The C₃-N Bond Cleavage of 2-Amino-3-(*N*-substituted-amino)-propionic Acids Catalyzed by L-Methionine γ -Lyase

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L-Methionine γ -lyase (EC 4.4.1.11) catalyzes α,β -elimination of L-2-amino-3-(*N*-methylamino)propionic acid and L-2-amino-3-(*N*-hydroxyethylamino)propionic acid to yield pyruvate, ammonia, and the corresponding amines. These amino acids also undergo the enzymatic β -replacement reaction with thiols to produce the corresponding *S*-substituted cysteines. Thus, L-methionine γ -lyase cleaves a C-N bond in addition to C-S, C-Se, and C-O bonds at the β position of amino acids by elimination and replacement reactions. A linear relationship between the reactivity, ($\log(V_{\max}/Km)$) and the pKa value of the conjugated acid of the leaving group has been found for *Se*-methyl-L-selenocysteine, *S*-methyl-L-cysteine, and *O*-methyl-L-serine. However, L-2-amino-3-(*N*-methylamino)propionic acid has shown lower reactivity than that expected from the pKa value of methylammonium ions.

Various non-protein amino acids containing nitrogen atoms in their side chains occur in nature. L-2,3-Diaminopropionic acid is a constituent of a polypeptide antibiotic, Viomycin, and occurs also in the free state in plant seeds.^{1,2)} 2-Amino-3-(*N*-oxalylamine)propionic acid has been found in plants, and shown to be neurotoxic to chicks, rats, and monkeys.³⁾ 2-Amino-3-ureidopropionic acid,⁴⁾ 2-amino-3-(*N*-acetamino)propionic acid,⁵⁾ and 2-amino-3-(*N*-methylamino)propionic acid⁶⁾ also have been demonstrated in plants. However, little is known about their metabolism. Only a few pyridoxal 5'-phosphate (pyridoxal-P) enzymes have been shown to act on them: cystathionine γ -lyase (EC 4.1.1.1) of rat liver⁷⁾ and diaminopropionate-ammonia lyase of *Salmonella typhimurium*⁸⁾ catalyze deamination of 2,3-diaminopropionic acid.

L-Methionine γ -lyase is a pyridoxal-P enzyme that cleaves C-S, C-Se, and C-O bonds at both β and γ positions of amino acids.⁹⁾ We have briefly reported its action of 2-amino-

3-(*N,N*-dimethylamino)propionic acid.¹⁰⁾ We here describe the enzymatic α,β -elimination and β -replacement reactions of 2-amino-3-(*N*-methylamino)propionic acid and 2-amino-3-(*N*-hydroxyethylamino)propionic acid to compare with those of S, Se, and O containing amino acids.

MATERIALS AND METHODS

Materials. L-Methionine γ -lyase (EC 4.4.1.11) was purified to homogeneity from an extract of *Pseudomonas putida* cells (ICR 3460) as reported previously.¹¹⁾ *S*-Methyl-L-cysteine, β -chloro-L-alanine, and lactate dehydrogenase of rabbit muscle were purchased from Sigma; *O*-methyl-DL-serine, dansyl chloride, and 2-acetaminoacrylic acid from Nakarai Chemicals.

L-Selenocystine was prepared as described previously.¹²⁾ L-Selenocystine was *Se*-methylated with methyl iodide after reduction with dithiothreitol.¹³⁾ The product was crystallized from 80% ethanol, and identified by ¹H-NMR (200 MHz, D₂O) and elemental analysis. ¹H-NMR (δ): 2.07 (3H, s, CH₃); 3.06 (1H, d, *J* = 6.5 Hz, CH₂); 3.07 (1H, d, *J* = 5.0 Hz, CH₂); 3.97 (1H, dd, *J* = 6.5 and 5.0 Hz, CH). Elemental anal. Calcd for C₄H₉NO₂Se: C, 26.39; H, 4.98;

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N, 7.69. Found: C, 26.23; H, 4.95; N, 7.66.

DL-2-Amino-3-(*N*-substituted-amino)propionic acids were synthesized by a modification of the method of Smith *et al.*¹⁴⁾ DL-2-Amino-3-(*N*-methylamino)propionic acid, DL-2-amino-3-(*N*-hydroxyethylamino)propionic acid, and DL-2-amino-3-(*N*-acetamino)propionic acid were synthesized from 2-acetaminoacrylic acid, and methylamine, ethanolamine and acetamide, respectively, as follows. 2-Acetaminoacrylic acid (1.5 g, 11 mmol) and amines (75 mmol) were dissolved in water (10 ml). The reaction mixture was allowed to stand at 40°C for 72 hr, and then dried *in vacuo*. The resulting pale yellow oil was freed from excess amine by repeated addition of ethanol and evaporation. Ether was added to the residue dissolved in ethanol until an oily product was separated from the solution. The oil was solidified at 4°C after several days. The crude solid was filtered and recrystallized from ethanol-ether. A solution of 2-acetamino-3-(*N*-substituted-amino)propionic acid (75 mmol) in 4*N* hydrochloric acid (140 ml) was refluxed for 5 hr. The solution was filtered, and the filtrate was dried *in vacuo*. The dark syrupy residue was dissolved in methanol. Ethylacetate was then added to the solution until it became slightly turbid. After chilling at 4°C, the crude material was formed. The product was purified by recrystallization from methanol-ethylacetate, and identified by ¹H-NMR and elemental analysis.

