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INACTIVATION OF KININS BY CHYMOTRYPSIN*

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Abstract—The inactivation rate of synthetic bradykinin (BK) by different samples of crystallized α-chymotrypsin at pH 7.0, 30° and a 1:10 enzyme/substrate molar ratio was reduced in the presence of 1,10-phenanthroline. Selective inactivation of the samples by preincubation with L-(1-tosylamido-2-phenyl) ethyl-chloromethylketone (TPCK) in 1:10 enzyme/TPCK ratio reduces the inactivation rate about 60 per cent. This residual BK-inactivating action is probably due to a carboxypeptidase-B-type enzyme present in the chymotrypsin commercially available. In a 1:200 enzyme/substrate ratio and in the presence of 1,10-phenanthroline, a few min of incubation at pH 7.0, 30°, is sufficient to inactivate bradykinin at a constant rate. This inactivation is due to the cleavage of the Phe–Arg bond by chymotrypsin, releasing free arginine. The rate of inactivation of BK by chymotrypsin in the presence of 3.3×10^{-3} M 1,10-phenanthroline was 43.9 nmoles/min/mg of enzyme, and the inactivation rates of the longer kinins, kallidin (lysylbradykinin) and Met-Lys-bradykinin, were 60.1 and 138.18 nmoles/min/mg of enzyme respectively.

It was reported in a previous paper [1] that the initial inactivation rates of bradykinin (BK), Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg, by some proteolytic enzymes varied from a relative rate of 100 for chymotrypsin (ChT) to 25,000 for carboxypeptidase B; preliminary observations had indicated that BK inactivation by the twice crystallized ChT preparation then used was partially inhibited by 1,10-phenanthroline (1,10-Phn). The present paper describes further experiments about this effect of 1,10-Phn on BK inactivation by ChT and compares the inactivation rates of BK, kallidin (lysylbradykinin, LBK), and methionyllysylbradykinin (MLBK) by this enzyme.

MATERIALS AND METHODS

Polypeptides. BK, LBK and MLBK, used as standards, were purchased from Schwarz Bio-Research, Orangeburg, N.Y. The polypeptides used in the experiments were synthesized by the solid phase method by Drs. A. C. M. Paiva and Mariana S. Araujo-Viel.| On paper electrophoresis, these three kinins revealed one main spot and traces of impurities. Assayed on the isolated guinea pig ileum, BK activity was about 70 per cent and LBK and MLBK activities were about 90 per cent of the respective standards. Phe–Arg was a kind gift from Dr. A. C. M. Paiva.

Enzymes. α-Chymotrypsin (EC 3.4.4.5.) of different origins was used; two batches were from Nutritional Biochemical Co., Ohio, lots 4091 and 6922, and two batches from Worthington Biochemical Co., Freehold, N.J., CDI 3AB and 671-74CDI. The determinations of the inactivation rates of bradykinin were made with the last batch unless otherwise stated. Carboxypeptidase B (EC 3.4.2.2.) was from Sigma Chemical Co., and it was a gift from Dr. F. G. Nobrega, Department of Biochemistry, USP.

N-acetyl-L-tyrosine ethyl ester (ATEE) and *N*-benzoylglycyl L-arginine were products from Sigma Chemical Co. 1,10-Phenanthroline, lot 702, was purchased from Eastman Kodak Co., Rochester, N.Y.; L-(1-tosylamido-2-phenyl) ethyl-chloromethylketone (TPCK), lot F 1159, was purchased from Cyclo Chemical Corp., Los Angeles, Calif. L(+)-Arginine for biochemical use and ninhydrin chemically pure were purchased from E. Merck, Darmstadt. Silica gel plates, for thin-layer chromatography, were purchased from Macherey–Nagel & Co., Duren.

Bioassay. Kinin activity was assayed on the isolated guinea pig ileum [2], suspended in atropinized Tyrode solution kept at 37°. Composition of this solution was: NaCl, 1.38×10^{-1} M; $CaCl_2$, 1.8×10^{-5} M; KCl, 2.68×10^{-3} M; $MgCl_2$, 4.90×10^{-4} M; $NaHCO_3$, 1.10×10^{-2} M; NaH_2PO_4, 3.60×10^{-4} M; and atropine sulfate (1 mg) and glucose (1 g) per liter. Single and double doses of standard kinin and unknown solutions were given every 3 min, to estimate the activity.

