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Effects of Halides on Dipeptidyl and Tripeptidyl Carboxypeptidase Activities of Kininase II

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The effects of halides on the dipeptidyl and tripeptidyl carboxypeptidase activities of kininase II (angiotensin-converting enzyme, EC 3.4.15.1) purified from hog kidney were investigated. The dipeptidyl carboxypeptidase activity of the enzyme for bradykinin in the absence of halides was found to be buffer-dependent, and was decreased by the anions in potassium phosphate, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), Tris-H_2SO_4, Tris-H_3PO_4 and Tris-acetate buffers with increasing concentration of these buffers (1—100 mM); however, the activity was unaffected by borate–sodium carbonate buffer in this concentration range. When borate–sodium carbonate buffer was used, F^- functioned as an activator by raising V (2-fold) without modifying K_m, while the other halides (Cl^-, Br^- and I^-) hardly influenced V/K_m (there were decreases of both V and K_m), despite the fact that all the halides functioned as activators in various buffers by raising V (maximum 14-fold) without markedly affecting K_m when angiotensin I was the substrate. The stimulatory effect of F^- on bradykinin hydrolytic activity was maximum at 10 mM, and it was reversed by various other anions (Cl^-, Br^-, I^-, PO_4^{3-}, SO_4^{2-}, Hepes and CH_3COO^-). The V value of the enzyme for Gly–Phe–Ser–Pro-Phe (des-Arg^9–bradykinin analogue), which is a substrate for the tripeptidyl carboxypeptidase activity of the enzyme (*Biochim. Biophys. Acta*, **662**, 300, 1981), was enhanced by the addition of halides in the order $Cl^- > Br^- > I^- > F^-$. On the other hand, the V value of the tripeptidyl carboxypeptidase activity of the enzyme for Gly–Pro–Ser–Pro–Phe, which has a proline residue at P_1, like bradykinin, was enhanced to a lesser degree by halides and the maximal activity was obtained by the addition of 100 mM F^- (which resulted in a 2-fold increase of V).

Keywords——dipeptidyl carboxypeptidase; tripeptidyl carboxypeptidase; kininase II; chloride effect; fluoride stimulation

Skeggs *et al.*[1] reported that kininase II requires Cl^- to generate angiotensin II from angiotensin I. However, the Cl^- dependence of the hydrolysis of bradykinin by the enzyme is unclear; for example, the hydrolysis of bradykinin proceeded at the same rate in the presence and absence of Cl^- in phosphate buffer,[2] while the hydrolysis was accelerated about 3-fold by the addition of a small amount of Cl^- in Hepes buffer.[3,4] We previously reported that kininase II can release C-terminal tripeptides from substrates having a proline residue in the penultimate position, such as des-Arg^9–bradykinin, and that the addition of Cl^- strongly accelerated the hydrolysis of des-Arg^9–bradykinin and its analogues, as well as the conversion of angiotensin I to angiotensin II.[5] Igic *et al.*[6] postulated that the effect of Cl^- on kininase II depends on the structure of the substrate used. Therefore, we speculated that the effects of halides on the dipeptidyl and tripeptidyl carboxypeptidase activities of kininase II may depend not only on the structure of the substrate used but also on the nature of the buffer employed.

In the present paper, we describe the effects of halides in various buffers on the dipeptidyl and tripeptidyl carboxypeptidase activities of kininase II.

Experimental

Materials——Bradykinin, des-Arg^9-bradykinin, angiotensin I, angiotensin II, Bz-Gly-His-Leu and His-Leu were obtained from the Protein Research Foundation, Minoh, Japan. Other peptides used were synthesized in this laboratory. All synthesized peptides were homogeneous on thin layer chromatography on silica gel (*n*-BuOH–AcOH–H_2O, 4:1:1 and *n*-BuOH–AcOH–pyridine–H_2O, 4:1:1:2) and on high performance liquid chromatography (HPLC).

