

RELATIONSHIP BETWEEN STRUCTURE AND CORRECTING
ACTIVITY OF BOVINE HIGH MOLECULAR WEIGHT
KININOGEN UPON THE CLOTTING TIME OF
FITZGERALD-TRAIT PLASMA*

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When normal plasma comes in contact with negatively charged surfaces such as glass, kaolin, and connective tissue, a group of plasma proteolytic enzymes is activated. The proteins that have been identified as being involved in this contact reaction are: Hageman factor (factor XII), Fletcher factor (prekallikrein), and Fitzgerald factor (high molecular weight kininogen, HMWK).¹ The interaction of these plasma proteins on a negatively charged surface results in the activation of factor XI, prekallikrein, and plasminogen (1-11). Congenital deficiencies in any of these proteins result in abnormalities of the intrinsic clotting and fibrinolytic pathways and in the formation of kinins (5-11).

Until the Fitzgerald trait (HMWK deficiency) (8) was discovered, the only known role of HMWK was as a substrate for plasma kallikrein. Now it is also recognized as a necessary component for the activation by contact of the above-mentioned proteolytic pathways. Data from our laboratory (12) and from others (13) have shown that purified bovine high molecular weight kininogen (bHMWK) partially corrects the prolonged activated plasma thromboplastin time (aPTT) and the kaolin-activated euglobulin-lysis time of Fitzgerald-trait plasma. Several investigators have found that, while hydrolysis of human HMWK (hHMWK) by human plasma kallikrein results in kinin release, the ability of kinin-free HMWK to correct the abnormalities of Fitzgerald-trait plasma is not affected (11, 14-16). Hydrolysis of bHMWK by bovine plasma kallikrein results in the release of a large peptide (fragment 1-2) as well as bradykinin. The bradykinin-fragment 1-2-free bHMWK had almost no correcting activity when tested on Fitzgerald-trait plasma (12, 13). These differences in the correcting activity of both human and bovine kininogens after hydrolysis may be due to a lack of fragment 1-2 cleavage from the hHMWK by plasma kallikrein.

To determine whether fragment 1-2 is necessary for bHMWK correcting activity,

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¹ *Abbreviations used in this paper:* aPTT, activated plasma thromboplastin time; bHMWK, bovine high molecular weight kininogen; Brk, bradykinin; HCH, heavy chain; HMWK, high molecular weight kininogen; hHMWK, human HMWK; LCH, light chain; LMWK, low molecular weight kininogen; SBTI, soy bean trypsin inhibitor.

kinin-free bHMWK with intact fragment 1-2 was tested for its correcting activity upon the aPTT of Fitzgerald-trait plasma. Also to examine whether bovine plasma kallikrein decreases the correcting activity of hHMWK, these two proteins were incubated and the correcting activity of the mixture was determined.

To clarify the relationship between the structure and the function of HMWK, the correcting activity of various isolated fragments of bHMWK was studied. Because we previously reported that fragment 2 (histidin-rich fragment) has an inhibitory effect upon the clotting time of normal human plasma, the inhibitory activity of fragment 1-2 was also studied.

Materials and Methods

Reagents and Chemicals. Sodium barbital, NaCl, acid-washed kaolin (Fisher Scientific Co., Pittsburgh, Pa.), CaCl₂ anhydrous (J. T. Baker Chemical Co., Phillipsburg, N.J.) were commercially obtained. HMWK-deficient plasma was obtained directly from Mr. Fitzgerald. Nine parts of blood were mixed with one part of 3.8% sodium citrate. Plasma was separated by centrifugation, distributed in 3-ml aliquots in polystyrene tubes, and kept frozen at -70°C.

