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Enzyme inhibition by dipeptides containing 2,3-methanophenylalanine, a sterically constrained amino acid

Tomohisa Ogawa, Haruko Yoshitomi, Hiroaki Kodama*, Michinori Waki, Charles H. Stammer° and Yasuyuki Shimohigashi

*Laboratory of Biochemistry, Faculty of Science, Kyushu University 33, Fukuoka 812, *Department of Chemistry, Faculty of Science and Engineering, Saga University, Honjo, Saga 840, Japan* and *°Department of Chemistry, School of Chemical Sciences, University of Georgia, Athens, GA 30602, USA*

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Both isomers of (E)-2,3-methanophenylalanine (▽^{E}Phe), a sterically restricted amino acid, were incorporated into peptides in order to examine their possible enzyme inhibitory activity. Both (2R,3S)- and (2S,3R)-▽^{E}Phe-Phe(or Leu)-OMe were found to inhibit effectively the hydrolysis of Ac-Tyr-OEt by chymotrypsin in a competitive manner. The ester groups of these dipeptides were quite resistant to chymotrypsin hydrolysis, and the ▽^{E}Phe-Phe peptide bond was also entirely stable. The inhibition constant (K_i) of the most potent dipeptide of H-(2R,3S)-▽^{E}Phe-Phe-OMe was 0.16 mM at 25°C. The inhibitory action of ▽Phe-containing peptides was found to depend on the configuration of the ▽Phe residue. The electrophilic nature of the cyclopropane ring which is conjugated with both the phenyl ring and the ester carbonyl group appears to be relevant to the inhibitory activity. Fully irreversible inactivation of chymotrypsin was achieved by its incubation with H-(2R,3S)-▽^{E}Phe-Leu-OMe. An enzyme carboxylate group is thought to be responsible for nucleophilic attack on the cyclopropane ring leading to irreversible inactivation.

Enzyme inhibition; Phenylalanine; Amino acid isomer

1. INTRODUCTION

Various chymotrypsin-like proteases have recently been discovered in cells from pathological lesions such as those present in carcinogenesis [1], muscular dystrophy [2], and rheumatoid arthritis [3]. For instance, a chymotrypsin-like intracellular protease involved in the expression of a transformed phenotype in in vivo carcinogenesis was isolated from mouse embryo fibroblast cells. It was characterized using the Bowman-Birk inhibitor, chymostatin, and TPCK. Interestingly, these inhibitors per se are anticarcinogenic [1]. A limited number of compounds are known, however, as highly specific inhibitors of chymotrypsin or chymotrypsin-like enzymes. Even such specific inhibitors as chymostatin, aldehyde or ketone type compounds, and boronic acid derivatives have disadvantages in kinetic and physiological investigations because of their poor water solubility and high toxicity [4]. It is thus important to design and synthesize new inhibitors which have properties without these drawbacks.

The interaction between a specific low molecular weight ligand and a protein receptor is usually very strong. This can be increased further by rigidifying the ligand so that it fits more tightly to the receptor. Since the interaction between ligand and receptor is essentially the same as that between enzyme and substrate or inhibitor, it is possible to obtain powerful low molecular inhibitors by designing a peptide with rigid structure. In the present study, we have designed dipeptides containing 2,3-methanophenylalanine, designated as ▽Phe, as potential enzyme inhibitors (fig.1).

2,3-Methanoamino acids (▽AA) are structural-

Correspondence address: Y. Shimohigashi, Laboratory of Biochemistry, Department of Chemistry, Faculty of Science, Kyushu University 33, Fukuoka 812, Japan

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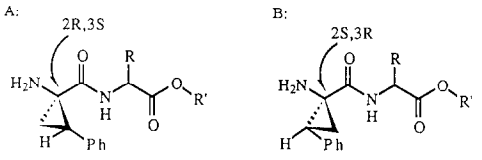

Fig.1. Chemical structure of ∇^{E}Phe-containing dipeptides. A, H-(2R,3S)-∇^{E}Phe-Phe(or Leu)-OH (or OMe); B, H-(2S,3R)-∇^{E}Phe-Phe(or Leu)-OH (or OMe).