Hog kidney kininase II was prepared as described previously.[7] The purified enzyme preparation gave a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis[8] and its molecular weight was estimated to be 300000 by Sephadex G-200 gel filtration. The specific activity (dipeptidyl carboxypeptidase) of the purified enzyme used in the present studies was 12.5 units/mg protein, which was measured as described previously.[5] A unit was defined as the amount of the enzyme cleaving 1 μmol Bz-Gly-His-Leu per min at 37 °C in 0.1 M Tris-HCl, pH 7.4, containing 0.1 M NaCl.

Enzyme Assays——In all experiments, incubation was carried out at 37 °C in 13 × 100 mm siliconized glass tubes. The following assay components, in a final volume of 1 ml, were incubated for 1—5 min: 1—100 mM buffer, pH 7.5, with or without halide/substrate/2.0—6.3 munits hog kidney kininase II which had been dialyzed against 1 mM borate-sodium carbonate buffer, pH 7.5, overnight at 4 °C. At the specified times following incubation, the reaction was stopped by boiling the mixture in a water bath for 5 min, and 100 μl of the incubation mixture was subjected to HPLC analysis as described previously.[5] The rates of hydrolysis were calculated directly from the peak heights of peptides released and were expressed as nmol peptide released per min in the 1 ml incubation mixture. The analytical system was calibrated with Phe-Arg when bradykinin was the substrate, with His-Leu when angiotensin I was the substrate, and with Ser-Pro-Phe when des-Arg^9-bradykinin, Gly-Phe-Ser-Pro-Phe or Gly-Pro-Ser-Pro-Phe was the substrate.

Enzyme Kinetic Studies——All values of K_m and V are averages of two independent determinations. K_m and V values in various buffers, pH 7.5, for each substrate were determined from Lineweaver-Burk plots. The HPLC assay method used in this study for hydrolysis of each substrate yielded velocities that were linear with time during the 5 min sampling period and enzyme concentrations that were in the range of 2.0—6.3 munits per ml.

Nomenclature——The nomenclature used to describe the position of amino acids in the substrate molecule is that of Schechter and Berger.[9] Starting from the scissile bond, the amino acid residues are P_1' and P_2' as one moves toward the COOH terminal residue of the original peptide, and P_1, $P_2 \cdots P_n$ as one moves toward the NH_2 terminal residue.

Results

Effects of Halides on Dipeptidyl Carboxypeptidase Activity of Kininase II

First, the effects of buffer concentrations on the hydrolysis of bradykinin by hog kidney kininase II in the absence of halide were investigated, as shown in Fig. 1. The results indicate that potassium phosphate, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes),

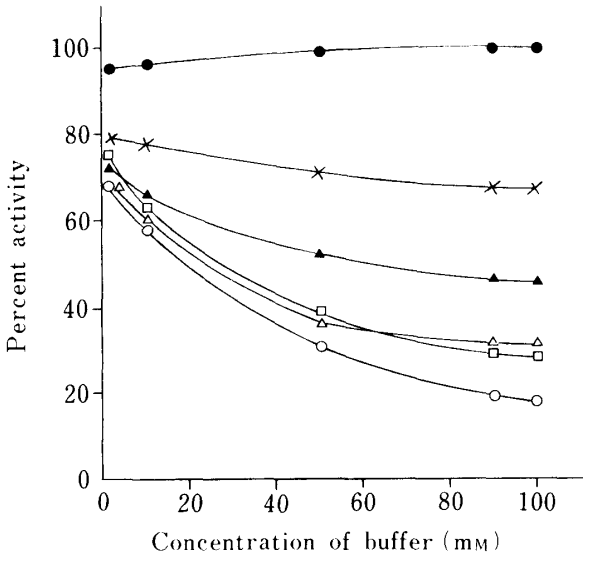

Fig. 1. Effect of Buffer Concentration on the Hydrolysis of Bradykinin by Kininase II

All experiments were carried out at pH 7.5. All values were calculated by taking the rate of hydrolysis of bradykinin in 100 mM borate-sodium carbonate buffer as 100%. Borate-sodium carbonate buffer (●), Hepes buffer (▲), Tris-acetate buffer (×), Tris-H_2SO_4 buffer (□), Tris-H_3PO_4 buffer (△), and potassium phosphate buffer (○). Assays were performed using 5.9 munits of hog kidney enzyme and 10 nmol of bradykinin.