Purified Bovine Proteins. bHMWK was purified and characterized as described elsewhere (17, 18). Prolonged incubation with bovine plasma kallikrein releases bradykinin, fragment 1-2, and, later on, fragments 1 and 2. These fragments have been characterized (18-20). Heavy chain and light chain were further obtained by reduction and alkylation of the bradykinin-fragment 1-2-free-bHMWK (21). Lys-bradykinin-free-bHMWK was obtained by treatment of bHMWK with human urinary kallikrein as previously published (22). Fragment 1-2-light chain was obtained by reduction and alkylation of the lys-bradykinin-free-bHMWK and characterized as described (22). Purified bHMWK was obtained by the method of Guimaraes et al. (23). 1 mg of lyophilized kininogen has 8.5 times more correcting activity on the Fitzgerald-trait plasma aPTT than 1 ml of a pool of normal human plasma. Assessed by polyacrylamide gel electrophoresis (20 µg), it revealed three major bands and two faint bands. When eluted from one unstained gel and tested on Fitzgerald-trait plasma aPTT, all three major bands revealed clotting activity. This hHMWK contained no detectable Hageman factor, prekallikrein, factor XI, plasminogen, or α₂-macroglobulin.

Kaolin aPTT. The correcting activity of HMWK and its fragments on the aPTT of Fitzgerald plasma was tested by adding the following in a polystyrene tube: 0.1 ml of Fitzgerald plasma, 0.1 ml of the test substance in Owren's buffer, and 0.1 ml of cephalin-kaolin suspension (10 mg of acid-washed kaolin suspended in 1.0 ml of cephalin). This mixture was preincubated at 37°C for 8 min, and then 0.1 ml of 0.2 M CaCl₂ was added and the clotting time determined.

Inhibition of the aPTT of Normal Plasma by Fragment 1-2 and Fragment 2. Cephalin-kaolin suspension (0.1 ml) was incubated for 1 min with an Owren's buffer solution of the fragment to be tested (0.1 ml). Normal plasma (0.1 ml) was added and the mixture incubated for 3 min. After the addition of 0.02 M CaCl₂ (0.1 ml), the clotting time was determined.

Comparison of the Correcting Activity of bHMWK and its Fragments on the aPTT of Fitzgerald-Trait Plasma on a Molar and on a Weight Basis. Intact bHMWK (mol wt 76,000); lys-bradykinin-free bHMWK (mol wt 75,000); bradykinin-fragment 1-2-free bHMWK (mol wt 66,000); and fragment 1-2-light chain (mol wt 48,500) were dissolved in Owren's buffer at concentrations of 1.32, 0.26, and 0.13 nmol/ml. The correcting activity of 0.1 ml of each solution was tested upon Fitzgerald-trait plasma aPTT. Simultaneously, the correcting activity of serial dilutions of a pool of normal human plasma and its fragments was determined. This curve was then used to calculate the correcting activity of bHMWK and its fragments in U/nmol. 1 U of correction is defined as the correcting activity of 200 µl of normal human plasma.

For comparison of the correcting activity of bHMWK and its fragments on a weight basis, 100 µg of each of the prelyophilized test materials were dissolved in 1.0 ml of Owren's buffer, and 0.1 ml was used to determine their correcting activity upon Fitzgerald plasma aPTT.

Effects of Bovine Plasma Kallikrein on the Correcting Activity of Human and Bovine HMWK. Purified bovine plasma kallikrein (0.28 µg) and hHMWK (95 µg) were dissolved in 1.0 ml of Owren's buffer (pH 7.5) and incubated at 37°C for 30 and 60 min. At these times, two aliquots of 20

TABLE I
Comparison on a Molar Basis of the Correcting Activity of Bovine HMWK and its
Fragments on Fitzgerald-Trait Plasma aPTT

Protein	Normal plasma	
	%	U/nmol
HMWK	2.5	0.10
Lys-bradykinin-free HMWK	5.6	0.22
Bradykinin and fragment 1-2-free HMWK	0.8	0.03
Fragment 1-2-light chain	2.5	0.10
Light chain	0.1	0.004
Heavy chain	No activity	0

All proteins were tested at 0.26 nmol/ml.

TABLE II
Comparison on a Weight Basis of the Correcting Activity of Bovine HMWK
and Fragments on Fitzgerald-Trait Plasma aPTT

Protein	U/mg
HMWK	1.2
Lys-bradykinin-free HMWK	1.7
Bradykinin and fragment 1-2-free HMWK	0.3
Fragment 1-2-light chain	2.6
Light chain	0.1
Heavy chain	0
Fragment 1-2	Inhibits
Fragment 2	Inhibits

All proteins were tested at 100 µg/ml.