Estimation of BK inactivation by α-ChT. A BK stock solution (3.4 mg/ml) in 1×10^{-3} M oxalic acid [3] was kept frozen; from this, a working-BK-solution (340 μg/ml) in 1×10^{-3} M oxalic acid was prepared daily. A 0.1-ml aliquot of BK-working-solution (32 nmoles) was diluted in 1.9 ml of 0.05 M, pH 7.0, phosphate buffer and preincubated for 10 min at 30°. To this was added at zero time 0.4 ml (3.2 nmoles) of an α-ChT solution (prepared daily in 1×10^{-3} M HCl) containing 200 μg/ml. In this case the enzyme/BK molar ratio was 1:10. Aliquots of 0.2, 0.4 and 0.8 ml were removed, respectively, at 2, 4 and 8 min from zero time and pipetted into a known volume of boiling Tyrode solution (to make up 2–3 ml) and

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kept in a boiling water bath for 5 min. After cooling, aliquots were assayed against respective kinin controls which had been subjected to the same procedure excluding enzyme addition. For the other kinins, essentially the same procedure was followed.

Effect of 1,10-Phn on BK inactivation by α-ChT. An aliquot of 1,10-Phn solution (1×10^{-2} M warm solution) was added to the α-ChT aliquot and preincubated for 10 min at 30°. Phosphate buffer 0.05 M, pH 7.0, was added to obtain a final 1,10-Phn concentration of 3.3×10^{-3} M and a further preincubation period of 10 min, at 30° was allowed. The BK aliquot was pipetted at zero time and the BK inactivation was measured as described above. Controls with α-ChT alone and 1,10-Phn alone were run simultaneously.

Determination of K_m and V_{max}. The incubation for the evaluation of the kinetic parameters was made in the presence of 1,10-Phn (3.3×10^{-3} M) as described above. The chymotrypsin concentration was held constant and the kinin concentrations varied from 9×10^{-6} to 30×10^{-6} M. The reaction was interrupted as previously described and the remaining kinin activity was measured on the guinea pig ileum. K_m and V_{max} were calculated by the method of Wilkinson [4] using a Varian 620/L-100 computer with a program in BASIC. The points were the average of three or four experiments.

Analysis of BK inactivation products by TLC. BK and α-ChT or carboxypeptidase B were incubated in 0.05 M ammonium acetate buffer, pH 7.0, for 5 min at 30°. The kinin concentration ranged from 1×10^{-4} to 4×10^{-4} M. The final incubation volume was 50 μl. The reaction was stopped by acidification with glacial acetic acid. An aliquot was removed and the remaining solution was dried *in vacuo*, resuspended in 10 μl of 2.0 M acetic acid, and applied to a Silica gel G plate. These plates were subjected to ascending chromatography with chloroform–methanol–17% (g/g) ammonia (2:2:1, v/v); butanol-1–ethylacetate–acetic acid–water (1:1:1:1, v/v); or butanol-1–acetic acid–water (2:3:5). Hydrolysis products were stained with 0.3% ninhydrin in butanol-1 containing 3% acetic acid.

Treatment of α-ChT with TPCK. α-ChT was treated with 10^{-3} M TPCK in 0.1 M Tris–maleate buffer, pH 6.0, in a 10:1 molar ratio of inhibitor/enzyme, for 2 hr, at room temperature [5]. The complete inhibition of α-ChT was followed by measuring its activity against ATEE (0.01 M) in a pH-stat [6]. The reaction was interrupted by lowering the pH to 3.0 and the enzyme solution was diluted to a final concentration of 3.2 nmoles in 0.4 ml.

Carboxypeptidase B activity. This was followed by the hydrolysis of 1.2 nM benzoylglycyl-arginine, in 0.025 M Tris–HCl, 0.1 M NaCl, pH 7.5, 30° in a final volume of 0.5 ml. The reaction was interrupted by glacial acetic acid, and the hippuric acid formed was extracted by ethylacetate [7], dried under reduced pressure, and dissolved in 2 ml water. The absorbance was read at 228 nm.