TABLE I. Kinetic Parameters of Hog Kidney Kininase II for the Hydrolysis of Bradykinin

Buffer		K_m	V	V/K_m
Borate–sodium carbonate	10 mM	1.45	2.61	1.80
	100 mM	1.45	2.70	1.86
Hepes	10 mM	1.10	1.78	1.62
	100 mM	1.50	1.24	1.21
Potassium phosphate	10 mM	1.64	1.59	0.97
	100 mM	1.77	0.51	0.29
Tris-H_3PO_4	10 mM	1.90	1.65	0.87
	100 mM	2.05	0.84	0.41
Tris-H_2SO_4	10 mM	2.05	1.70	0.83
	100 mM	1.66	0.78	0.47
Tris-acetate	10 mM	1.82	2.11	1.16
	100 mM	1.90	1.84	0.97

K_m and V values are expressed as μM and μmol·min^{-1}·mg^{-1} of enzyme, respectively. Enzyme assays were carried out at pH 7.5 over the bradykinin concentration range from 0.5×10^{-6} to 1×10^{-5} M.

Tris-H_2SO_4, Tris-H_3PO_4 and Tris-acetate buffers decreased the activity of kininase II toward bradykinin with increasing concentration of buffer; however, borate–sodium carbonate buffer had no effect. The inhibitory effect of phosphate buffer was similar to those of sodium phosphate buffer and Tris-H_3PO_4 buffer. Therefore, it is clear that the inhibitory effect of phosphate buffer on the hydrolysis of bradykinin by the enzyme was caused by phosphate anions. Thus, the decrease of the rate of hydrolysis of bradykinin by the various buffers described above seem to be caused by the anions (in the order $PO_4^{3-} \geqq SO_4^{2-} >$ Hepes > CH_3COO^-). Table I shows that increase of the concentrations of potassium phosphate, Hepes, Tris-H_2SO_4, Tris-H_3PO_4 and Tris-acetate buffers resulted in a decrease of V for bradykinin without much affecting K_m; however, in the case of borate–sodium carbonate buffer, V and K_m were unaffected.

Figure 2 shows that the inhibitory effects of PO_4^{3-}, SO_4^{2-} and Hepes on the hydrolysis of bradykinin by hog kidney kininase II were considerably eliminated by the addition of a small amount of Cl^-. The effect of Cl^- reached a maximum at 10 mM and then declined. These phenomena are observed as an activation of kininase II by Cl^-.[3,4] In borate–sodium carbonate buffer, which has no inhibitory effect on the hydrolysis of bradykinin, the rate was decreased by Cl^-, Br^- and I^-, and stimulated by only F^- (Figs. 2 and 3). The effect of F^- reached a maximum at 10 mM and then declined. Thus, bradykinin hydrolytic activity of kininase II was highest in borate–sodium carbonate buffer, pH 7.5, containing 10 mM F^- (Fig. 3). However, the increase in the rate of hydrolysis of bradykinin in the presence of 10 mM F^- was totally prevented by the addition of other halides (Cl^-, Br^- and I^-) at above 10 mM (Fig. 4).

On the other hand, the rate of hydrolysis of angiotensin I by hog kidney kininase II in borate–sodium carbonate buffer was greatly enhanced by all halides in the order $Cl^- = Br^- > I^- > F^-$ (Fig. 5). The enhancements by halides were also in the same order in phosphate, Hepes, Tris-H_2SO_4 and Tris-acetate buffers (data not shown).

