Studies on Inhibition of Enzymatic Arginyltransfer Reaction

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Received February 6, 1998; accepted March 17, 1998

Synthetic polyamines and various derivatives of aspartic acid and glutamic acid were examined *in vitro* for their inhibitory activity on arginyl-tRNA-protein transferase. All the polyamines tested showed non-specific activation or inhibition at 0.1 or 10 mM, respectively, suggesting an interaction of polyamines with tRNA. Of the newly prepared active site directed compounds including the inhibitory peptides so far reported, L-aspartic acid α-[(*S*)-(−)-naphthylethylamide] was found to inhibit the enzyme activity most potently with a slight substrate activity, whereas the *R*-isomer showed very weak inhibition, giving information on the active site of the enzyme.

Key words arginyl-tRNA-protein-transferase; inhibition; polyamine analog; aspartic acid derivative

Arginyl-tRNA-protein transferase (arginyltransferase) is an enzyme which catalyzes the post-translational transfer of arginine from arginyl-tRNA to the α-amino group of the *N*-terminal aspartic or glutamic acid residue of proteins and peptides.[1,2] The physiological significance of the enzyme is still unestablished, but the enzyme seems to participate in protein degradation relevant to *N*-end rule in mammalian cells, as it has been demonstrated by gene manipulation that arginylated acceptor proteins followed by ubiquitination were easily degraded by cellular proteasome.[3–5]

However, there is no information about the cellular acceptor proteins probably because they are immediately degraded after arginylation, and thus difficult to detect. Inhibitors for the transferase, therefore, would be useful for the detection of the acceptors as well as to clarify their physiological significance. Compounds so far reported for inhibiting arginyltransfer reactions *in vitro* are serine protease inhibitors[6] (by interfering with the charging of tRNA), phenylarsenoxide[7] (by reacting with the sulfhydryl group of the transferase from *Saccharomyces cerevisiae*), polyamines,[8] and small peptides.[9] The present paper deals with further confirmation of the polyamine effect on the enzyme and the inhibitory effect of newly prepared compounds with reference to a tripeptide, Glu–Val–Phe, which potently inhibited the enzyme activity.[9]

Experimental

Chemicals Hydrochloride salts of putrescine (Put), spermidine (Spd) and spermine (Spm) were purchased from Sigma and used after recrystallization from aqueous ethanol. Other tetraamines, pentaamines and hexaamine were prepared in this laboratory according to the methods reported.[10–13] Their purity (as the hydrochloride salts) was supported by elemental analysis. The synthetic polyamines are *sym*-norspermine tetrahydrochloride (333), *N*,*N*′-bis(aminopropyl)cadaverine tetrahydrochloride (353), tris(aminopropyl)amine tetrahydrochloride (3(3)3), N^1,N^{12}-bis(ethyl)spermine (Et343Et), 1,17-diamino-5,9,13-triazaheptadecane pentahydrochloride (4334), 1,17-diamino-4,9,14-triazaheptadecane pentahydrochloride (3443), 1,17-bis(ethylamino)-5,9,13-triazaheptadecane pentahydrochloride (Et4334Et), 1,17-bis(ethylamino)-4,9,14-triazaheptadecane pentahydrochloride (Et3443Et) and 1,21-diamino-4,9,13,18-tetraazaheneicosane hexahydrochloride (34343).

The tripeptide, Glu–Val–Phe, L-aspartic acid α-(β-naphthylamide) (**7**) and L-glutamic acid α-(4-methoxy-β-naphthylamide) (**9**) were obtained from Sigma, and L-glutamic acid α-(β-naphthylamide) (**8**) from Funakoshi Co. 3-Aminoquinoline, phenethylamine, L-pyroglutamic acid, (*R*)-(+)-1-(naphthyl)ethylamine, (*S*)-(−)-1-(naphthyl)ethylamine and benzylamine were purchased from Tokyo Kasei Co., and cyclohexylamine was from Wako Pure Chemicals Ind. 1,2,3,4-Tetrahydro-1-naphthylamine and 4-phenylbutylamine were purchased from Aldrich. Succinic anhydride and *n*-heptylamine were obtained from Kanto Chemicals.