µl each of the incubation mixture were taken. One of the aliquots was diluted by mixing it with 80 µl of Owren's buffer. The correction upon the aPTT of Fitzgerald-trait plasma was tested immediately. The second aliquot was mixed with 1.980 µl of a solution of 0.1 M SBTI in 0.1 M Tris-HCl buffer (pH 7.4). This mixture was immersed in a boiling water bath for 10 min, centrifuged, and the kinins measured. The same procedure was used with bHMWK (50 µg) except that 100 µl of undiluted incubation mixture was used for testing its Fitzgerald correcting activity. When human and bovine HMWK were incubated with trypsin (24), they released 8.7 and 8.0 µg of kinins/mg of protein, respectively. Kinins were tested by a radioimmunoassay (25).

Results

The correcting activity of the different purified bovine proteins, when expressed in U/nmol, was not affected by the three different concentrations tested. The exception to this result was lys-bradykinin-free bHMWK, in which the specific activity doubled with a 10-fold dilution.

Table I shows the correcting activity of intact bHMWK and its different fragments expressed in U/nmol of proteins, when tested at a concentration of 0.26 nmol/ml. Table II shows the correcting activity expressed as U/mg of proteins. Table III shows the effect of fragment 1-2 and fragment 2 on the aPTT of normal human plasma. Table IV shows the effects of bovine plasma kallikrein on the Fitzgerald correcting activity of human and bovine HMWK.

TABLE III
Inhibitory Effects of Fragment 1-2 and of Fragment 2 on Normal Human Plasma aPTT

	aPTT	
	nmol/ml	
Fragment 2 (mol wt 4,600)	21.7	320
Fragment 1-2 (mol wt 12,600)	19.8	139

Normal plasma aPTT was 45 s.

TABLE IV
Effect of Bovine Plasma Kallikrein on the Correcting Activity of Human and Bovine HMWK on Fitzgerald-Trait Plasma aPTT

Time of incubation	Human HMWK	Human HMWK and bovine kallikrein	Bovine HMWK	Bovine HMWK and bovine kallikrein
min	—	—	—	—
0	73	71	91	103
30	72	74 (240)	—	149 (265)
60	76	72 (300)	98	200 (375)

Number indicates aPTT in seconds. Numbers in parentheses indicate ng of kinins released by bovine plasma kallikrein.

Discussion

High molecular weight kininogen seems to be essential for the contact activation of the intrinsic coagulation pathway (26). We have tried to determine which region of the HMWK molecule is responsible for the correction of Fitzgerald trait plasma aPTT. For this we have used bHMWK, which is known to partially correct the abnormalities in the aPTT of HMWK-deficient human plasma (12, 13).

Intact bHMWK has a mol wt of $\approx 76,000$ daltons. Of these, 48,500 are contributed by the heavy chain; 1,000 by bradykinin; 12,600 by fragment 1-2; and 16,000 by the light chain (17). Because the fragments of bHMWK are present in the intact molecule in equimolar concentration, their activity must be expressed on a molar base to compare their relative correcting activity. Fig. 1 shows a schematic representation of the structure of bHMWK and its fragments and the relative correcting activity of equimolar concentration of each protein. Intact bHMWK was arbitrarily assigned a correcting activity of 100%.

Our findings indicate that a peptide formed by fragment 1-2-light chain is responsible for the Fitzgerald correcting activity of bHMWK. Fragment 1-2-light chain is as active as intact bHMWK on a molar basis, and virtually more than twice as active on a weight basis.

As expected, we found that the heavy chain of bHMWK has little or no correcting activity, because it is chemically and immunologically common to both low and high molecular weight kininogen; and low molecular weight kininogen lacks correcting activity (12). Although the heavy chain is not essential for the expression of the coagulant activity of bHMWK, it may play a permissive role, because lys-bradykinin-free bHMWK (which includes both the heavy chain and the fragment 1-2-light chain) is more active on a molar basis than fragment 1-2-light chain (Table I). It is also more active than intact bHMWK. Lys-bradykinin-free bHMWK may have a