Kinetic parameters for the hydrolysis of bradykinin or angiotensin I in the presence of halides are shown in Table II. All the halides functioned as activators by raising V (maximum 14-fold) without markedly affecting K_m in either 50 mM borate–sodium carbonate buffer or 50 mM Hepes buffer when angiotensin I was the substrate. When 50 mM borate–sodium carbonate buffer used for the measurement of the hydrolysis of bradykinin, only F^-

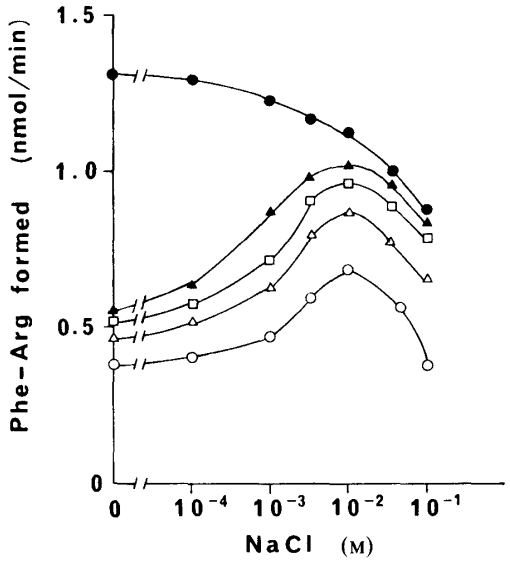

Fig. 2. Hydrolysis of Bradykinin by Kininase II as a Function of NaCl Concentration

All experiments were carried out at pH 7.5; 100 mM borate–sodium carbonate buffer (●), 50 mM Hepes buffer (▲), 50 mM Tris-H_2SO_4 buffer (□), 50 mM Tris-H_3PO_4 buffer (△), and 50 mM potassium phosphate buffer (○). Assays were performed using 5.9 munits of hog kidney enzyme and 10 nmol of bradykinin.

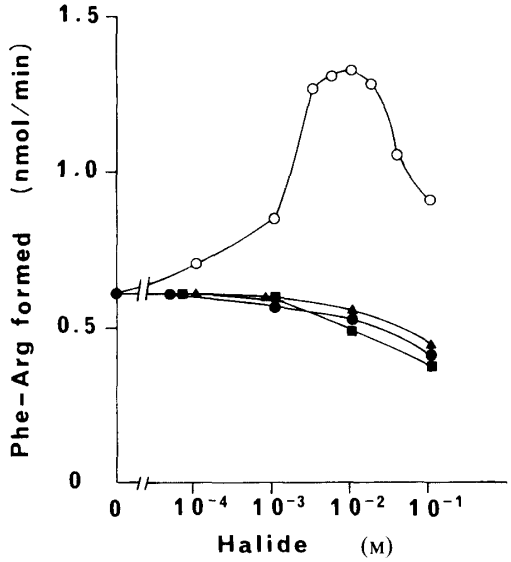

Fig. 3. Effects of Halides on the Hydrolysis of Bradykinin by Kininase II

NaF (○), NaCl (●), NaBr (■) or NaI (▲) was added to 100 mM borate–sodium carbonate buffer, pH 7.5. Assays were performed using 2.8 munits of hog kidney enzyme and 10 nmol of bradykinin.

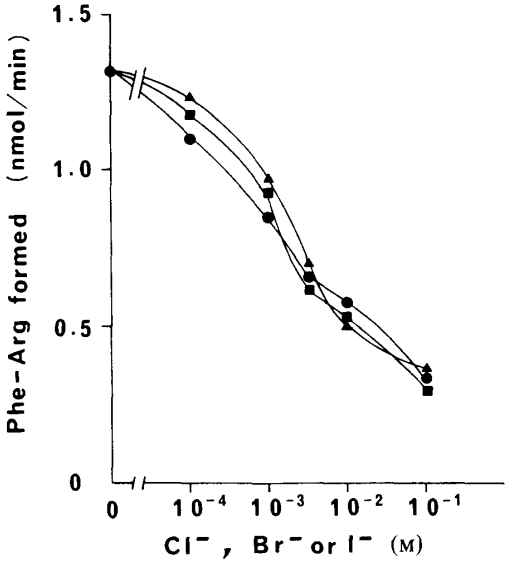

Fig. 4. Antagonism of the Effect of F^- with Cl^-, Br^- or I^- in the Hydrolysis of Bradykinin by Kininase II