The aspartic acid derivatives listed in Table 2 except for compounds **7**—**9** were prepared similarly in this laboratory as follows: commercially available *tert*-butyloxycarbonyl-L-aspartic acid β-benzyl ester (Kokusan Chemicals Co.) (2 mmol) and *N*-hydroxysuccinimide (Tokyo Kasei Co.) (2.6 mmol) were reacted in dioxane in the presence of *N*,*N*′-dicyclohexylcarbodiimide (Tokyo Kasei Co.) (2.2 mmol) for 20 h at room temperature. To the filtrate the appropriate primary amino compound (2 mmol) was added, and the reaction mixture was kept at room temperature for a further 20 h. After filtration, the resulting solution was evaporated and the residue (dissolved in benzene) was subjected to silica gel column chromatography using a solvent system of benzene and acetone. The purified amide compound usually obtained in the yield of more than 80% was deprotected by the usual manner: trifluoroacetic acid treatment to remove the *tert*-butyloxycarbonyl group followed by catalytic hydrogenation in acetic acid to remove the benzyl group. The resultant L-aspartic acid α-amide compounds were recrystallized from aqueous ethanol. L-Aspartic acid α-(benzylamide) (**1**): *Anal.* Calcd for $C_{11}H_{14}N_2O_3 \cdot 0.55CF_3COOH$: C, 51.00; H, 5.17; N, 9.83. Found: C, 50.63; H, 5.61; N, 9.57. L-Aspartic acid α-(phenethylamide) (2): *Anal.* Calcd for $C_{12}H_{16}N_2O_3 \cdot 0.9CF_3COOH$: C, 48.91; H, 5.03; N, 8.27. Found: C, 49.27; H, 5.12; N, 8.38. L-Aspartic acid α-(phenylbutylamide) (**3**): *Anal.* Calcd for $C_{14}H_{20}N_2O_3 \cdot CF_3COOH$: C, 50.79; H, 5.59; N, 7.40. Found: C, 50.64; H, 5.54; N, 7.49. L-Aspartic acid α-(heptylamide) (**4**): *Anal.* Calcd for $C_{11}H_{22}N_2O_3 \cdot CF_3COOH$: C, 45.35; H, 6.73; N, 8.14. Found: C, 45.01; H, 6.52; N, 8.31. L-Aspartic acid α-(cyclohexyl amide) (**5**): *Anal.* Calcd for $C_{10}H_{18}N_{26}O_3 \cdot CF_3COOH$: C, 43.90; H, 5.83; N, 8.53. Found: C, 43.69; H, 5.73; N, 8.71. L-Aspartic acid α-(1,2,3,4-tetrahydro-1-naphthylamide) (**6**): *Anal.* Calcd for $C_{14}H_{20}N_2O_3 \cdot CF_3COOH$: C, 51.07; H, 5.09; N, 7.44. Found: C, 51.07; H, 5.13; N, 7.56. L-Aspartic acid α-(3-quinolinamide) (**10**): *Anal.* Calcd for $C_{13}H_{13}N_3O_3 \cdot CF_3COOH \cdot 2H_2O$: C, 44.02; H, 4.43; N, 10.27. Found: C, 43.70; H, 4.69; N, 10.42. L-Aspartic acid α-[(*R*)-(+)-naphthylethylamide] (**11**): *Anal.* Calcd for $C_{16}H_{18}N_2O_3 \cdot 0.7CF_3COOH$: C, 57.08; H, 5.15; N, 7.65. Found: C, 57.36; H, 5.30; N, 7.29. L-Aspartic acid α-[(*S*)-(−)-naphthylethylamide] (**12**, Asp(S)NEA): *Anal.* Calcd for $C_{16}H_{18}N_2O_3 \cdot H_2O$: C, 63.14; H, 6.62; N, 9.20. Found: C, 62.80; H, 6.57; N, 9.13. D-Aspartic acid α-[(*S*)-(−)-naphthylethylamide] (**13**): *Anal.* Calcd for $C_{16}H_{18}N_2O_3 \cdot 0.5CH_3COOH \cdot 0.7CF_3COOH$: C, 55.58; H, 5.27; N, 7.07. Found: C, 55.94; H, 5.63; N, 7.07. L-Pyroglutamic acid α-[(*S*)-(−)-naphthylethylamide] (**14**): *Anal.* Calcd for $C_{17}H_{18}N_2O_2$: C, 72.32; H, 6.43; N, 9.92. Found: C, 72.04; H, 6.58; N, 9.93. *N*-α-*tert*-Butyloxycarbonyl-L-aspartic acid β-benzyl ester α-[(*S*)-(−)-naphthylethylamide] (**15**): *Anal.* Calcd for $C_{28}H_{32}N_2O_5$: C, 70.57; H, 6.77; N, 5.88. Found: C, 70.43; H, 6.83; N, 5.81. L-aspartic acid β-benzyl ester α-[(*S*)-(−)-naphthylethylamide] (**16**): *Anal.* Calcd for $C_{23}H_{24}N_2O_3 \cdot 2H_2O$: C, 66.68; H, 6.84; N, 6.76. Found: C, 66.58; H, 6.12; N, 6.70. *N*-α-Acetyl-L-aspartic acid α-[(*S*)-(−)-naphthylethylamide] (**17**): *Anal.* Calcd for $C_{18}H_{20}N_2O_4 \cdot 0.1H_2O$: C, 65.48; H, 6.17; N, 8.48. Found: C, 65.25; H, 6.13; N, 8.48. *N*-Succinyl-