2-Amino-3-(*N*-methylamino)propionic acid monohydrochloride. ¹H-NMR (δ): 2.83 (3H, s, CH₃); 3.47 (1H, d, *J* = 6.1 Hz, CH₂); 3.49 (1H, d, *J* = 8.4 Hz, CH₂); 4.06 (1H, dd, *J* = 8.4 and 6.1 Hz, CH). Elemental anal. Calcd. for C₅H₁₁ClN₂O₂: C, 31.07; H, 7.17; N, 18.12. Found: C, 30.63; H, 7.01; N, 18.07.

2-Amino-3-(*N*-hydroxyethylamino)propionic acid. ¹H-NMR (δ): 3.04 (2H, t, *J* = 5.4 Hz, O—C—CH₂); 3.14 (1H, dd, *J* = 12.9 and 7.2 Hz, 3-CH₂); 3.23 (1H, dd, *J* = 12.9 and 6.3 Hz, 3-CH₂); 3.77 (1H, dd, *J* = 7.2 and 6.3 Hz, 2-CH); 3.81 (2H, t, *J* = 5.4 Hz, O—CH₂—C). Elemental anal. Calcd. for C₅H₁₂N₂O₃: C, 40.53; H, 8.16; N, 18.91. Found: C, 40.20; H, 8.03; N, 18.75.

2-Amino-3-(*N*-acetamino)propionic acid. ¹H-NMR (δ): 2.02 (3H, s, CH₃); 3.58 (1H, dd, *J* = 14.8 and 6.6 Hz, CH₂); 3.80 (1H, dd, *J* = 14.8 and 3.6 Hz, CH₂); 3.90 (1H, dd, *J* = 6.6 and 3.6 Hz, CH). Elemental anal. Calcd. for C₅H₁₀N₂O₃: C, 41.09; H, 6.90; N, 19.17. Found: C, 39.82; H, 6.68; N, 18.78.

Enzyme assay.

Method A. The enzymatic α , β -elimination of amino acids were routinely followed by measurement of the pyruvate formed. The standard assay system contained 200 μ mol of Tricine/KOH buffer (pH 8.0), 50 μ mol of amino acids, 0.01 μ mol of pyridoxal-P, and enzyme in a final volume of 1.0 ml. After incubation at 37°C for 10 min, the reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid. After centrifugation, pyruvate in the supernatant solution was measured with 3-methyl-2-

benzothiazolone hydrazone (MBTH) as described previously.¹⁵⁾

Method B. We used this method to measure initial velocities of α , β -elimination of amino acids producing thiols and selenols. The rate of α , β -elimination is decreased with an increase in the amounts of thiols and selenols accumulated, which leads to a β -replacement reaction. The reaction mixture contained the same ingredients as method A except that 0.1 μ mol of NADH and 1 unit of rabbit muscle lactate dehydrogenase were added. The reaction was started by the addition of L-methionine γ -lyase, and a decrease in absorption at 340 nm was measured. The amount of pyruvate was calculated from the molar absorption coefficient of NADH, 6,220 M⁻¹ cm⁻¹.

Method C. For the β -replacement reaction, the reaction system contained 200 μ mol of Tricine/KOH buffer (pH 8.0), 0.01 μ mol of pyridoxal-P, 10 μ mol of amino acids, 0.5 μ mol of thiols, and enzyme in a final volume of 1.0 ml. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid. The remaining thiol was extracted with ether, and the *S*-substituted cysteine was measured with a Hitachi high-performance amino acid analyzer (Model 835).

