Role of Charged Groups in Factor XI/XIa Activity*

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To elucidate the role of charged groups in expression of factor XI coagulant activity, the charged groups of purified human blood coagulation factor XI/XIa containing 125I-XI/XIa were derivatized: free amino groups by succinylation, guanido groups of arginine by reaction with phenylglyoxal hydrate, and free carboxyl groups by reaction with ethylenediamine. The modified proteins were tested for: 1) ability to adsorb to glass, 2) ability to be cleaved by trypsin or factor XII-high molecular weight kininogen, 3) coagulant activity. The amino group-modified factor XI had a significantly decreased ability to bind to glass; modification of arginine or carboxyl groups did not affect adsorption. Trypsin cleaved factor XI with modified free amino, guanido, or carboxyl groups. Factor XII-high molecular weight kininogen could cleave only the arginine-modified factor XI. Amino group-modified factor XI and carboxyl group-modified factor XI lost all their factor XI assay activity, whereas arginine-modified factor XI retained 50% of the original activity. Amino group-modified factor XI could not be activated by trypsin, but arginine-modified and carboxyl group-modified factor XI could be activated by trypsin to 50% of the original activity. Succinylation of the amino groups of factor XI destroyed all its factor XIa activity. Arginine-modified and carboxyl group-modified factor XIa retained 50% of their factor XIa activity. We conclude that ε-amino and carboxyl but not guanido groups; free amino, carboxyl, and guanido groups in factor XIa all appear to be critical for interaction of factor XIa with factor IX.

Blood clotting factor XI is a trace plasma protein which circulates as a zymogen consisting of two apparently identical polypeptide chains, each of molecular weight of about 80,000, linked by disulfide bonds (1–5). Factor XI in plasma undergoes activation by interacting with clotting factors XII and high molecular weight kininogen at an activating surface such as kaolin or glass (6–8). Activating surfaces which are negatively charged (9) are essential. Factor XI is adsorbed onto the surface from plasma (10–14) or from purified solutions of the protein (2, 13). Activation results from proteolytic cleavage of each of the two chains of factor XI without a change in molecular weight. The cleavage occurs both in plasma systems (2, 12, 14) and in mixtures of purified proteins (2, 3, 4, 13). Each chain is proteolytically cleaved into a heavy chain of molecular weight of about 46,000 containing the surface adsorption sites (13) and a light chain of molecular weight of about 37,000 containing the active site (2, 3). In the laboratory, activation cleavage can also be catalyzed by trypsin in the absence of factor XII, HMWK1 or surface (1, 5, 15, 16). In isolated purified systems, activated factor XI (factor XIa) yields an additional smaller chain(s) of molecular weight 23,000–26,000 (5, 14). Factor XIa in the presence of calcium ions catalyzes the activation of factor IX which leads to subsequent clot formation (11, 17, 18). Thus, there are three separate steps in which factor XI in plasma participates in order to fully express its coagulant activity: adsorption at the activating surface, proteolytic cleavage to factor XIa, and activation of factor IX by factor XIa. In the present study, we evaluate the role of the charged groups of lysine, aspartic and glutamic acids, and arginine in each of these aspects of factor XI activity.

MATERIALS AND METHODS

All chemicals obtained from commercial sources were the best grade available. Protein concentration was determined by the Lowry method (19) using human serum albumin (Miles) as reference. All experiments with purified proteins were carried out in NUNC plastic tubes (11 x 70 mm) (Intermed, Denmark) unless otherwise stated. Purified Proteins—Human factor XI was purified as described previously (6). Its specific activity was 155 units/mg of protein. Factor XII was purified as described by Griffin and Cochrane (20). Its specific activity was 32.6 units/mg. HMWK was purified as described previously (21) and its specific activity was 15 units/mg. Activated factor XI reagent was prepared by tryptic activation as described previously (13).

