[Chem. Pharm. Bull. 36(3)1205—1209(1988)]

β-Naphthylamides of Guanidinophenyl Amino Acids as Substrates of Aminopeptidases

HIDEAKI TSUNEMATSU,*,[a] HIDEKAZU ARATANI,[b] KOICHI MIZUSAKI,[b] YOSHIHIRO HATANAKA,[b] SHUJI KAWATA,[b] MAGOBEI YAMAMOTO,[a] and SATORU MAKISUMI[b]

Faculty of Pharmaceutical Sciences, Fukuoka University,[a] Jonan-ku, Fukuoka 814–01, and Department of Chemistry, Faculty of Science, Kyushu University,[b] Higashi-ku, Fukuoka 812, Japan

(Received August 3, 1987)

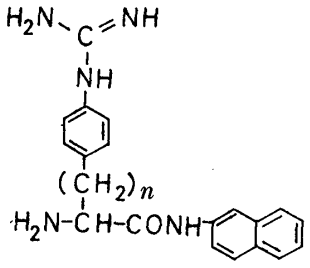
β-Naphthylamides of *p*-guanidino-L-phenylalanine (GPA) and *p*-guanidino-DL-phenylglycine (GPG) were synthesized and tested as substrates of bovine leukocyte aminopeptidase (BL-APase) and porcine liver aminopeptidase B (PL-APaseB) in comparison with L-arginine β-naphthylamide (Arg-βNA). BL-APase-catalyzed hydrolysis of GPA-βNA proceeded as fast as that of Arg-βNA, while the rate of hydrolysis of GPG-βNA was much slower. The specificity constant (V_{max}/K_m) for the hydrolysis of GPA-βNA by BL-APase was somewhat larger than that for the hydrolysis of Arg-βNA. The benzene ring in the side chain of GPA-βNA is considered to contribute to the binding of this substrate to the specificity site of this enzyme, based on a comparison of the K_m values for the two β-naphthylamide substrates. Substrate inhibition was observed with BL-APase in the hydrolysis of GPA-βNA in the substrate concentration range higher than about 0.1 mM. Neither GPA-βNA nor GPG-βNA was hydrolyzed by PL-APaseB and they inhibited the hydrolysis of Arg-βNA by this enzyme. GPA-βNA is expected to be a useful substrate in the study of the binding and catalytic specificities of aminopeptidases.

Keywords——aminopeptidase; guanidinophenyl amino acid; *p*-guanidinophenylalanine; *p*-guanidinophenylglycine; β-naphthylamide substrate; kinetics

Introduction

We have reported that esters, amides, anilides and β-naphthylamides of N^{α}-substituted *p*-guanidino-L-phenylalanine (GPA), in which the alkyl methylenes in the arginyl residue are replaced by a benzene ring, are excellent substrates of trypsin, and showed that GPA derivatives are useful for the study of the specificity of trypsin-like enzymes.[1-5] L-Arginine β-naphthylamide (Arg-βNA) has been widely used as a substrate for the study of the specificity of aminopeptidases. To evaluate the contribution of the side chains of the guanidinophenyl-amino acids to the binding and kinetic properties as substrates for aminopeptidases, we synthesized β-naphthylamides of GPA and *p*-guanidino-DL-phenylglycine (GPG).

Various aminopeptidases which are specific to the basic L-amino acids have been purified and characterized. Kawata *et al.* purified porcine liver aminopeptidase B from porcine liver and reported that this enzyme is highly specific for the hydrolysis of β-naphthylamides of arginine and lysine.[6,7] Aratani *et al.* have purified an aminopeptidase from bovine leukocytes and showed that this enzyme has a higher affinity for amino acids with basic or longer aliphatic side chains such as lysine, arginine and leucine.[8] We found in the present work that bovine leukocyte aminopeptidase (BL-APase)-catalyzed hydrolysis of GPA-βNA proceeded as fast as that of Arg-βNA, but GPA-βNA was not hydrolyzed by porcine liver aminopeptidase B (PL-APase B) at all. It is of interest, therefore, to analyze the kinetics of hydrolysis of the β-naphthylamides of guanidinophenyl-amino acids by the two aminopeptidases, and to



GPG-βNA ($n=0$)
GPA-βNA ($n=1$)

Fig. 1. Chemical Structures of GPA-βNA and GPG-βNA

compare the geometry of the active sites of both enzymes. We describe here the synthesis of GPA-βNA and GPG-βNA, and the hydrolysis of the two β-naphthylamides by BL-APase and PL-APaseB.