RESULTS

BK inactivation by α-ChT, TPCK-treated α-ChT (TPCK-α-ChT) and effect of 1,10-Phn. Figure 1 shows the per cent inactivation of BK by α-ChT and by TPCK-α-ChT, both in the presence and absence of 1,10-Phn. It is evident that 1,10-Phn inhibited partially BK inactivation by α-ChT. The inactivation rate of BK by TPCK-α-ChT was lower than that of the non treated α-ChT. In the presence of 1,10-Phn, this inactivation was completely abolished. Adding up the inactivation rate of BK by α-ChT in the presence of 1,10-Phn and the inactivation rate by TPCK-α-ChT, one gets the rate of inactivation by the nontreated α-ChT (Table 1). Only one sample of α-ChT tested (lot 6922, Nutritional Biochemical Co.) was not inhibited partially by 1,10-Phn. The other samples showed inhibitions of the same order of magnitude. When benzoylglycyl-arginine was used as substrate, the chymotrypsin showed a carboxypeptidase activity corresponding to 2–6 μg carboxypeptidase B/mg of chymotrypsin.

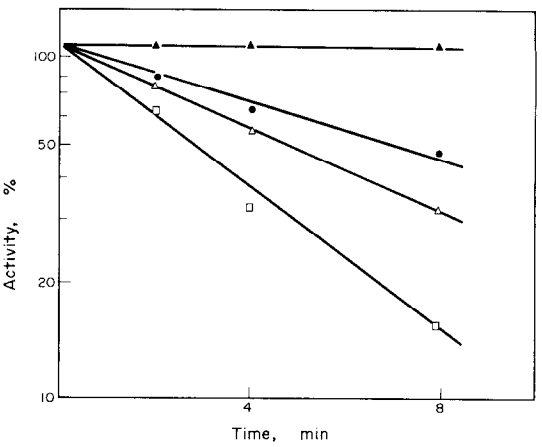

Fig. 1. Per cent inactivation of bradykinin by α-ChT. Incubation of a 1.3×10^{-5} M bradykinin solution in a 1:10 enzyme substrate molar ratio, pH 7.0, 30°, for 5 min. Key: (□---□) α-ChT; (△——△) α-ChT preincubated for 10 min at 30° with 3×10^{-3} M 1,10-phenanthroline; (●——●) TPCK-α-ChT; and (▲——▲) TPCK-α-ChT plus 3×10^{-3} M 1,10-phenanthroline.

Site of cleavage by α-ChT. The kinin peptide bond cleaved by α-ChT was investigated on thin-layer chromatography plates. The earliest bond to be cleaved in bradykinin, as revealed by three different solvent systems, was the Phe^8–Arg^9 bond; arginine was thus released (Fig. 2). Increasing the substrate/enzyme ratio from 1:200 to 1:10, other fragments appeared, probably due to a Phe–Ser hydrolysis. α-ChT also releases a free Arg from LBK and MLBK in the presence of 1,10-Phn (3.3×10^{-3} M). It may be seen

Table 1. Effect of 1,10-Phn on BK inactivation by α-ChT and TPCK-α-ChT

Incubation*	BK inactivation (nmoles BK/mg enzyme/min)	Per cent
α-ChT	68.8	100
α-ChT + 1,10-Phn	40.4	58.7
TPCK-α-ChT	27.2	39.9
TPCK-α-ChT + 1,10-Phn	0	0

* Conditions are as described in Fig. 1.