NaCl (●), NaBr (■) or NaI (▲) was added to 100 mM borate–sodium carbonate buffer, pH 7.5, containing 10 mM NaF. Assays were performed using 2.8 munits of hog kidney enzyme and 10 nmol of bradykinin.

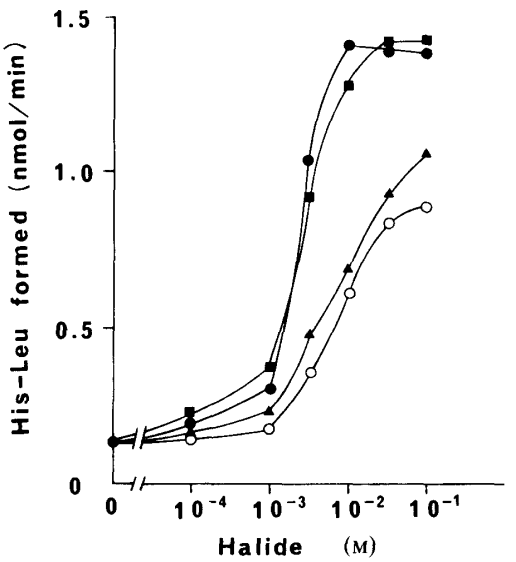

Fig. 5. Effects of Halides on the Hydrolysis of Angiotensin I by Kininase II

NaF (○), NaCl (●), NaBr (■) or NaI (▲) was added to 100 mM borate–sodium carbonate buffer, pH 7.5. Assays were performed using 2.8 munits of hog kidney enzyme and 100 nmol of angiotensin I.

functioned as an activator by raising V (2-fold) without affecting K_m, and the other halides (Cl^-, Br^- and I^-) hardly influenced V/K_m (there were decreases of both V and K_m). On the other hand, since both V and K_m for bradykinin in 50 mM Hepes buffer were increased by the addition of F^-, V/K_m was not increased (Table II). Similar results were obtained in

TABLE II. Kinetic Parameters for the Hydrolysis of Bradykinin or Angiotensin I in the Presence of Halides

Buffer	Bradykinin			Angiotensin I		
	K_m	V	V/K_m	K_m	V	V/K_m
50 mM Borate–sodium carbonate	1.45	2.70	1.86	25.0	0.82	0.033
+10 mM NaCl	1.19	2.26	1.90	27.0	5.32	0.197
+100 mM NaCl	0.97	1.75	1.80	30.0	5.30	0.177
+10 mM NaF	1.30	5.69	4.38	31.0	2.32	0.075
+100 mM NaF	1.40	3.91	2.79	41.0	3.42	0.083
+10 mM NaBr	1.15	2.36	2.05	30.0	4.76	0.159
+100 mM NaBr	1.00	1.79	1.79	33.0	4.80	0.146
+10 mM NaI	1.47	2.20	1.50	27.0	2.63	0.097
+100 mM NaI	1.30	1.80	1.39	29.0	3.56	0.123
50 mM Hepes	1.25	1.40	1.12	26.0	0.47	0.018
+10 mM NaCl	1.23	2.59	2.10	34.0	6.57	0.193
+100 mM NaCl	0.87	1.70	1.95	30.0	6.50	0.127
+10 mM NaF	3.05	4.12	1.35	25.0	2.89	0.116
+100 mM NaF	4.07	4.48	1.10	23.0	4.27	0.186
+10 mM NaBr	1.28	2.05	1.60	28.0	6.02	0.215
+100 mM NaBr	0.95	1.56	1.64	29.5	6.10	0.204
+10 mM NaI	1.16	1.68	1.45	29.5	3.21	0.109
+100 mM NaI	1.10	1.38	1.26	30.0	4.52	0.151

K_m and V values are expressed as μM and μmol·min^{-1}·mg^{-1} of hog kidney kininase II, respectively. Enzyme assays were carried out at pH 7.5 over the substrate concentration ranges from 0.5×10^{-6} to 1×10^{-5} M bradykinin and 1×10^{-5} to 1×10^{-4} M angiotensin I.