Experimental

p-Nitro-DL-phenylglycine was prepared as reported previously.[1] N^{α}-Benzyloxycarbonyl(Z)-*p*-guanidino-L-phenylalanine β-naphthylamide hydrochloride(HCl) was synthesized by the method described.[4]. L-Arg-βNA·HCl was purchased from Sigma Chemical Co. All chemicals were of analytical or reagent grade. Optical rotations were measured on a Union Giken high-sensitivity polarimeter, type PM-71. All melting points are uncorrected. The purity of each compound was checked by thin-layer chromatography (TLC) as described previously.[3] The structures of the synthesized compounds were confirmed with the aid of fast atom bombardment mass spectrometry (FABMS) using a JEOL JMS-DX/JMA 3500 data analysis system. Sakaguchi and nitroprusside-ferricyanide reagents were prepared as described by Makisumi.[9]

BL-APase and PL-APaseB were purified described previously.[6,7] Protein concentrations were determined by the method of Lowry *et al.*[10] using bovine serum albumin as a standard.

GPA-βNA·HCl——A solution of Z-GPA-βNA·HCl (400 mg, 0.77 mmol) in methanol (MeOH) (25 ml) was reduced with hydrogen at room temperature for 24 h in the presence of palladium black. The catalyst was removed by filtration, the filtrate was concentrated to a small volume, and ether was added. The resulting precipitate was filtered off, washed with ether and dried over P_2O_5. The crude product was dissolved in MeOH and the solution was applied to a column (2.8 × 100 cm) of Sephadex LH-20, which was eluted with MeOH (flow rate, 20 ml/h; fraction volume, 4 ml). Fractions of eluate containing the desired material as judged by paper chromatography by staining with nitroprusside-ferricyanide reagent were combined, the solvent was concentrated, and the product was crystallized by addition of ether. Yield 260 mg (88%), mp 173—174 °C (dec.), $[\alpha]_D^{22}+43.3°$ ($c=1$, MeOH). *Anal.* Calcd for $C_{20}H_{21}N_5O \cdot HCl$: C, 62.57; H, 5.79; N, 18.25. Found: C, 62.26; H, 6.01; N, 18.01. FABMS *m/z*: 348 $[M+H]^+$.

***tert*-Butoxycarbonyl-*p*-nitro-DL-phenylglycine(Boc-NO_2-PG)**——This compound was synthesized from *p*-nitro-DL-phenylglycine (3.5 g, 15.3 mmol) and 2-butoxycarbonyloxyimino-2-phenylacetonitrile(Boc-on) (4.2 g, 16.8 mmol) by the method described previously for the synthesis of Boc-*p*-nitro-L-phenylalanine.[2] Yield 3.2 g (70%), mp 88—90 °C. *Anal.* Calcd for $C_{13}H_{16}N_2O_6$: C, 52.72; H, 5.40; N, 9.46. Found: C, 53.01; H, 5.64; N, 9.62.

Boc-GPG——This compound was prepared by catalytic reduction of Boc-NO_2-PG (3.0 g, 10 mmol) followed by guanidination with 1-amidino-3,5-dimethylpyrazole nitrate. Yield 1.7 g (61%), mp 167—168 °C (dec.). *Anal.* Calcd for $C_{14}H_{20}N_4O_4$: C, 54.52; H, 6.55; N, 18.17. Found: C, 54,80; H, 6.70; N, 18.37. This product was stained with nitroprusside-ferricyanide reagent (red-violet) and Sakaguchi reagent (violet) on a thin-layer plate.

