-containing substrate for α-amino-ε-caprolactam racemase

Syed Ashrafuddin Ahmed, Nobuyoshi Esaki, Hidehiko Tanaka and Kenji Soda

Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan

Received 25 june 1984

A one-step synthesis of a new sulfur-containing compound, L-α-amino-β-thio-ε-caprolactam from L-cysteine methyl ester and 2-chloroethylamine has been described. This intramolecular cyclic amide of *S*-(β-aminoethyl)-L-cysteine serves as a good substrate for α-amino-ε-caprolactam racemase. L-α-Amino-β-thio-ε-caprolactam is racemized more than 3 times faster and binds about 4-fold stronger to the enzyme than L-α-amino-ε-caprolactam itself. Optimum pH for racemization of this new compound is about 10.0.

L-α-Amino-β-thio-ε-caprolactam *S-(β-Aminoethyl)-L-cysteine-ε-lactam* *α-Amino-ε-caprolactam* *racemase* *Amine racemase* *Pyridoxal 5'-phosphate enzyme*

1. INTRODUCTION

α-Amino-ε-caprolactam (ACL) racemase is a unique enzyme among racemases and pyridoxal 5'-phosphate (pyridoxal-P) dependent enzymes in acting exclusively on ACL and α-amino-δ-valerolactam (AVL), the intramolecular cyclic amides of lysine and ornithine, respectively [1,2]. *Achromobacter obae* produces this cytoplasmic enzyme most abundantly, when it was grown in a medium containing various organic and inorganic nitrogen sources [3]. We have purified it to homogeneity [4] to study enzymological properties [1,2]. Inability of the enzyme to racemize amino acids and peptides, and competitive inhibition of the enzyme by ε-caprolactam, a substrate analog suggest that the substrate should have a free amino group adjacent to an amide bond in a cyclic structure [2].

S-(β-Aminoethyl)-L-cysteine in which the γ-methylene group of lysine is replaced by a sulfur atom acts as an antimetabolite of lysine for certain bacteria [5–7]. We found that this lysine analog serves as a poor substrate for L-lysine-α-ketoglutarate ε-aminotransferase of *Flavobacterium fuscum* [8], L-lysine decarboxylase of *Bacterium cadaveris* [9] and L-lysine α-oxidase of *Trichoderma viride* [10]. It is thus very interesting to investigate whether the intramolecular cyclic amide of *S*-(β-aminoethyl)-L-cysteine can act as a substrate or inhibitor of ACL racemase. We have found that α-amino-β-thio-ε-caprolactam (*S*-(β-aminoethyl)-L-cysteine-ε-lactam; SACL) is utilized as a good substrate by the enzyme. We here report the chemical synthesis of this new sulfur compound and its behavior as a substrate for ACL racemase.

2. MATERIALS AND METHODS

D- and L-ACL, D-α-monomethylamino-ε-caprolactam and D-α-dimethylamino-ε-caprolactam were kindly supplied by Dr Kyosuke Yotsumoto of Toray Ind., Japan. Pyridoxal-P was obtained from Kyowa Hakko Kogyo, Japan, and 2-chloroethylamine·HCl and L-cysteine methyl ester·HCl from Nakarai Chemicals, Japan.

L-SACL was synthesized as follows from cysteine methyl ester and 2-chloroethylamine. L-Cysteine methyl ester·HCl (15 g; 87.4 mmol) was dissolved in 150 ml absolute methanol, to which was added 74 ml (530 mmol) triethylamine. To this solution was added dropwise 15 g (130 mmol) 2-chloroethylamine·HCl dissolved in 150 ml ab-

Published by Elsevier Science Publishers B.V.

