RELATIONSHIP BETWEEN TERTIARY STRUCTURE AND KININ YIELDING ABILITY OF BOVINE KININOGEN

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Kininogen is a protein precursor of hypotensive polypeptides in plasma, plasma kinin, such as bradykinin and kallidin. As the first step towards the understanding of the kinin liberation system in bovine blood plasma, the purification of kininogen was undertaken by our group, by Habermann et al. (1963) and Greenbaum and Hosoda (1963). The author's studies (Suzuki et al. 1965, 1966, Nagasawa et al. 1966) showed that bovine kininogen was a glycoprotein with a molecular weight of 49,500 and with NH_2-terminal serine and COOH-terminal leucine as end groups. We further studied the relationship between the structure and the kinin yielding ability of bovine kininogen physicochemically and immunologically. This paper presents evidence that the tertiary structure of kininogen is essential for the liberation of kinin by enzymatic hydrolysis with pancreatic kallikrein.

MATERIALS AND METHODS

Kininogen was prepared from bovine plasma as reported previously, except that heat treatment was replaced by zinc acetate precipitation (unpublished). The snake venom bradykinin releasing enzyme was purified from the venom of Agkistrodon halys blomhoffii by the method of Iwanaga et al. (1965). The kinin

activity was assayed as described previously (Suzuki et al. 1965). Pancreatic kallikrein given by Teikoku Zoki Co. was further purified by the use of DEAE- and CM-cellulose columns to remove some proteolytic enzymes. For immunological studies, rabbits were immunized with purified kininogen with the use of Freund-type complete adjuvant. Immuno-γ-globulin was prepared from the serum by the Kekwick's method. The antigen-antibody reaction was carried out according to the method of Ouchterlony.

RESULTS AND DISCUSSION

As a part of a series of studies on the gross structure of kininogen, the sulfhydryl and disulfide groups in the protein were determined. Kininogen was reacted with C^{14}-monoiodoacetic acid in the presence of 8 M urea. The reaction mixture was applied on a column of Sephadex G-25 and the radioactivity in each fraction was measured. No radioactivity was found in the protein fraction eluted. Moreover, on spectrophotometric titration with p-chloromercuribenzoate in the presence and absence of 8 M urea, no shoulder in the curves was found, indicating the absence of a sulfhydryl residue. On the other hand, when C^{14}-monoiodoacetic acid was added after kininogen had been reduced with β-mercaptoethanol, the protein became labeled with radioactivity. The number of disulfide groups was determined by the following methods: (i) amino acid analyses of native, and reduced and carboxymethylated kininogen (RCM-kininogen), (ii) incubation of reduced kininogen with C^{14}-monoiodoacetic acid, (iii) spectrophotometric titration of reduced kininogen with p-chloromercuribenzoate. As shown in Table I, six or seven disulfide groups were found in kininogen. These results show that kininogen has no free sulfhydryl groups but consisted of a single polypeptide chain bridged with six or seven disulfide groups.

Table I

Summary of Estimation of Disulfide Bonds of Kininogen

Methods	Half cystine	Carboxymethyl-cysteine
(1) Amino acid analysis native kininogen	13.0	---
RCM-kininogen	---	11.3
(2) C^{14}-Monoiodoacetic acid method after reduction	---	14.3
(3) p-Chloromercuribenzoate method after reduction	11.3	---

Each value represents the number of sulfhydryl residue per mole of protein.

When all the disulfide groups of kininogen were reduced with β-mercaptoethanol and successively converted to their carboxymethyl derivatives with monoiodoacetic acid, kinin was not released from either reduced kininogen or RCM-kininogen by pancreatic kallikrein. However, trypsin and snake venom bradykinin releasing enzyme could release kinin from these modified kininogens as well as from native kininogen. It was also found that pancreatic kallikrein could not release kallidin from performic acid oxidized kininogen but could release kallidin from kininogen treated with 8 M urea. When reduced kininogen was slowly reoxidized by standing at pH 8.6 at room temperature, its ability to yield kinin with pancreatic kallikrein was progressively restored with decrease in the number of sulfhydryl residues, as shown in Fig. 1. After 12 hours standing, the kinin yielding ability of reoxidized kininogen was restored to about 50 per cent of native kininogen. However, when the reoxidation was carried out in the presence of a catalytic amount of cupric ion

(7×10^{-5}M), no kinin release from this reoxidized kininogen with pancreatic kallikrein was observed. These results are shown in Table II.