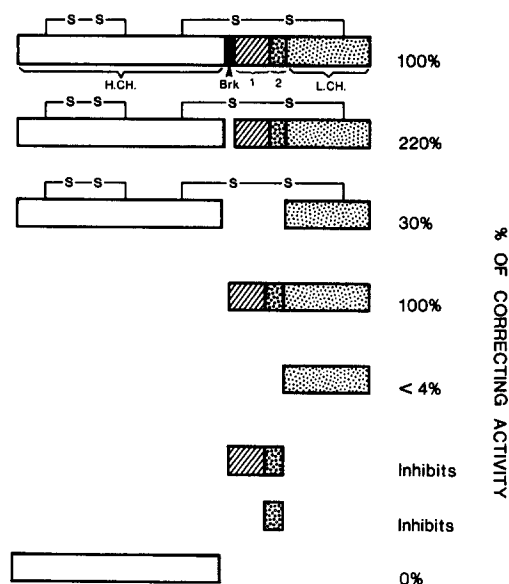


FIG. 1. Relative correcting activity of equimolar concentration of bovine HMW kinogen and its fragments. The correcting activity of intact bovine HMW kinogen was arbitrarily assigned 100%.

better steric relationship with Hageman factor and/or prekallikrein and factor XI, than fragment 1-2-light chain or bHMWK. This would favor a more effective interaction on the surface.

We have observed an increase in the specific activity of lys-bradykinin-free bHMWK with higher dilutions. This increase may be due to the presence of trace amounts of a contaminant with inhibiting capacity, such as fragment 1-2 or fragment 2, in the preparation.

Fragment 1-2-light chain was obtained by reduction of the disulfide bonds of the lys-bradykinin-free bHMWK, followed by alkylation of the thiol groups. It retains full activity in the coagulation assay, which suggests that intact disulfide bonds are not necessary for the Fitzgerald correcting activity of bHMWK.

The light chain without fragment 1-2 lacks functional activity, indicating that the fragment 1-2 peptide, or part of it (fragment 2), plays a crucial role in the initiation of the intrinsic coagulation system. It was also observed that fragment 1-2 and, to a greater degree, fragment 2 have a marked capacity to inhibit the surface activation of the coagulation pathways of normal human plasma, but only if the fragment 1-2 or fragment 2 was added before or during the incubation of normal plasma with kaolin. This observation suggests that when fragment 1-2 and fragment 2 bind to the negatively charged surface, they competitively inhibit the binding and activation of the proteins involved in the intrinsic coagulation pathway.

Considering these results, we propose that the fragment 1-2 portion serves to bind HMWK to the negatively charged surfaces, and that the light chain, or part of it, interacts with Hageman factor and/or prekallikrein and factor XI. Furthermore, because Wiggins et al. (27) have clearly shown that the activation of factor XI occurs only on the surface, we propose that the light chain without fragment 1-2 (binding site) lacks activity because it will remain in the fluid phase. It may be speculated that HMWK complexed with prekallikrein and factor XI in the fluid phase (28, 29) can

carry these proteins to negatively charged surfaces. On the surface, the interaction between Hageman factor, HMWK, prekallikrein, and factor XI will activate all the Hageman-dependent pathways, such as intrinsic coagulation, fibrinolytic, kallikrein-kinin, and complement systems.

There are some differences as well as similarities between bovine and human HMWK. Bovine plasma kallikrein destroys the correcting activity of bHMWK, while human plasma kallikrein does not affect the activity of hHMWK. This difference could be due to an enzymatic contamination in the bovine plasma kallikrein preparation. When hHMWK was incubated with bovine kallikrein, kinins were released although there was no loss of functional activity. It is therefore unlikely that a contaminating enzyme is present in the bovine plasma kallikrein preparation, unless the hypothetical contaminating enzyme has species specificity. It is more probable that the reason for the different behavior of bHMWK and hHMWK after hydrolysis with homologous plasma kallikrein can be explained by structural variations between both substrates. The fact that the bovine plasma has only 7–12% (12, 30) of the correcting activity of human plasma also points to partial structural differences between bHMWK and hHMWK. Furthermore, we have found that if fragment 1-2 remains attached to the light chain of bHMWK, the Fitzgerald correcting activity is also retained. Because hHMWK retains full correcting activity after hydrolysis with bovine or human plasma kallikrein, we conclude that neither enzyme is able to separate the basic peptide, equivalent to fragment 1-2, from hHMWK.