solute methanol over a period of 3 h. The resulted solution was refluxed for 11 h and then concentrated to dryness. Chloroform (200 ml) was added to the dry mass which was suspended by sonication. The precipitate obtained by filtration was again taken in 200 ml ethanol and suspended by heating and sonication. The precipitate collected by filtration was dried to give 2.4 g SACL, which was still contaminated by traces of *S*-(β-aminoethyl)cysteine and its methyl ester. The crude SACL was dissolved in 6 ml of water and was applied to a column (34 × 2.7 cm) of silica gel (Wakogel C-200, Wako, Japan) equilibrated previously with a solvent system of acetone–methanol–water (45:45:10, v/v/v), followed by elution with the same solvent system. SACL, which was eluted in the early fractions was free from the contaminants as checked by silica gel thin layer chromatography, and was concentrated to dryness under reduced pressure to yield a silky white residue. The material was further dried at 50°C under vacuum to give 1.83 g (10 mmol) of pure SACL · HCl. The yield was 11.5%. The compound gave a single spot upon silica gel thin layer chromatoplates in solvent systems, *n*-butanol–acetate–water (6:2:2, v/v/v), acetone–methanol (1:1, v/v), and methyl ethyl ketone–ethanol–ammonia–water (3:1:1:1, v/v/v/v), when visualized with ninhydrin. The structure of the compound was confirmed by ^{1}H-NMR, infrared, mass spectroscopic and elemental analyses. Fine white needle-shaped crystals of the compound could also be obtained from acetone–methanol–water (15:15:10, v/v/v). Analytical data for SACL synthesized are shown in table 1.

ACL racemase was purified from *A. obae* FERM-P 776 as in [4]. One unit of enzyme was defined as the amount of enzyme that catalyzes the racemization of 1 μmol D-ACL per min. Racemization of L-SACL was determined at 25°C by polarimetry with a Perkin-Elmer 241 polarimeter as described for ACL [4]. The standard reaction mixture contained 100 μmol L-ACL or L-SACL, 40 nmol pyridoxal-P, 100 μmol potassium phosphate buffer (pH 8.0) and enzyme in a final volume of 2.0 ml. The ^{1}H-NMR spec-

Table 1

Analytical data for α-amino-β-thio-ε-caprolactam · HCl

Melting point (°C)	222–225
$[\alpha]_D^{20}$ (*c*, 1.82, H_2O)	−17.85°
IR_{max}^{KBr} (cm^{-1})	3170 (vs, NH), 3100–2900 (br, vs, NH_3^+)
	1660 (vs, C = 0), 1600 (m, NH_3^+)
^{1}H-NMR (δ)	(in DMSO-d_6)
	8.25 (4H, br, s, NH and NH_3^+)
	4.40 (1H, t, CH)
	3.50 (2H, t, N-CH_2)
	2.6–2.8 (4H, q, -CH_2-S-CH_2-)
	(in D_2O)
	4.6 (1H, t, CH)
	3.64 (2H, t, N-CH_2)
	2.88–3.16 (4H, m, -CH_2-S-CH_2-)
MS (*m/e*)	146 (20, M^+-HCl)
	130 (01, 146-NH_2)
	104 (18, 146-NCO)
	88 (14, 104-NH_2)
	72 (55, 88-CH_4)
	43 (100, 104-C_2H_4S)
	36 (78, HCl)
Elemental analysis (%)	Found C, 32.61; H, 6.28; N, 15.36
For $C_5H_{11}ON_2SCl$	Calcd C, 32.87; H, 6.07; N, 15.33

IR (infrared): br, broad; m, medium; s, strong; vs, very strong. ^{1}H-NMR: br, broad; s, singlet; t, triplet; q, quartet; m, multiplet

trum was taken with a JEOL JNM-FX 100 spectrometer, mass spectrum with a JEOL JMS-DX 300 spectrometer operated at 70 eV, and infrared spectrum with a Hitachi IR spectrometer (Type 260-50).