Reduced kininogen and RCM-kininogen failed to cross-react with antibody against native kininogen, although reoxidized kininogen and the dekininated protein derived by dekinination with snake venom bradykinin releasing enzyme or pancreatic kallikrein, cross-reacted with the antibody.

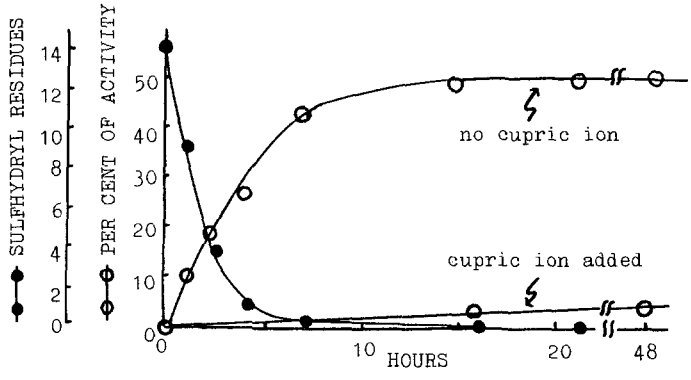


Fig. 1 Kinin yielding ability and sulfhydryl content during reoxidation of reduced kininogen.
Reduced kininogen was slowly reoxidized in 0.05 M Tris buffer, pH 8.6 (1.1 mg/ml) with and without cupric ion. At intervals, aliquots were taken and their kinin yielding ability with pancreatic kallikrein was examined. Sulfhydryl contents were estimated by adding C14-monoiodoacetic acid (A product of Radiochemical Center, England) to the aliquots.

These results suggest that the rigid conformation of kininogen is necessary for the formation of kinin by enzymatic hydrolysis with pancreatic kallikrein, but not with trypsin or snake venom bradykinin releasing enzyme. The above results were confirmed by optical rotatory dispersion and the difference spectrum, and the results of these studies indicated that the tertiary structure of RCM-kininogen was greatly changed from that of the native protein, but that the dekininated protein had

a similar conformation to the native protein (Kato et al.).

Table II

Kinin Yielding Ability of Kininogen and Its Derivatives

Kininogen	Kinin yielding ability*	Antigen-antibody reaction
Native	100	+
Reduced	< 10	-
Reduced and carboxy-methylated	< 10	-
8 M urea treated	100±10	
Performic acid oxidized	<10	
Dekininated	-	+
Reoxidized		
no cupric ion	50±5	+
cupric ion added	<10	+

* Percentages of the amount of kinin liberated from native kininogen by pancreatic kallikrein.

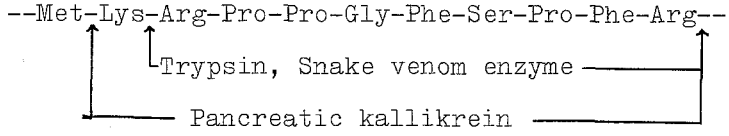
Recently, we obtained a kinin yielding fragment from kininogen by cyanogen bromide treatment (unpublished). Axén et al. also reported kinin release by cyanogen bromide treatment of human kininogen (Axén et al., 1966). From this peptide isolated by us, pancreatic kallikrein, trypsin and the snake venom bradykinin releasing enzyme could release kinin by cleaving only the C-terminal arginine residue in the fragment. Studies on the full structure of this fragment are now under progress. But, considering the structure of pepsitocin isolated hy Habermann (1966), its structure can be written as follows.

Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-

-Ser-Val-GluNH_2-Val-Homoser

On the basis of these facts, it is speculated that the ter-

tiary structure of kininogen is essential for hydrolysis at the methionyl-lysyl bond in the kininogen molecule by pancreatic kallikrein.



It is probable that pancreatic kallikrein hydrolyses the arginyl peptide bond, because it has TAMe esterase activity. But the reason why pancreatic kallikrein hydrolyses the methionyl-lysyl bond, is not yet clear. It seems that the amino acid sequences or an unknown conformation around the NH_2-terminal structure of kinin in kininogen may contribute in the interact to the hydrolysis by pancreatic kallikrein.

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