GPG·2HCl——Boc-GPG (1.9 g, 6.2 mmol) was dissolved in 20 ml of HCl/dioxane–formic acid–anisole (5 : 3 : 2, v/v/v) and the solution was left to stand for 20 h at 0°C. The resulting precipitate was filtered off, washed with ether, and dried over P_2O_5. The crude product was recrystallized from MeOH–dioxane. The yield of hygroscopic product was 1.5 g (86%), mp 185—187 °C (dec.). *Anal.* Calcd for $C_9H_{14}N_4O_2 \cdot 2HCl \cdot 1/2H_2O$: C, 37.25; H, 5.22; N, 19.31. Found: C, 37.51; H, 5.58; N, 19.38.

Z-GPG——This compound was prepared from GPG·2HCl (2.4 g, 6 mmol) and benzyloxycarbonyl chloride (1.02 g, 6 mmol) according to the same method as described for the synthesis of Z-GPA.[3] Yield 1.5 g (70%), mp 160—161 °C. *Anal.* Calcd for $C_{17}H_{18}N_4O_4$: C, 59.63; H, 5.31; N, 16.37. Found: C, 59.35; H, 5.46; N, 16.52.

Z-GPG-βNA·HCl——This compound was synthesized by coupling Z-GPG (900 mg, 2.6 mmol) to β-naphthylamine (564 mg, 3.9 mmol) by the same method as used for the synthesis of Z-GPA-βNA.[4] Yield 460 mg (35%), mp 138—140 °C (dec.). *Anal.* Calcd for $C_{27}H_{25}N_2O_3 \cdot 1/2H_2O$: C, 63.21; H, 5.32; N, 13.65. Found: C, 63.10; H, 5.50; N, 13.38. FABMS *m/z*: 468$[M+H]^+$.

GPG-βNA·HCl——This compound was prepared by catalytic reduction of Z-GPG-βNA (400 mg, 7.9 mmol) as described for the synthesis for GPA-βNA above. Yield 270 mg (92%), mp 161—163 °C (dec.). *Anal.* Calcd for $C_{19}H_{19}N_5O \cdot HCl$: C, 61.69; H, 5.46; N, 18.94. Found: C, 61.42; H, 5.61; N, 18.69. FABMS *m/z*: 334 $[M+H]^+$.

Determination of Kinetic Parameters——The ultraviolet (UV) and visible absorption spectra of GPA-βNA and GPG-βNA in phosphate buffer (0.057 M, pH 7.0) were measured. It was found that the absorption of the two substrates is negligible at 340 nm. The rate of hydrolysis of β-naphthylamides of GPA, GPG and Arg by BL-APase was followed in terms of the increase in absorbance at 340 nm, at pH 7.0 and 37°C, according to Lee *et al.*[11] using a Shimadzu UV-200 spectrophotometer equipped with a thermostated cell compartment and a U-125 MU-recorder. The rate of hydrolysis was calculated using $\Delta\varepsilon = 1780$. The substrate concentration ranges were 0.005 to 0.100 mM for GPA-βNA and 0.005 to 0.200 mM for Arg-βNA. The steady-state kinetic parameters, K_m and V_{max}, for hydrolysis of the two β-naphthylamides by BL-APase were determined by the method of Lineweaver and Burk.[12] The rates of hydrolysis of the β-naphthylamides by PL-APaseB were measured as described previously.[6]

Results and Discussion

Hydrolysis of GPA-βNA, GPG-βNA and Arg-βNA by BL-APase

Before determining the kinetic constants, the pH–activity relationship for the hydrolysis of GPA-βNA by BL-APase was examined. This enzyme has a pH optimum at around 7.0 for hydrolysis of this substrate as well as for that of Arg-βNA reported previously.[8]