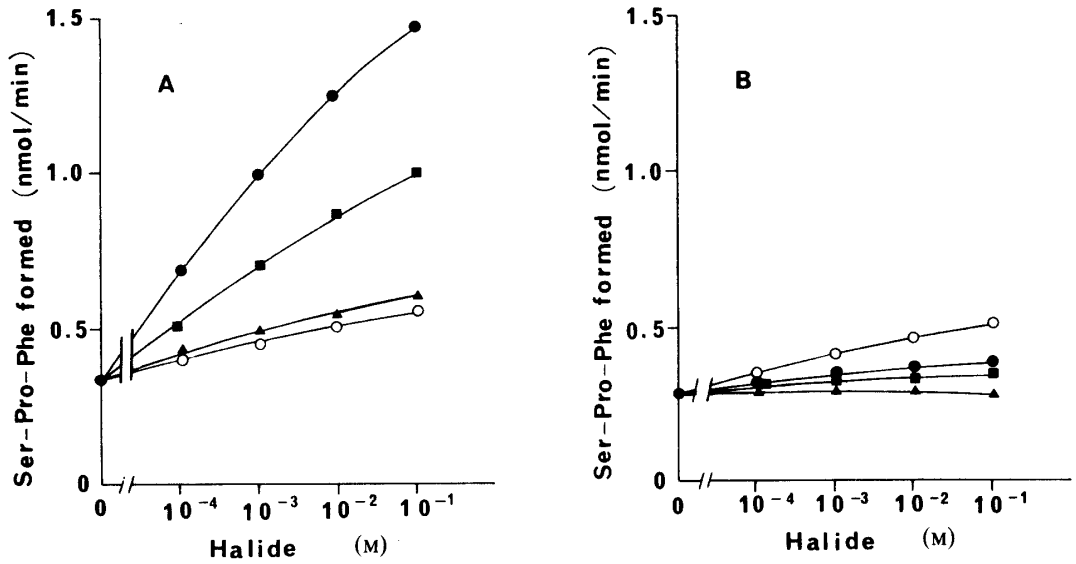

Fig. 6. Effects of Halides on the Hydrolysis of Gly–Phe–Ser–Pro–Phe or Gly–Pro–Ser–Pro–Phe by Kininase II

NaF (○), NaCl (●), NaBr (■) or NaI (▲) was added to 100 mM borate–sodium carbonate buffer, pH 7.5. Assays were performed using 2.8 munits of hog kidney enzyme and 1 μmol of Gly–Phe–Ser–Pro–Phe (A) or Gly–Pro–Ser–Pro–Phe (B).

phosphate, Tris-H_2SO_4 and Tris-acetate buffers at the same concentration (data not shown).

Effects of Halides on Tripeptidyl Carboxypeptidase Activity of Kininase II

The relationship between hydrolysis of Gly–Phe–Ser–Pro–Phe (des-Arg^9–bradykinin

analogue) or Gly–Pro–Ser–Pro–Phe and halide concentration is shown in Fig. 6. The rate of hydrolysis of Gly–Phe–Ser–Pro–Phe by hog kidney kininase II in borate–sodium carbonate buffer was enhanced by the addition of halides in the order $Cl^- > Br^- > I^- > F^-$ (Fig. 6A). The enhancements by halides also occurred in the same order in phosphate, Hepes, Tris-H_2SO_4 and Tris-acetate buffers (data not shown). On the other hand, the rate of hydrolysis of Gly–Pro–Ser–Pro–Phe, which has a proline residue at P_1, like bradykinin, was enhanced to a lesser degree by the addition of halides and the maximal activity of the enzyme was found in borate–sodium carbonate buffer, pH 7.5, containing 100 mM F^- (Fig. 6B). Enhancements by halides hardly occurred in phosphate, Hepes, Tris-H_2SO_4 and Tris-acetate buffers at the concentration of 50 mM (data not shown).