RESULTS

α , β -Elimination of 2-amino-3-(*N*-substituted-amino)propionic acids

When L-methionine γ -lyase reacted with DL-2-amino-3-(*N*-methylamino)propionic acid, pyruvate, ammonia, and methylamine were formed. DL-2-Amino-3-(*N*-hydroxyethylamino)propionic acid also served as a substrate to produce pyruvate, ammonia, and ethanolamine. DL-2-Amino-3-(*N*-acetamino)propionic acid, however, was inert. Pyruvate was identified spectrophotometrically with L-lactate dehydrogenase, and ammonia was with glutamate dehydrogenase as described previously.¹⁶⁾ Amines formed were identified by ¹H-NMR and thin-layer chromatography (TLC) by a modification of the method of Greveling and Daly¹⁷⁾ as follows. The reaction was done with a 20-times larger scale reaction mixture in a bottle sealed with a rubber cap to prevent the amine from evaporating. After incubation at 37°C for 12 hr, dansyl chloride (13.5 mg, 30 mmol) dissolved in 10 ml of acetone was added to the reaction mixture through the cap with a syringe, and the mixture was shaken vigorously at room

TABLE I. IDENTIFICATION OF DANSYL DERIVATIVES OF METHYLAMINE AND ETHANOLAMINE

Analysis	Methylamine	Ethanolamine
TLC		
Solvent system 1 ^a	$R_f=0.44$	$R_f=0.10$
Solvent system 2 ^b	$R_f=0.32$	—
Solvent system 3 ^c	—	$R_f=0.48$
¹ H-NMR	$\delta=2.57$ (3H; d ^d ; $J=5.4$ Hz; S-N-CH ₃)	$\delta=3.02$ (2H; t ^e ; $J=5.0$ Hz; S-N-CH ₂) $\delta=3.59$ (2H; t ^e ; $J=5.0$ Hz; N-C-CH ₂ -O)

^a Ethylacetate-cyclohexane (3:4, v/v).^b Benzene-triethylamine (8:1, v/v).^c Chloroform-methanol (10:1, v/v).^d Doublet.^e Triplet.

TABLE II. STOICHIOMETRY OF REACTION

Substrate	Substrate disappeared ^a (μ mol)	Pyruvate formed ^b (μ mol)	Ammonia formed ^c (μ mol)
DL-2-Amino-3-(<i>N</i> -methylamino)-propionic acid	2.3	2.5	2.4
DL-2-Amino-3-(<i>N</i> -hydroxyethylamino)-propionic acid	3.1	3.1	3.2

^a Measured by amino acid analysis.^{b,c} Measured spectrophotometrically as described in text.

temperature for 4 hr. Acetone was evaporated from the solution, and the residual aqueous solution was treated with ethyl acetate. The ethyl acetate layer was concentrated, then analyzed by TLC and ¹H-NMR (Table I). Balance studies show that 1 mol of 2-amino-3-(*N*-substituted-amino)propionic acid is converted into an equimolar amount of pyruvate and ammonia (Table II).

*β -Replacement reaction of DL-2-Amino-3-(*N*-substituted-amino)propionic acids*

We have found that L-methionine γ -lyase

catalyzes β -replacement reactions between the substituted amino groups at C₃ of 2-amino-3-(*N*-substituted-amino)propionic acids and thiols. The products were identified as the corresponding *S*-substituted cysteines with a Hitachi amino acid analyzer (Model 835). DL-2-Amino-3-(*N*-substituted-amino)propionic acids underwent only the β -replacement reaction in the presence of thiols; no α , β -elimination occurred.

β -Replacement reactions of cysteine derivatives with amines

We have attempted to synthesize 2-amino-3-(*N*-substituted-amino)propionic acids from various amines and β -substituted alanines. However, none of the 2-amino-3-(*N*-substituted-amino)propionic acids were produced enzymatically in the following systems. *N*-Substituent acceptors: *S*-methyl-L-cysteine, β -chloro-L-alanine, *O*-methyl-DL-serine, and L-cysteine, and *N*-substituent donors: methylamine, ethylamine, 1-propylamine, dimethylamine, and trimethylamine. The reactions were done under the same conditions as for the β -replacement reactions of 2-amino-3-(*N*-substituted-amino)propionic acids with thiols except that 20, 100, and 250 mM *N*-substituent donors were used.

Kinetics

The kinetic parameters for α , β -elimination of DL-2-amino-3-(*N*-methylamino)propionic acid and DL-2-amino-3-(*N*-hydroxyethylamino)propionic acid were measured by Method A (Table III). Tanaka *et al.*¹⁸⁾ showed that L-methionine γ -lyase acts only on the L-isomers of methionine and cysteine: their D-isomers do not affect the reactions. Therefore, the *K_m* values for DL-2-amino-3-(*N*-substituted-amino)propionic acids were estimated on the assumption that only the L-forms are active. The affinity for DL-2-amino-3-(*N*-hydroxyethylamino)propionic acid is lower than that for DL-2-amino-3-(*N*-methylamino)propionic acid. All the substrates efficiently eliminated by L-methionine γ -lyase are hydrophobic. The distal hydroxy group

TABLE III. KINETIC CONSTANTS^a

Substrate	K_m (mM)	V_{max} (μ mol/min/mg)	V_{max}/K_m
Se-Methyl-L-seleno-cysteine ^a	0.13	0.36	2.78
S-Methyl-L-cysteine ^a	1.4	0.98	0.70
O-Methyl-L-serine ^b	2.8	0.14	0.049
DL-2-Amino-3-(N-methylamino)-propionic acid ^b	2.3	0.082	0.036
DL-2-Amino-3-(N-hydroxyethyl)-propionic acid ^b	12.3	0.014	0.0011

^a The activity was measured by the pyruvate formed with L-lactate dehydrogenase (method B).