Recent evidence indicates that human kininogen shares the same basic structures with its bovine counterpart. Nagasawa et al. (31) have reported that hHMWK is a linear glycoprotein in which the bradykinin moiety is included in the inner portions. Human kallikrein acts upon hHMWK, releasing bradykinin and leaving a kinin-free HMWK which has a heavy and a light chain linked by a disulfide loop as in bHMWK. It has been shown that antibodies against human low molecular weight kininogen cross-react with human high molecular weight kininogen (28–32). Similarly, antibodies against bovine low molecular weight kininogen (bLMWK) cross-react with bHMWK (21).

It is known that both bLMWK and human LMWK (hLMWK) lack correcting activity upon Fitzgerald-trait plasma aPTT (10–13). Antibodies against hHMWK, after adsorption with Fitzgerald-trait plasma (which has LMWK), no longer cross-react with hLMWK. When tested against hHMWK, a precipitation line is observed (14, 33). This suggests the presence of antigenic determinants in the HMWK molecule, which are not present in the LMWK. It is logical to assume that the antigenic determinants of the HMWK not present in the LMWK are responsible for the hHMWK coagulating activity.

Komiya et al. (17) and Kato et al. (21) have reported that the bLMWK and bHMWK differ in their light chains, and we have shown that the correcting activity of the bHMWK resides precisely in the fragment 1-2 light chain. To complete the resemblance between the bovine and human kininogen, it has been reported (33) that the light chain enables the hHMWK to correct the aPTT of Fitzgerald-trait plasma. However, a positively charged peptide similar to the bovine fragment 1-2 has yet to be identified in the light chain of the hHMWK.

It is interesting that plasma of patients such as Flaujeac and Williams (10, 11) lacks both LMWK and HMWK, functionally and immunologically, although Fitzgerald

plasma lacks only HMWK. It seems unlikely that the first group is deficient in more than one gene. It is more likely that both kininogens are synthesized by the expression of different but closely related genes. We speculate that the heavy chain, which is probably common to both forms of kininogen, might be synthesized through a gene (A) which might be linked to a regulatory gene in a lac operon. The addition of the fragment 1-2 light chain may be regulated by a gene (B), whereas the addition of the light chain of LMWK may be regulated by a gene (C). Genes (A), (B), and (C) might be structural genes. Within this context, a lack of expression of gene (A) will produce a deficiency in both LMWK and HMWK. A deficiency in the structural gene (B) might still allow the synthesis of LMWK (Fitzgerald-trait). Therefore, it can be predicted that a deficiency of LMWK, but not of HMWK, will eventually be found.

Summary

Bovine high molecular weight kininogen (bHMWK) partially corrects the activated plasma thromboplastin time (aPTT) of Fitzgerald trait plasma which is congenitally deficient in HMWK. The relationship between the structure and activity of HMWK was clarified by studying the effects of different fragments of bHMWK on the aPTT of Fitzgerald-trait plasma. The peptides studied were lys-bradykinin-free HMWK, bradykinin-fragment 1-2-free HMWK, heavy chain, fragment 1-2-light chain, and light chain. All fragments were tested in equimolar concentrations. Bradykinin-fragment 1-2-free HMWK, heavy chain, and light chain have little or no correcting activity upon Fitzgerald-trait plasma aPTT. Fragment 1-2 light chain has the same correcting activity as intact bHMWK, while that of lys-bradykinin-free HMWK appears to be higher. Both fragment 1-2 and fragment 2 inhibit the clotting time of normal human plasma. When compared on a molar basis, fragment 2 is a more active inhibitor than fragment 1-2. When the effects of bovine plasma kallikrein upon bHMWK and hHMWK were studied, it was found that it released kinins from both kininogens. However, while the correcting activity of bHMWK was completely destroyed after 60 min of incubation, that of hHMWK was fully retained. These data suggest that: (a) the active part of bHMWK is comprised of the fragment 1-2 light chain portion; (b) fragment 1-2 or fragment 2 is the binding site to negatively charged surfaces, while the light chain interacts with other components of the surface-mediated reactions; and (c) bovine plasma kallikrein releases kinins, but probably does not cause the release of fragment 1-2 from human HMWK.

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