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(*S*)-(−)-naphthylethylamine (**18**): *Anal.* Calcd for $C_{16}H_{17}NO_5$: C, 70.83; H, 6.32; N, 5.16. Found: C, 70.72; H, 6.41; N, 4.88.

An authentic sample of the arginylated product of compound **12** [*N*-α-Arginyl-L-aspartic acid α-[(*S*)-(−)-naphthylethylamide)] [ArgAsp(S)-NEA] was synthesized as follows: Asp(OBzl)(S)NEA (2 mmol) prepared by trifluoroacetic acid (TFA) treatment of Boc-Asp(OBzl)(S)NEA and Z-Arg(di-Z)–OSu (2 mmol) obtained from Nova Biochem Co. were dissolved in *N*,*N*-dimethylformamide (DMF) and kept at room temperature for 20 h. After the solvent was evaporated in vacuo, the residue was purified by silica gel column chromatography using the solvent system of $CHCl_3$–MeOH (9:1). The purified compound was then deprotected by catalytic hydrogenation followed by silica gel column chromatography using a solvent system of $CHCl_3$–MeOH–AcOH (1:1:0.1), to get ArgAsp(S)NEA. (Ionspray-MS *m/z*: 442.4 (Calcd for $C_{22}H_{30}N_6O_4$: 442.4)).

Assay Conditions for Arginyltransferase Activity The enzyme activities were measured according to the method reported by Horinishi *et al.*[14] The standard incubation mixture (100 μl) contains 10 μmol Tris–HCl (pH 8.0), 5 μmol of 2-mercaptoethanol and 9 μmol of potassium chloride and 0.3 μmol of ATP, 150 μg of tRNA and 3 nmol of α-lactoalbumin obtained from Sigma, and 2 nmol of L-[U-^{14}C]-arginine (1.85 kBq/nmol) obtained from American Radiolabeled Chemicals, 10 μl of arginyl-tRNA synthetase in *E. coli* partially purified by the method of Zubay,[15] and 20 μl of sample solution containing arginyltransferase. After incubation at 37 °C for 20 min, ^{14}C-arginine-labeled α-lactoalbumin was measured by the paper disk method reported by Mans and Novelli.[16]

Enzyme Preparation Arginyltransferase was partially purified from hog kidneys to the step of chromatographic separation by DEAE-cellulofine and hydroxyapatite (obtained from Seikagaku Co.), principally based on the methods by Kato and Nozawa[17] and Ciechanover *et al.*[18] The second active fraction by hydroxyapatite chromatography was used as the enzyme source in the present experiments.

Conditions for HPLC HPLC was carried out as follows: column, TOSOH TSKgel ODS-80$_{Tm}$; elution solvent, a mixture of 2 volumes of methanol and 3 volumes of citrate-phosphate buffer made by mixing 48.5 vol% of 0.1 M citric acid and 51.5 vol% of 0.2 M sodium monohydrogen phosphate; flow rate, 0.6 ml/min; fluorescence detection, Ex 280 nm, Em 325 nm.