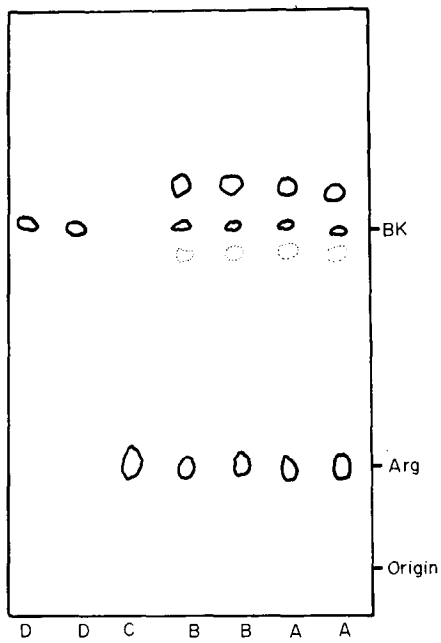

Fig. 2. Thin-layer chromatography of bradykinin hydrolyzed by α-ChT at 30°. Hydrolysates in 0.05 M ammonium acetate buffer, pH 7.0, of 10 nmoles BK with α-ChT in a 1:200 enzyme/substrate molar ratio in a final volume of 50 μl (A). The same incubation in the presence of 1,10-Phn (B); 10 μl of 0.01 M arginine (C); and 10 μg bradykinin standard (D). (Chloroform–methanol–ammonia). Top spots are (des Arg^9) bradykinin.

that the migration of free arginine is different from the peptide Phe–Arg. (Fig. 3). It was also observed that carboxypeptidase B and TPCK-α-ChT release the C-terminal arginine from bradykinin, and from this experiment the top spot in Fig. 2 was identified as (des Arg^9)-BK.

Hydrolysis of MLBK and LBK by α-ChT and K_m *determinations.* Experiments run under the same conditions as those described for BK but using MLBK

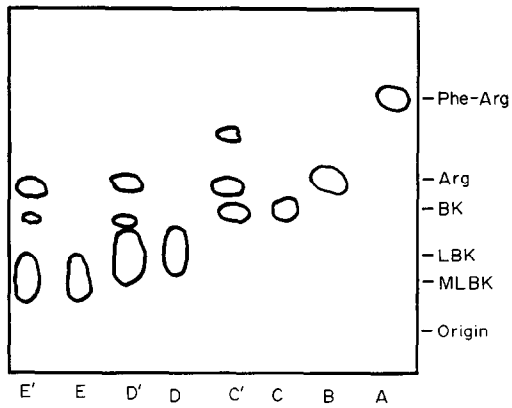

Fig. 3. Thin-layer chromatography of BK, LBK and MLBK hydrolyzed by α-ChT at 30°. Hydrolysis in the same conditions as described in Fig. 2 but at a 1:25 enzyme/substrate molar ratio. This solvent system was butanol–1–ethanol–ethyl-acetate–acetic acid–water; 5 μl of a 0.01 M solution of: Phe–Arg (A) and Arg (B); 10 μg of: BK (C), LBK (D) and MLBK (E); chymotryptic incubates: BK (C'), LBK (D') and MLBK (E').

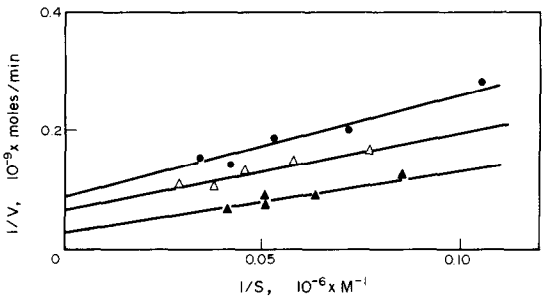

Fig. 4. Kinetics of the inactivation of kinin. Lineweaver–Burk plots of BK (●——●), LBK (△——△) and MLBK (▲——▲) inactivation by α-ChT (0.33 mg/ml in 0.05 M phosphate buffer, pH 7.0, 30°). The ChT concentration was held constant and the substrate concentration varied from 9 to 30 × 10^{-6} M.

or LBK as substrates, in the presence of 3.3 × 10^{-3} M 1,10-Phn (Fig. 4), showed that the inactivation rate of longer kinins was faster than that of BK but the differences of K_m (Table 2) for the three substrates were not significant.

DISCUSSION

The inactivation rate of BK by α-ChT decreased about 40 per cent by preincubation of this enzyme with 1,10-Phn, an observation which confirms and extends preliminary experiments made in this laboratory [1]; this behavior was observed for three out of four commercial samples of the enzyme. On the other hand, selective inhibition of the ChT sample by preincubating the enzyme with TPCK, a specific ChT inhibitor, reduced the BK inactivation rate by about 60 per cent. These two observations indicated that the BK inactivation was due to two different enzymatic activities: one was ChT and the other, which was susceptible to the chelating agent 1,10-Phn, we believe to be contaminating carboxypeptidase B. It is known that carboxypeptidase B is much more active than ChT as a BK-inactivating enzyme [8], reaching a value as high as 250-fold more active [1]. Using this last estimate, a 0.4 per cent contamination of ChT with carboxypeptidase B, which was measured by the hydrolysis of benzoylglycyl-arginine, would explain our observations regarding both the degree of BK inactivation and the effect of 1,10-Phn on this inactivation. After the TPCK treatment of α-ChT, the remaining BK-inactivating enzyme released Arg from