3. RESULTS AND DISCUSSION

When L-SACL was incubated at 25°C with ACL racemase, the optical rotation decreased with time and reached zero after about 5 h (fig.1). The change in optical rotation followed a pseudo first-order kinetics (fig.1, inset) as observed for racemization of ACL [1]. Thin-layer chromatography of the reaction mixture showed that no ninhydrin-positive compound was produced from L-SACL. These indicate that L-SACL is racemized by ACL racemase. We have reported that the α-hydrogen of ACL and AVL is exchanged with solvent deuterium in ^2H_2O during the enzymatic racemization [2,11]. L-SACL (100 μmol) was incubated in the reaction mixture containing 40 nmol pyridoxal-P and the enzyme (5 units) in 2 ml ^2H_2O (99.85%) (Merck) (pD 8.0) at 30°C for 9 h. After removal of the enzyme by ultrafiltration through an Amicon PM-10 membrane, the filtrate was concentrated to dryness and the residue was dissolved in 0.05 ml DMSO-d_6. ^{1}H-NMR spectra

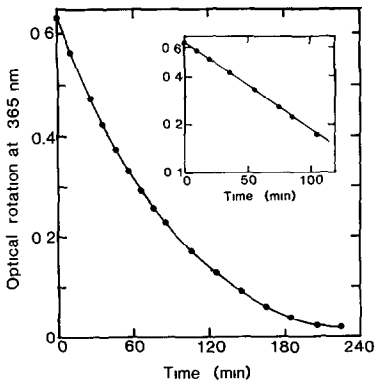

Fig.1. Racemization of L-α-amino-β-thio-ϵ-caprolactam catalyzed by α-amino-ϵ-caprolactam racemase. The reaction mixture (2.0 ml, pH 8.0) containing 100 μmol L-α-amino-β-thio-ϵ-caprolactam, 20 nmol pyridoxal 5'-phosphate and enzyme (0.4 unit) was incubated at 25°C. Inset, the same optical rotation change of the substrate is plotted in a semilogarithmic scale.

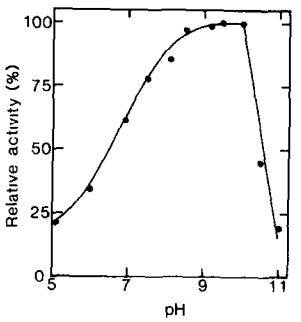

Fig.2. Effect of pH on the racemization of L-α-amino-β-thio-ϵ-caprolactam. The reaction mixture (2.0 ml) contained 40 μmol substrate, 40 nmol pyridoxal 5'-phosphate and the enzyme (0.17 unit). pH was adjusted with 0.5 N NaOH. The activities were measured by the optical rotation change at 365 nm.

of the reaction mixture showed that almost all the α-hydrogen (at δ 4.4 ppm) of SACL was replaced by deuterium during incubation with ACL racemase.

The rate of racemization of L-SACL by the enzyme was compared with that of L-ACL by polarimetry under the same conditions. L-SACL was found to be racemized more than 3-times faster than L-ACL. Reciprocals of the reaction velocities were plotted *vs* reciprocals of the substrate concentrations to determine its K_m value. The apparent K_m value calculated was 1.5 mM, which is much smaller than the K_m (6 mM) for L-ACL [4]. Except for acetyl CoA : *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase of *Aerobacter aerogenes* [12], *S*-(β-aminoethyl)-L-cysteine is a poor substrate for a number of lysine-metabolizing enzymes [5–7,13,14]. ACL racemase is thus quite different from these enzymes: the lactam of *S*-(β-aminoethyl)-L-cysteine is better as a substrate than that of L-lysine in terms of both reaction velocity and affinity to the enzyme. Since the ring size of L-SACL is similar to that of L-ACL the sulfur atom of L-SACL probably interacts noncovalently with a nucleophile on the binding site of the enzyme to exhibit enhanced binding of the substrate and so higher reaction velocity. In analogy to this assumption, we have observed that methylation of the α-amino group of D-ACL abolishes its substrate property and behaves as a competitive inhibitor: D-α-monomethylamino-ϵ-caprolactam and D-α-dimethylamino-ϵ-caprolactam have K_i values of

about 3 and 6 mM, respectively, with either D- or L-ACL as a substrate (unpublished).