The dependence of the observed initial rate of BL-APase-catalyzed hydrolysis of GPA-βNA upon substrate concentration was expressed in the form of a Lineweaver–Burk plot[12] as represented in Fig. 2. The plot afforded a straight line up to the substrate concentration of about 0.1 mM, indicating that the enzymatic process obeys simple Michaelis–Menten kinetics in this low concentration range. The upward curvature of the graph in the substrate concentration range higher than about 0.1 mM may be explained as the result of substrate inhibition. Such substrate inhibition was also observed in the *Streptomyces griseus* tryptic hydrolysis of N^{α}-protected *p*-nitroanilide substrates of Arg and GPA.[5,13] Several mechanisms for substrate inhibition or activation in the enzyme reactions have been proposed.[14,15] Further experiments will be required to elucidate the mechanism for the substrate inhibition in the hydrolysis of GPA-βNA by BL-APase. However, our results indicate the existence of a secondary substrate binding site on BL-APase. It is likely that the binding of the benzene ring of GPA-βNA to the auxiliary binding site of BL-APase is better than that of methylene

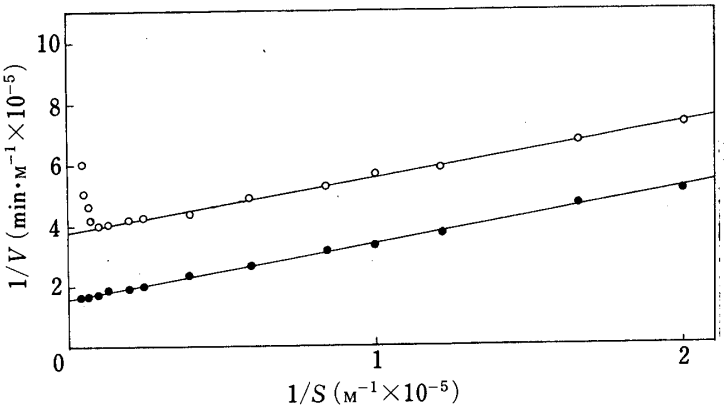


Fig. 2. Double-Reciprocal Plots for the Hydrolysis of GPA-βNA (○) and Arg-βNA (●) Catalyzed by BL-APase at 37 °C in Phosphate Buffer (0.057 M, pH 7.0)

The solid straight line was drawn according to the simple Michaelis–Menten equation with the values calculated by the least-squares method.

TABLE I. Kinetic Parameters for the Hydrolysis of GPA-βNA and Arg-βNA by BL-APase at pH 7.0 at 37 °C

	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m (min^{-1}·mg^{-1})
GPA-βNA	2.37±0.15	3.15±0.13	251±12
Arg-βNA	5.45±0.27	11.1 ±0.7	164± 9

groups of Arg-βNA, since no inhibition was observed in the BL-APase-catalyzed hydrolysis of Arg-βNA.

The action of BL-APase on β-naphthylamides followed Michaelis-Menten kinetics in the range of substrate concentration below 0.1 mM for GPA-βNA and 0.2 mM for Arg-βNA. The kinetic parameters for BL-APase with GPA-βNA and Arg-βNA are presented in Table I. Based on the second-order rate constants (V_{max}/K_m), the value for GPA-βNA is somewhat larger than that for Arg-βNA, which is a good substrate for this enzyme. The V_{max} value for GPA-βNA is about half that for Arg-βNA, while the K_m value is about 3 times smaller. If the binding affinity of these substrates to BL-APase is related to K_m, it is clear that GPA-βNA is preferable to Arg-βNA with respect to binding ability to the specificity site of this enzyme. This suggests that binding of a benzene ring in the side chain of GPA-βNA is better than that of methylene groups of Arg-βNA in the hydrolysis of β-naphthylamide substrates by this enzyme. On the other hand, the small V_{max} value for GPA-βNA may be due to inappropriate orientation of the scissile bond to the catalytic site of this enzyme.