Table 2. Inactivation of BK, LBK and MLBK by α-ChT at pH 7.0, 30°, in the presence of 1,10-Phn*

Substrate	K_m† (mM)	V_{max} (nmoles kinin/min/mg enzyme)	Relative inactivation rate
BK	2.06 ± 1.20	43.9 ± 5.1	1
LBK	2.23 ± 0.78	60.1 ± 3.6	1.36
MLBK	4.09 ± 1.22	138.2 ± 11.1	3.14

* Substrate concentration varied from 9 to 30 μM. Enzyme concentration was 0.33 mg/ml. 1,10-Phn (3.3 × 10^{-3} M) was used. The final volume of incubation was 2.4 ml.

† K_m: averages and standard deviations of three or four determinations for each kinin.

bradykinin and was completely inhibited by 3.3×10^{-3} M 1,10-Phn. This concentration of 1,10-Phn inhibited also the carboxypeptidase B activity on benzoylglycyl-arginine; thus our observations are compatible with the interpretation that carboxypeptidase B is the enzyme contaminating α-ChT.

This interpretation raised doubts about the reported release of Arg^9 from BK by ChT. It was found that in a 1:100 molar ratio ChT splits the Phe^8–Arg^9 bond of BK more rapidly than the Phe^5–Ser^6 bond, forming as main products free arginine and an octapeptide; at a 1:40 molar ratio the Phe^8–Ser^9 bond was also split [9]. It was also reported by Boissonas *et al.* [10] that in a 1:10 weight ratio ChT attacked more rapidly the Phe^8–Arg^9 bond in BK and more slowly the Phe^5–Ser^6 bond. It is accepted, however, that ChT is an endopeptidase, and location of a normally susceptible peptide bond penultimate to the C-terminal end of a peptide appears to be a major reason for lack of hydrolysis [11]. We observed in the present paper that in a 1:200 enzyme/substrate molar ratio a 5-min incubation at 30° of ChT with BK inactivates the kinin and releases free arginine. Considering that this splitting was observed under conditions in which carboxypeptidase B could not be the enzyme responsible for the arginine release, we conclude that the Phe^8–Arg^9 bond is really more easily attacked by ChT than the Phe^5–Ser^6 bond, and its rupture is probably responsible for the rapid kinin inactivation by ChT. These findings agree with the model proposed for the active site of chymotrypsin [12] and would explain the faster splitting of the Phe^8–Arg^9 bond. The tripeptide Ser^6.Pro^7.Phe^8 would fit in the subsites of the active site of chymotrypsin, while the presence of Pro in the tripeptide Pro^3.Gly^4.Phe^5 would cause difficulties for the proper binding. The bond split in LBK and MLBK at the beginning of the reaction was also the C-terminal Phe.Arg, in the same way as shown for BK.

It was surprising to find that the inactivation rates of LBK and MLBK were higher than the BK inactivation rate. It is known that residues adjacent to those involved in the peptide bond split may enhance the binding of proteins to carboxypeptidase A [13]. Although in our case the increase in sensitivity to ChT was due to one or two extra residues located eight amino acid residues apart from the bond to be split, the new residues seem to be altering the peptide, making it more susceptible to the proteolytic attack by ChT. A similar fact was observed for the inactivation of kinins by angiotensin converting enzyme from rat and hog lung; in this case the longer kinins are more resistant to inactivation [14, 15]. It is tempting to suppose that a change in the polypeptide conformation produced by the extra residue(s) would either expose or hide the bonds to be split; on the other hand it is not possible to exclude an extended active site for chymotrypsin. In the case of trypsin, recent observations in this laboratory have shown a dramatic difference in the susceptibility of the Arg.Ser bond in bradykinin and lysylbradykinin analogs [16].

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