Results and Discussion

Activation and Inhibition of Arginyltransfer Reaction by Polyamines Spermidine and spermine, major polyamines in mammalian tissues, have been reported to affect arginyltransferase activity *in vitro*, showing a moderate activation at submillimolar concentrations and significant inhibition at higher concentrations.[8] So, we first examined whether or not the two-phase effect was also observed in various synthetic tetraamines, pentaamines and a hexaamine. The results are summarized in Table 1, in which the values are expressed based on the control activity as 100. All the polyamines tested showed more or less similar effects, a moderate activation at 0.1 millimolar concentration and a significant inhibition at 10 millimolar concentration. The inhibitory effect seemed to increase in the order of diamine, triamine, tetraamine, pentaamine and hexaamine. The structurally non-specific inhibition by polyamines might be brought about through the interaction of polyamines with tRNA^{Arg} leading to a conformational change, rather than through an interaction with the enzyme protein. As was described before,[8] it can be concluded that polyamines at physiological concentrations play primarily a positive role in *in vivo* arginyl transfer reaction. We then planned to find some compounds acting directly at the enzyme protein to inhibit the activity.

Inhibition of Arginyltransferase with Active Site-Directed Compounds The general concept to inhibit enzyme reaction specifically is to target the active site of the enzyme. In this line of research, Soffer[9] reported a tripeptide, Glu–Val–Phe, as the most potent competitive inhibitor for arginyltransferase. This indicated a simple model for the active site-directed compound such as *N*-terminal aspartic acid or glutamic acid connected to some hydrophobic groups. According to this model, a number of compounds were prepared, and tested for their inhibitory effects. The results are summarized in Table 2. Looking over the data, the presence of an *N*-terminal L-aspartic acid or glutamic acid seemed to be necessary for reducing enzyme activity, as can be seen in the weak inhibitory effects in compound **13** to **17** except for compound **12**, and the enhancement of hydrophobicity of compounds seemed to inhibit the enzyme activity more effectively, *e.g.* see the different inhibition of compound **1** to **6**, supporting the model. β-Naphthylamine derivatives (compound **7**—**9**) and the quinoline derivative (compound **10**) also showed more potent inhibition than Glu–Val–Phe. Of the compounds tested, compound **12** (Asp(S)NEA) inhibited the enzyme activity most potently, whereas, the absolute configuration *R* isomer (compound **11**) showed weak inhibition. These results suggested that there is a fairly large hydrophobic region in the active site of the enzyme, which stereo-specifically accepts a variety of hydrophobic groups.

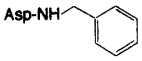
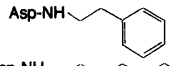
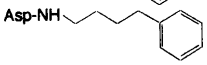
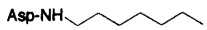
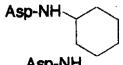
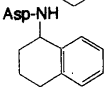
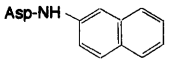
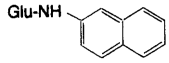
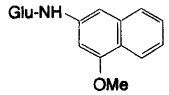
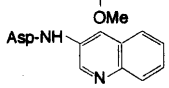
We then examined the inhibition mechanism of Asp(S)NEA to see whether it really acted in the active site of the enzyme. As can be seen in Fig. 1, the plot shows a competitive inhibition with a K_i value of 140 μM that is about ten times smaller than for Glu–Val–Phe (K_i = 1.3 mM) measured under the same conditions. This evidence for Asp(S)NEA entering the active site led us to examine whether it could be subjected to arginylation as a substrate. After calibrating the HPLC conditions to separate authentic Asp(S)NEA and ArgAsp(S)NEA, the incubated mixture containing Asp(S)NEA in the absence of α-lactoalbumin was applied to the HPLC, and the chromatograms are shown in Fig. 2. The formation of ArgAsp(S)NEA was clearly demonstrated, although the rate was extremely low. With the suggestion that di and tripeptides having *N*-terminal aspartic acid or glutamic acid are arginine acceptors,[9] we hypothesize that the other inhibiting compounds tested here might also have accepted

Table 1. Effects of Polyamines on Arginyltransferase Activity

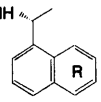
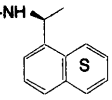
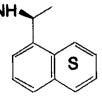
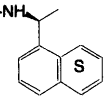
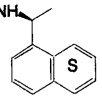
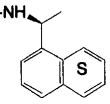
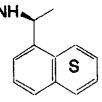
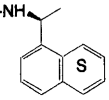
Compound	Concentrations (mM)		
	0.1	1	10
Put	116	105	88
Spd	136	101	55
Spm	111	125	42
333	118	91	41
353	120	93	36
Et343Et	101	109	48
3(3)3	103	103	60
4343	110	96	22
3443	122	88	20
Et4334Et	109	85	24
Et3443Et	107	91	11
34343	112	75	7

Control activity = 100.