ly constrained, having only two possible side chain rotatory angles, $\chi_1 = 0°$ and 120°, which correspond to the Z- and E-configurations, respectively. In addition, the incorporation of ∇AA into a peptide constrains effectively the proximal peptide conformation due to the small ϕ and ψ angles at the ∇AA conformational energy minima. α,β-Dehydroamino acids (ΔAA) are another interesting type of sterically restricted compounds which also increase peptide rigidity due to limitation of ϕ and ψ angles. Utilizing these ∇AA and ΔAA as constituents, various agonist and antagonist analogs of biologically active peptides have been reported [5–8]. We have recently found that enkephalin analogs containing ∇AA and ΔAA are strongly resistant to enzymes such as chymotrypsin and carboxypeptidase Y [7,8]. Since these structurally constrained amino acid derivatives have additional interesting electrophilic properties due to the π-character of cyclopropane ring, we thought that the combined effects of conformational rigidity and electrophilicity might lead, not only to stabilization toward enzyme cleavage, but also to enzyme inhibition. Herein, we describe the inhibitory activity of some ∇AA dipeptides on the serine protease, chymotrypsin, and suggest a possible inhibitory mechanism.

2. MATERIALS AND METHODS

2.1. *Materials*

The synthesis of 2,3-methanophenylalanine and H-(2R,3S)- or (2S,3R)-∇^{E}Phe-Leu-OH (or OMe) were reported previously [8]. Optically pure dipeptide derivatives, Cbz-(2R,3S)- and Cbz-(2S,3R)-∇^{E}Phe-Phe-OMe, were obtained from an isomeric mixture of Cbz-(±)-∇^{E}Phe-Phe-OMe by separation on a silica gel column. The benzyloxycarbonyl group (Cbz) was removed with trifluoroacetic acid in the presence of thioanisole to avoid the destruction of the cyclopropane ring [8]. The purity of the water soluble dipeptide esters was verified by HPLC, CD and ^{1}H-NMR measurements.

Bovine chymotrypsin was purchased from Worthington Biochemical Co. (Freehold, NJ) and Ac-Tyr-OEt was purchased from the Peptide Institute Inc. (Osaka).

2.2. *Enzyme assays*

The stability of the synthetic peptides to chymotrypsin hydrolysis was monitored by HPLC at certain time intervals. The peptide (1 mM) was incubated in phosphate buffer (total volume, 3.0 ml; pH 7.0) with chymotrypsin (1.3 μg/ml) at 25°C. An aliquot (10 μl) of the incubation mixture was added to acetic acid (10 μl), and the solution was injected into the HPLC to determine the % hydrolysis. Analytical HPLC was performed on a Hitachi no.3063 ODS reversed-phase column (4 × 150 mm) in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile (10–60%) for 60 min.

∇Phe-containing dipeptides were assayed for chymotrypsin inhibition using Ac-Tyr-OEt as a substrate. Inhibition constants (K_i) were determined by Dixon plots [9] and are summarized in table 1.

2.3. *^{1}H-NMR measurements*

All ^{1}H-NMR spectra were recorded on a JEOL JNM-GX-270 spectrometer (270 MHz) and the chemical shifts were determined using tetramethylsilane as an internal standard at 30°C. The NMR samples contained 6 mg of peptide in 0.5 ml DMSO-d_6 and the signal assignments were made using two-dimensional COSY and NOE experiments.

3. RESULTS AND DISCUSSION

The compounds examined have both peptide and ester bonds, and we examined the enzymatic stability of these linkages prior to the inhibition assay. Fig.2 shows the stability of ∇Phe-containing peptides to chymotrypsin. While Ac-Tyr-OEt was hydrolyzed completely within 15 min, H-∇^{Z}Phe-OMe was fully resistant to the enzyme, showing no hydrolysis even after 2 h (fig.2) indicating that the linkage at the carbonyl side of the ∇Phe residue was completely stable. The dipeptide, H-∇^{E}Phe-Leu-OMe, was also quite stable since only 15% of acid, H-∇^{E}Phe-Leu-OH, after 1 h and no amino acids (∇^{E}Phe and Leu) were detected indicating that the peptide bond was unhydrolyzed. The hydrolysis of the ester bond during the first 5 min, which is the time necessary for the inhibition assay, was almost negligible. These results clearly confirm the strong resistance of ∇Phe peptides to hydrolysis by chymotrypsin [7,8].