Discussion

Igic *et al.*[6] previously reported that the effect of Cl^- on kininase II varied from substrate to substrate. Our results also show that the effects of halides and other anions on kininase II depend on the substrate used. It seems that the effects of halides on the hydrolysis of bradykinin are dependent on the nature and concentration of anions in the buffer employed. Increase of the concentrations of phosphate, Hepes, Tris-H_2SO_4, Tris-H_3PO_4 and Tris-acetate buffers resulted in a decrease of V for bradykinin without markedly affecting K_m, but in the case of borate–sodium carbonate buffer, V and K_m were not affected (Table I). When the bradykinin hydrolytic activity of the enzyme was measured in borate–sodium carbonate buffer, F^- functioned as an activator by raising V (2-fold) without altering K_m, while the other halides (Cl^-, Br^- and I^-) hardly influenced V/K_m (there were decreases of both V and K_m), despite the fact that all the halides functioned as activators by raising V/K_m (increase of V without much change of K_m) when angiotensin I was the substrate (Table II). Thus, the bradykinin hydrolytic activity of hog kidney kininase II was highest in borate–sodium carbonate buffer, pH 7.5, containing 10 mM F^- (Fig. 3). The increase of the rate of hydrolysis of bradykinin by 10 mM F^- was totally prevented by the simultaneous addition of Cl^-, Br^- and I^- at above 10 mM (Fig. 4). In phosphate, Tris-H_2SO_4 and Tris-acetate buffers, the V/K_m for bradykinin was not increased by the addition of F^- (data not shown), as in Hepes buffer (Table II). Therefore, the affinity of F^- for the enzyme may be lower than those of the other anions. The results in Fig. 2 suggest that the affinity of Cl^- for the enzyme is greater than those of the other anions, so that the inhibitory effects of phosphate, Hepes and sulfate ions on the hydrolysis of bradykinin are largely eliminated by the addition of a small amount of Cl^-.

Oshima *et al.*[10] have studied the ultraviolet (UV) spectra of the kininase II from hog kidney. Their results indicated that kininase II has different conformations in the absence and presence of Cl^-. Thus, Cl^- appears to serve as an allosteric modifier, changing the shape of the active site of the enzyme; such a change may make the active site more suitable for angiotensin I hydrolysis, and more unsuitable for bradykinin hydrolysis.

We reported previously that kininase II releases C-terminal tripeptides from peptides consisting of five or more residues and N-protected tetrapeptides which contain a proline residue at P_2' and a serine or an alanine residue at P_1'.[5] Tripeptidyl carboxypeptidase activity of kininase II on des-Arg^9–bradykinin and its analogues (for example; Gly–Phe–Ser–Pro–Phe) was greatly accelerated by halides, as was the conversion of angiotensin I to angiotensin II. Substitution of a proline residue for the phenylalanine residue at P_1 in Gly–Phe–Ser–Pro–Phe, to give Gly–Pro–Ser–Pro–Phe, resulted in a significant decrease of both the effects of halides and the rate of hydrolysis (Fig. 6). The maximal activity for Gly–Pro–Ser–Pro–Phe, which has a proline residue at P_1, like bradykinin, was obtained by the addition of 100 mM F^-, which gave a 2-fold increase of V (Fig. 6).

The results obtained in the present studies indicate that substrates with a proline residue at P_1 have low V values for both kininase II activities (dipeptidyl and tripeptidyl carboxypeptidase), and replacement of the phenylalanine residue at P_1 in the substrates by a proline residue led to a substantial decline in the halide ion-sensitivity of kininase II.

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