^b The activity was assayed by the amount of pyruvate formed with MBTH method as described in the text as method A.

FIG. 1. Semilogarithmic Plot of V_{max}/K_m against pK_a of Conjugated Acids Produced from 3-Substituted Alanines. CH_3Se^- , 3-Se-methyl-L-selenocysteine; CH_3S^- , 3-S-methyl-L-cysteine; CH_3O^- , 3-O-methyl-L-serine.

of 2-amino-3-(N-hydroxyethylamino)propionic acid probably interferes with the binding to the hydrophobic region at the active site of the enzyme. DL-2-Amino-3-(N-methylamino)propionic acid and DL-2-amino-3-(N-hydroxyethylamino)propionic acid showed similar V_{max} values in α , β -elimination. However, the values were about 10 times lower than those for other substrates such as S-methyl-L-cysteine and Se-methyl-L-selenocysteine (Table III). We have calculated the second-order rate constant, V_{max}/K_m , to compare the reactivity of the substrates. V_{max} is influenced by various factors: accumulation of intermediates, strain in the enzyme-substrate complex, and induced-fit conformation

change of enzyme. However, such ambiguous factors are canceled in V_{max}/K_m .¹⁹⁾ We have observed a linear relationship between $\log(V_{max}/K_m)$ values of β -substituted alanines and pK_a values for CH_3SeH , CH_3SH , and CH_3OH (Fig. 1). However, the $\log(V_{max}/K_m)$ value obtained for 2-amino-3-(N-methylamino)propionic acid (-1.44) was between the values expected from the pK_a s of methylamine (about 35.0) and methylammonium ions (about 10.7).

DISCUSSION

2,3-Diaminopropionic acid is deaminated by the cystathionine γ -lyase (EC 4.1.1.1) of rat liver and diaminopropionate-ammonia lyase of *Salmonella typhimurium*. We have found that L-2,3-diaminopropionic acid is inert as a substrate of L-methionine γ -lyase.¹⁰⁾ However, L-methionine γ -lyase catalyzes α , β -elimination and β -replacement reactions of 3-N-methyl-, 3-N,N-dimethyl-, and 3-N-hydroxyethyl derivatives of 2,3-diaminopropionic acid. Thus, L-methionine γ -lyase differs from cystathionine γ -lyase and diaminopropionate ammonia-lyase in respect to the substrate specificity.

Pyridoxal-P enzymes which catalyze replacement reactions are important in the biosynthesis of amino acids. However, no information has been available on the enzymes that catalyze the introduction of nitrogen atoms into non-carbonyl carbon of the side chain of amino acids. We have shown that L-methionine γ -lyase cannot use amines as substituent donors in replacement reactions. Snell and DiMari²⁰⁾ and Davis and Metzler²¹⁾ proposed that β - and γ -replacement reactions by pyridoxal-P enzymes proceed through nucleophilic addition of a substituent donor to the intermediate derived from the substrate amino acid: the nucleophilicity of a substituent donor decides its reactivity. Amines are poor nucleophiles at neutral pH in aqueous solution, because of protonation of the nitrogen atom.

Snell and DiMari²⁰⁾ and Davis and Metzler²¹⁾ have reported that the rate-limiting

step in the α , β -elimination and β -replacement reactions catalyzed by pyridoxal-P enzymes are the removal of an electronegative C_3 -substituent. We have observed a linear relationship between the reactivity, $\log (V_{\max}/Km)$ values and the pK_a values of conjugated acid of C_3 -substituents for 3-*Se*-methyl-, 3-*S*-methyl-, and 3-*O*-methyl-substituted 2-amino-propionic acids. However, this was not the case for 2-amino-3-(*N*-methylamino)propionic acid. Protonated amines are released much more readily than the non-protonated forms, because iminium ions, RNH^+ , are too unstable to be produced. Therefore, 2-amino-3-(*N*-substituted-amino)propionic acids, which are nonprotonated at the C_3 nitrogen atom are essentially inert in α , β -elimination. Presumably, C_3 nitrogen atoms of 2-amino-3-(*N*-methylamino)propionic acid substantially are non-protonated at the active site of L-methionine γ -lyase. Thus, the reactivity of the substrate is lower than that expected from the pK_a value for methylammonium ion.

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