Table 2. Inhibitory Effects of Synthetic Compounds on Arginyltransferase Activity

	Compound	Concentration (mM) 0.1	1
	Glu–Val–Phe	n.s.	23
1		n.s.	n.s.
2		n.s.	10
3		26	56
4		25	48
5		15	53
6		25	59
7		n.s.	39
8		n.s.	31
9		n.s.	26
10		n.s.	26

n.s. = negligibly small.

	Compound	Concentration (mM) 0.1	1
11		n.s.	28
12		43	91
13		n.s.	n.s.
14		n.s.	n.s.
15		n.s.	16
16		n.s.	26
17		n.s.	17
18		n.s.	10

Each compound dissolved in 5 μl of DMSO was added to 95 μl of the standard incubation mixture. Control activity measured in the presence of 5 μl of DMSO was estimated as 100, and DMSO did not affect the enzyme activity.

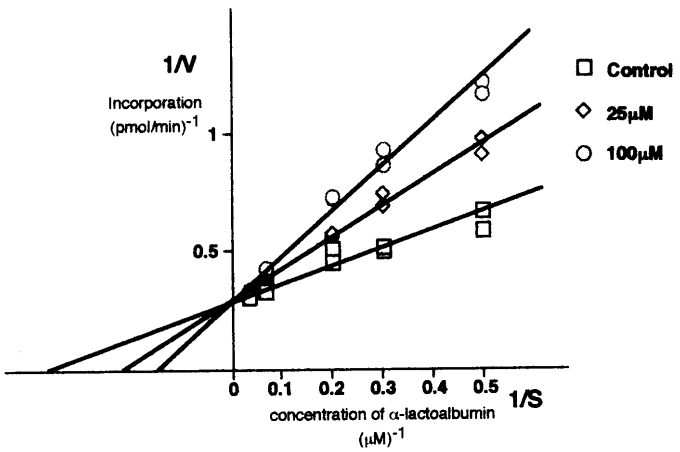


Fig. 1. Competitive Inhibition of Arginyltransferase by Asp(S)NEA

Incubation was carried out in modified standard mixture (100 μl), which contained 0 nmol (□) or 2.5 nmol (◇) or 10 nmol (○) of Asp(S)NEA in 5 μl of dimethyl sulfoxide (DMSO) at various concentrations (2—30 μM) of α-lactoalbumin.

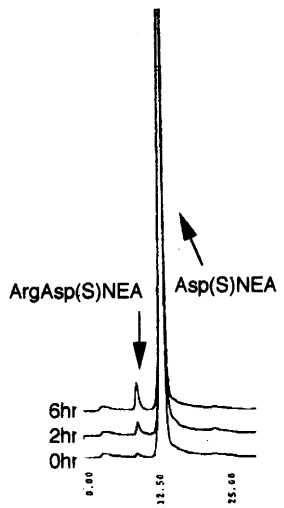


Fig. 2. Time-Dependent Increase of Arginylated Asp(S)NEA during Incubation with Arginyltransferase

α-Lactoalbumin (3 nmol) in the standard incubation mixture was replaced by Asp(S)NEA (100 nmol) dissolved in 5 μl of DMSO. After incubation for 0, 2, and 6 h, the incubation mixtures were deproteinized with 100 μl of 20% trichloroacetic acid, and aliquots were applied to the HPLC. Conditions for the HPLC are described in the Experimental.

arginine. The present results show that arginyltransferase has a wide acceptability to compounds having *N*-terminal acidic amino acids, and hence it may be possible to find some low molecular weight compounds with either potent inhibitory activity or significant substrate activity.

Acknowledgements We express our thanks to Dr. Hiroo Horinishi, Nihon Kayaku Co., Ltd.

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