When these peptides were assayed for inhibition of chymotrypsin, we found that, as expected, they did inhibit the enzyme. By analyses of Dixon plots, the inhibition was found to be competitive, with inhibition K_i values of 0.16–1.3 mM (table 1).

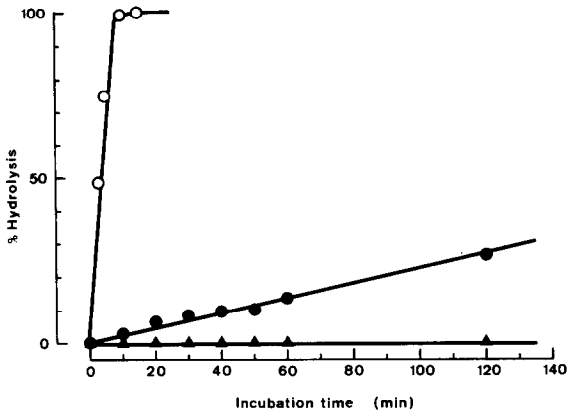

Fig.2. Chymotrypsin hydrolysis of ∇Phe-containing compounds. (○—○) Ac-Tyr-OEt; (●—●) H-(2R,3S)-∇^{E}Phe-Leu-OMe; (▲—▲) H-∇^{E}Phe-OMe.

Among the peptides tested, H-(2R,3S)-∇Phe-Phe-OMe was the best with K_i = 0.16 mM, while H-(2S,3R)-∇Phe-Leu-OH showed no inhibition. Since H-∇Phe-OMe inhibits Ac-Tyr-OEt hydrolysis effectively while the corresponding free acid does not (table 1), a peptide or ester bond at the carbonyl side of ∇Phe appears to be necessary for its interaction with chymotrypsin.

The dipeptides synthesized in the present study possess two aromatic or hydrophobic moieties, either of which possibly occupies the chymotrypsin S_1 site adjacent to the catalytic site. If ∇^{E}Phe[1] fits the S_1 hydrophobic pocket, inhibition of Ac-Tyr-OEt hydrolysis may be caused by both the steric and electrophilic character of ∇^{E}Phe moiety. The rigidly fixed phenyl ring may energetically favor the interaction with the enzyme S_1 site. However, in the case of the simple amino acid derivatives, H-∇^{Z}Phe or ∇^{E}Phe-OMe, there was essentially no difference in the inhibition potency of the two stereoisomers (table 1), a fact seemingly inconsistent with the above 'fit' argument.

Table 1

K_i values of inhibitors containing 2,3-methanophenylalanine

Inhibitors	K_i (mM)
H-(2R,3S)-∇^{E}Phe-Phe-OMe	0.16
H-(2S,3R)-∇^{E}Phe-Phe-OMe	0.60
H-(2R,3S)-∇^{E}Phe-Phe-OH	0.60
H-(2S,3R)-∇^{E}Phe-Phe-OH	1.4
H-(2R,3S)-∇^{E}Phe-Leu-OMe	0.30
H-(2S,3R)-∇^{E}Phe-Leu-OMe	1.3
H-(2R,3S)-∇^{E}Phe-Leu-OH	0.75
H-(2S,3R)-∇^{E}Phe-Leu-OH	no inhibition
H-(±)-∇^{E}Phe-OMe	0.43
H-(±)-∇^{E}Phe-OH	no inhibition
H-(±)-∇^{Z}Phe-OMe	0.36
H-(±)-∇^{Z}Phe-OH	no inhibition

When Phe^2 or Leu^2 occupies the S_1 site, other structural elements stabilizing the enzyme-inhibitor complex should exist. When the inhibition potencies are considered in light of the configurations of the ∇Phe residue, changes in the amino acid at position 2, and changes in carboxylic acid function, the following points became clear: (i) (2R,3S)-configuration of ∇^{E}Phe is preferred to the (2S,3R)-configuration, (ii) Phe^2 is more favorable than Leu^2, and (iii) the methyl ester is more effective than free acid. When all three favorable conditions are met, the most potent inhibitor is obtained, indicating that it is likely that the amino acid at position 2, namely Phe^2 or Leu^2, is the residue interacting with the chymotrypsin S_1 site rather than the ∇Phe in the 1-position.

