EVIDENCE FOR THE LOCATION OF METHIONYL-LYSYL-BRADYKININ MOIETIES AT THE CARBOXYL TERMINUS AND INSIDE OF BOVINE KININOGEN-I

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SUMMARY: The location of the kinin moiety in high molecular weight (HMW) bovine kininogen (kininogen-I), a sensitive substrate for serum kallikrein, was investigated. Treatment with carboxypeptidase B destroyed about one-half of the initial kinin-yielding ability of kininogen-I, and selective cleavage of the methionyl peptide bonds in kininogen-I with cyanogen bromide liberated free kallidin (lysyl-bradykinin) and an inactive kallidin-containing peptide. These results show that there is a methionyl-lysyl-bradykinin sequence located both at the carboxyl terminus and inside of the polypeptide chain of bovine kininogen-I.

The presence of two kininogens with different molecular weights and susceptibilities to plasma kallikrein has been shown in human, dog, guinea pig, rabbit and rat plasma by Jacobsen (1,2); in horse plasma by Henriques *et al.* (3); and in bovine plasma by Yano *et al.* (4). More recently, both HMW and LMW (low molecular weight) kininogens have been highly purified from human plasma (5). LMW bovine·kininogen (kininogen-II) has been highly purified by the groups of Habermann (6) and of Suzuki (7,8). Its kinin segment--which is released by trypsin, snake venom kininogenases or hog pancreatic kallikrein (but not by bovine plasma kallikrein)--was thought to lie in the central part of a single polypeptide chain bridged by disulfide bonds (8,9). On the other hand, Pierce and Webster (10) isolated two LMW human kininogens with the same molecular weight of about 50,000 but with their kinin sequences carboxyl-terminal in one case and internal in the other. However, until now no

definitive results have been published about the position of the kinin sequence in HMW kininogens. We wish to report the results of our experiments to determine the location of the kinin moiety in HMW bovine kininogen (kininogen-I).

MATERIALS AND METHODS

Bovine kininogen-I, previously isolated by the authors (4), was further purified by a combination of heat treatment and chromatography on DEAE-Sephadex A-50 and CM-Sephadex C-50 columns. Details of this procedure will be published elsewhere. This preparation did not contain kininogen-II or its dimeric artifact isolated by Habermann (11) and by Nagasawa *et al.* (8). Bovine serum kallikrein activated with casein was purified as described earlier (12). Twice-crystallized trypsin (Armour Pharmaceutical Co., U.S.A.) was treated with L-(1-tosylamido-2-phenyl)-ethyl-chloromethyl ketone. CPase A (carboxypeptidase A) and CPase B (Sigma Chemical Co., U.S.A.) were treated with diisopropyl fluorophosphate. Synthetic bradykinin and kallidin were prepared by the Peptide Center of this Institute. Methionyl peptide bonds were selectively cleaved by cyanogen bromide (BrCN) by the method of Gross and Witkop (13). Quantitative determination of amino acids was performed on an autoanalyzer, Model JLC-5AH, Japan Electric Optics Lab. Ltd. The BrCN peptides were chromatographed on CM-Sephadex C-50 columns as previously described for kinins (14).

RESULTS AND DISCUSSION

Effect of CPase B on kininogen-I --- Kininogen-I was incubated with CPase B at 30° overnight. The reaction was terminated by the addition of excess *o*-phenanthroline. Kininogen-I, before and after CPase B treatment, was incubated with trypsin or serum kallikrein, and the kinin activity so

Table I

Kinin activities from kininogen-I before and after CPase B treatment

Kininogenase	Kinin activity* produced from	
	Untreated kininogen-I	CPase B-treated kininogen-I
Serum kallikrein	1,240	560
Trypsin	1,200	540

* Kinin activity is expressed by defining the activity equivalent of 1 x 10^{-9} gram of synthetic bradykinin as 1 unit.

The reaction mixture containing purified kininogen-I (total A_{280}=0.045) 100 µl, 1M Tris buffer, pH 8.5, 0.05 ml, saline 0.05 ml and CPase B 10 µl (0.45 µg) was incubated overnight at 30°. The reaction was terminated by addition of 0.1 ml of 40 mM o-phenanthroline. After 30 min, 0.19 ml of serum kallikrein (0.057 TAME unit) was added and incubation was continued at 30° for 40 hours. In an experiment with trypsin, kininogen-I was treated with CPase B in an identical condition except for the volume of saline; 0.235 ml. Then the CPase B-treated kininogen-I was incubated with 5 µl of trypsin (1.45 TAME unit) at 30° for 2 hours. Fully liberated kinin was assayed by measuring its effect on the contraction of isolated rat uterus (15). One TAME unit is defined as the amount of enzyme which hydrolyzed 1 µmole of TAME (N-α-tosyl-L-arginine methyl ester) per min at 37° at pH 8.5 in 0.1 M Tris buffer.

generated was measured. After treatment with CPase B, the kinin-yielding ability of kininogen-I decreased to 45% of that before treatment, as shown in Table I. Even when the incubation time was shortened to 8, 30 or 60 min, the kinin-yielding ability of kininogen-I decreased significantly.

This experiment shows that at least one-half of the kinin is at the carboxyl terminus of kininogen-I, since removal of the carboxyl-terminal arginine residue from a kinin moiety so located would destroy its ability to give active kinin. The observation that another half of the kinin-yielding ability of kininogen-I is not destroyed by CPase B suggests two possibili-

ties: either this kinin moiety is also carboxyl-terminal but is inaccessible to CPase B, or it lies within the polypeptide chain. The following experiment discriminates between these alternatives.

Cleavage of kininogen-I with BrCN --- Kininogen-I (40 mg) was treated with BrCN (113 mg) in 5 ml of 70% HCOOH at 15° for 24 hours. After a ten-fold dilution with water, the reaction mixture was lyophilized and filtered through a Sephadex G-50 column (2 x 98 cm) equilibrated with 5% CH_3COOH. The BrCN peptide fraction was further purified by chromatography on a CM-Sephadex C-50 column (1 x 12 cm). Two peptides were isolated, one with kinin activity and the other with potential kinin activity. The former peptide appeared at the same point of the chromatogram as authentic kallidin, and its amino acid analysis agrees with that expected for kallidin (Table II). The yield was about 230 μg. The second BrCN peptide gave kinin

Table II

Amino acid composition of the BrCN-fragment with kinin activity

Amino acid	μmoles	Residues* per mole	Kallidin**
Serine	0.0079	0.754 (1)	1
Proline	0.0344	3.271 (3)	3
Glycine	0.0137	1.308 (1)	1
Phenylalanine	0.0161	1.534 (2)	2
Lysine	0.0095	0.903 (1)	1
Arginine	0.0221	2.107 (2)	2

* 0.0105 μmole was taken as 1 μmole and the most probable integer of residues is indicated in parentheses.

** For comparison the amino acid composition of kallidin is shown.

activity when treated with CPase A, trypsin or bovine serum kallikrein. The yield was 340 μg as kallidin when trypsin was used. A preliminary amino acid analysis was consistent with the sequence Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Val-Gln-Val-Homoser, as found for the BrCN kinin peptide obtained from LMW bovine kininogen by Habermann (16) and by Kato et al. (9,17).

The isolation of two BrCN kinin peptides, one active and the other inactive until treated with certain enzymes, agrees with the CPase B experiment and, further, shows conclusively that methionyl-lysyl-bradykinin moieties are located both at the carboxyl terminus and inside of the polypeptide chain of bovine kininogen-I. However, whether the two kinin sequences reside in the same or different molecules remains to be ascertained.

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