The optimum pH for racemization of L-SACL by ACL racemase is shown in fig.2. The optimum pH is about 10.0, while L-ACL is racemized at a rate of only 30% of the maximum velocity at this pH [2]. The optimum pH for racemization of L-ACL is about 8.8 [2]. This difference in the pH optima of the two substrates could merely reflect the differences in the ionization of certain group(s) at the enzyme binding site, or a different reaction could also be catalyzed by the enzyme during racemization at a particular pH. A similar difference among substrates in the optimum pH values has been reported for arginine racemase of *Pseudomonas graveolens* [15] and amino acid racemase with low substrate specificity of *Ps. putida* [16]. As in arginine racemase, which transaminates between the enzyme bound pyridoxal-P and ornithine, a substrate for racemization [17], we have found that the difference between the pH optimum for racemization of L-ACL and L-AVL [2] catalyzed by ACL racemase also is based on the transamination of the enzyme bound pyridoxal-P with AVL [18]. Further investigation on the effect of pH in the racemization of L-SACL is currently in progress.

ACKNOWLEDGEMENTS

We thank Dr Kyosuke Totsumoto of Toray Ind. Inc., Japan, for generously supplying the samples of D- and L-ACL, D-α-monomethylamino-ϵ-caprolactam and D-α-dimethylamino-ϵ-caprolactam, and Dr Fumio Kimura for his helpful advice in the synthesis of L-SACL.

REFERENCES

[1] Ahmed, S.A., Esaki, N., Tanaka, H. and Soda, K. (1983) Agric. Biol. Chem. 47, 1887–1893.
[2] Ahmed, S.A., Esaki, N., Tanaka, H. and Soda, K. (1983) Agric. Biol. Chem. 47, 1149–1150.
[3] Ahmed, S.A., Esaki, N., Tanaka, H. and Soda, K. (1982) Bull. Inst. Chem. Res. Kyoto Univ. 5-6, 342–346.
[4] Ahmed, S.A., Esaki, N. and Soda, K. (1982) FEBS Lett. 150, 370–374.
[5] Shiota, T., Folk, J.E. and Tietze, F. (1958) Arch. Biochem. Biophys. 77, 372–377.
[6] Shiota, T., Mauraon, J. and Folk, J.E. (1961) Biochim. Biophys. Acta 53, 360–365.
[7] Sano, K. and Shiio, I. (1970) J. Gen. Appl. Microbiol. 16, 373–391.
[8] Soda, K., Misono, H. and Yamamoto, T. (1969) Biochim. Biophys. Acta 177, 364–367.
[9] Soda, K. and Moriguchi, M. (1969) Biochem. Biophys. Res. Commun. 34, 34–39.
[10] Kusakabe, H., Kodama, K., Kuninaka, A., Yoshino, H., Misono, H. and Soda, K. (1980) J. Biol. Chem. 255, 976–981.
[11] Ahmed, S.A., Esaki, N., Tanaka, H. and Soda, K. (1983) Seikagaku 55, 1250–1251.
[12] Tanaka, H. and Soda, K. (1974) J. Biol. Chem. 249, 5285–5289.
[13] Work, E. (1962) Biochim. Biophys. Acta 62, 173–174.
[14] Hope, D.B. (1965) Ciba Found. Study Group 19, 88.
[15] Yorifuji, T., Ogata, K. and Soda, K. (1971) J. Biol. Chem. 246, 5085–5092.
[16] Osumi, T. (1973) Doctoral Dissertation, Kyoto University.
[17] Yorifuji, T., Misono, H. and Soda, K. (1971) J. Biol. Chem. 246, 5093–5101.
[18] Ahmed, S.A., Esaki, N., Tanaka, H. and Soda, K. (1983) in: The Proceedings of the Annual Meeting of the Agricultural Chemical Society of Japan (Sendai), p.191.