GPG-βNA was hydrolyzed by BL-APase much more slowly than GPA-βNA. This result indicates that the β-methylene group in the derivative of GPA plays an important role in the hydrolysis of β-naphthylamide substrates by BL-APase. Similar results were obtained in the bovine and *Streptomyces griseus* tryptic hydrolysis of N^{α}-protected esters of GPA and GPG.[1] However, the present data do not exclude the possibility that the D-isomer of GPG-βNA may inhibit the activity of BL-APase, since this substrate was used in racemic form. Optical resolution of this substrate will be necessary to examine this point.

Hydrolysis of GPA-βNA, GPG-βNA and Arg-βNA by PL-APaseB

Kawata *et al.*[6] reported that PL-APaseB [EC 3.4.11.6.] is highly specific for the hydrolysis of β-naphthylamides of basic L-amino acids, especially Arg-βNA. We therefore examined the hydrolysis of both GPA-βNA and GPG-βNA by this enzyme. Neither GPA-βNA nor GPG-βNA was hydrolyzed by PL-APaseB at 37°C, pH 7.5, and neither inhibited the hydrolysis of Arg-βNA by PL-APaseB up to a concentration of 2.0 mM of these two guanidinophenyl amino acid substrates. It was reported that there is a hydrophobic region that binds the side chain of substrates or inhibitors in the specificity site of PL-APaseB and that the binding of methylene groups in the side chain of the arginyl residue to the hydrophobic region of this enzyme is much better than that of alkyl or aryl side chains of aliphatic and aromatic amino acid residues.[7] It is likely that the geometry of the hydrophobic region of PL-APaseB is a better fit for the methylene groups of alkylguanidine derivatives than for the benzene ring of guanidinophenyl-amino acid ones.

GPA-βNA was found to be a good substrate for BL-APase, but not for PL-APaseB. New information about the active centers of the two aminopeptidases was obtained by comparison of the hydrolysis of GPA-βNA and Arg-βNA by these aminopeptidases. Consequently, GPA-βNA should be useful in the study of the binding and catalytic specificities of various aminopeptidases which are specific for basic L-amino acids.

References

1) H. Tsunematsu and S. Makisumi, *J. Biochem.* (Tokyo), **88**, 1773 (1980).
2) H. Tsunematsu, T. Imamura, and S. Makisumi, *J. Biochem.* (Tokyo), **94**, 123 (1983).
3) H. Tsunematsu, H. Nishimura, K. Mizusaki, and S. Makisumi, *J. Biochem.* (Tokyo), **97**, 617 (1985).
4) H. Tsunematsu, K. Ando, Y. Hatanaka, K. Mizusaki, R. Isobe, and S. Makisumi, *J. Biochem.* (Tokyo), **98**, 1597 (1985).
5) Y. Hatanaka, H. Tsunematsu, K. Mizusaki, and S. Makisumi, *Biochem. Biophys. Acta*, **832**, 274 (1985).
6) S. Kawata, S. Takayama, K. Ninomiya, and S. Makisumi, *J. Biochem.* (Tokyo), **88**, 1025 (1980).
7) S. Kawata, S. Takayama, K. Ninomiya, and S. Makisumi, *J. Biochem.* (Tokyo), **88**, 1601 (1980).
8) H. Aratani, S. Kawata, S. Tsuruyama, Y. Yoshida, and S. Makisumi, *J. Biochem.* (Tokyo), **96**, 107 (1984).

9) S. Makisumi, *Nippon Kagaku Zasshi*, **73**, 737 (1952).
10) O. H. Lowry, N. J. Rosebrough, A. J. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) H. J. Lee, J. N. LaRue, and I. B. Wilson, *Anal. Biochem.*, **41**, 397 (1971).
12) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
13) H. Nakata, N. Yoshida, Y. Narahashi, and S. Ishii, *J. Biochem.* (Tokyo), **71**, 1085 (1972).
14) C. G. Trowbridge, A. Krehbiel, and M. Laskowski Jr., *Biochemistry*, **2**, 843 (1963).
15) M. Dixon and E. C. Webb, "Enzymes," 3rd ed., Longman Group Ltd., London, 1979, pp. 126—133.