^{1}H-NMR spectra of the series of H-∇^{E}Phe-Leu dipeptides discussed here showed remarkable differences between the (2R,3S)- and (2S,3R)-isomers and between the methyl ester and free acid, not only in the chemical shifts of ∇Phe signals but also those in the Leu^2 side chain. Detailed conformational analysis from NMR studies suggested that the Leu^2 side chain of (2R,3S)-∇^{E}Phe peptide is more strongly fixed than that of the (2S,3R)-∇^{E}Phe compound and this rigidity may be related to the somewhat greater (4 times) inhibitory activity of the former. The NMR spectrum of H-(2S,3R)-∇Phe-Leu-OH, which has no inhibitory activity, was completely different from that of the other peptides. The X-ray structure of the (2R,3S)-dipeptide ester reported earlier [10] shows a remarkable twist of the cyclopropane ring (the dihedral angle between the carbonyl and phenyl groups is 10.7° instead of the theoretical 0°) possibly weakening the ring and increasing the ease with which it is opened by nucleophiles. Details of the conformational analyses by NMR will be reported elsewhere.

Suckling et al. [11,12] recently reported the partial irreversible inhibition of carboxypeptidase A by cyclopropane derivatives and suggested that enzyme nucleophiles may attack the electrophilic cyclopropane ring of an inhibitor. As a

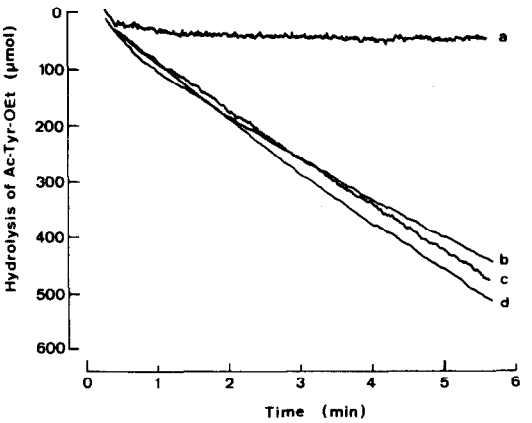

Fig.3. Chymotrypsin activity in Ac-Tyr-OEt hydrolysis after incubation with and without ∇^{E}Phe-containing dipeptide inhibitor. a, enzyme preincubated with H-(2R,3S)-∇^{E}Phe-Leu-OMe for 48 h; b, enzyme preincubated without inhibitor; c, enzyme plus inhibitor not preincubated; d, enzyme not preincubated.

preliminary examination in the present study, when chymotrypsin (1.3 μg) was incubated with 1 mM H-(2R,3S)-∇^{E}Phe-Leu-OMe containing such a ring for 48 h, no hydrolysis of Ac-Tyr-OEt by the enzyme was observed (fig.3, curve a), while the enzyme was almost fully active when preincubated without inhibitor (fig.3, curve b). These results indicated that the enzyme was completely inactivated during incubation with the ∇Phe-peptide. When the preincubation period was omitted (fig.3, curve c) using the same peptide concentration, about 90% of the hydrolysis of Ac-Tyr-OEt occurred, showing only about 10% inhibition. Since the active site of chymotrypsin contains aspartic acid residues (Asp-102 and 194), the carboxylate group of one of these residues may be placed for nucleophilic attack on the cyclopropane ring forming an ester at the active site and thereby causing irreversible inactivation of the enzyme.

In conclusion, this work indicates that the incorporation of 2,3-methanoamino acids into specific enzyme substrates may generate peptidic enzyme inhibitors.

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