Radiolabeling of factor XI was carried out with Na125I (Amersham) by the lactoperoxidase method (22) with slight modifications. Factor XI (5 µg in 60 µl), dialyzed against 0.5 mM sodium phosphate buffer, pH 7.0, at room temperature, was reacted with 10 µl of Na125I (0.5 mCi) in 0.5 mM sodium phosphate buffer, pH 7.0, 2 µl of lactoperoxidase (4 µg), and 2 µl of HzO2 (300 ng) for 3 min with mixing every 30 s. The reaction was then stopped by adding 500 µl of 0.02 M Tris-HCl, 0.13 M NaCl, pH 8.0. The unbound 125I and lactoperoxidase were removed on a Sephadex G-100 column (1.5 x 60 cm) equilibrated with 0.02 M Tris-HCl, 0.13 M NaCl, pH 8.0, containing purified human serum albumin (60) to yield A280<0.1. The final product retained its procoagulant activity. It had the same gel filtration, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and trypsin activation properties as unlabeled factor XI.

Modification of free amino groups with succinic anhydride was carried out by a variation of the method of Habeet et al. (23). A crystal of succinic anhydride (about 0.5 mg) was added to 75 µl of a mixture of factor XI and 125I-factor XI (clotting activity 0.8 unit/ml, 0.4 µg of protein) in 0.02 M Tris-HCl, 0.13 M NaCl, pH 8.0. This mixture was incubated at 4°C. At intervals, aliquots were removed, immediately diluted 20-fold in buffer appropriate for subsequent analysis, and tested either for clotting activity, binding to glass, activation/cleavage by trypsin, or activation/cleavage by factors XII.

The abbreviations used are: HMWK, high molecular weight kininogen; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; factor XIa, the activated form of factor XI; factor XIa, the activated form of factor XII; SDS, sodium dodecyl sulfate.

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HMWK in the presence of kaolin.

Modification of arginine guano groups with phenylglyoxal hydrate was carried out by a variation of the method of Takahashi (24). To a mixture of 50 μl of factor XI and 125I-factor XI (clotting activity, 1.5 units/ml; protein, 0.5 μg) in 0.02 M Tris-HCl, 0.13 M NaCl, pH 8.0, were added 5 μl of 0.01 M phenylglyoxal hydrate in the same buffer. The reaction was carried out at 4 °C. Aliquots were removed at desired intervals and immediately diluted 20-fold in buffer appropriate for subsequent analysis.

Modification of the carboxyl groups with ethylenediamine in the presence of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide was carried out by a variation of the method developed by Hoare and Koshland (25). EDC (0.5 mg) was added to 75 μl of a mixture of factor XI and 125I-factor XI (clotting activity, 0.7 unit/ml; protein, 0.35 μg) in citrate saline. Then, 7.5 μl of 0.01 M ethylenediamine dihydrochloride solution in water was added. The pH of the mixture was 6.5. The reaction was carried out at 4 °C. Aliquots were removed at intervals and immediately diluted 20-fold in buffer appropriate for subsequent analysis.

Clotting Assay—Factor XI or factor XII assay was measured in a modified, activated partial thromboplastin time assay using heparitin factor XI or factor XII-deficient plasma as substrate, as described previously (16). Factor XIa was measured by eliminating kaolin in the factor XIa assay and carrying the assay in polyethylene tubes without the 3-min incubation. High molecular weight kininogen clotting assay was measured in a modified, activated partial thromboplastin time assay using Fitzgerald-trait plasma under the same conditions as the factor XI assay.

Surface Binding—Test substance (50 μl) containing a mixture of factor XI and 125I-factor XI (about 2000 cpm/tube) in 0.1 M sodium phosphate buffer containing 0.05 M NaCl, pH 6.0, was incubated at room temperature in new, unwashed, borosilicate glass tubes (12 × 75 mm) for 30 min during which time the radioactivity was measured in a Tracor Analytic Model 1190 γ-spectrometer. The tubes were then washed twice with 1 ml of the same buffer, drained, and counted again. % factor XI bound = washed tube cpm/original cpm × 100.

Factor XII activation of factor XI or its derivatives was carried out by adding 0.15 unit of factor XII and 0.07 unit of HMWK to 0.07 unit of factor XI/125I-factor XI or its derivative in 0.02 M Tris-HCl, 0.13 M NaCl, pH 7.35, in a total volume of 290 μl. This was incubated for 10 min at 37 °C, then 10 μl of kaolin (2 mg/ml) were added. After 30 min at 37 °C, it was tested for factor XIa activity; at this time, unmodified factor XI was maximally activated. Aliquots of 60 μl were further incubated at room temperature overnight to allow maximum cleavage, then reduced by adding 10 μl of 225 mM dithiothreitol in 18% SDS and incubating for 1 h at 37 °C. SDS-polyacrylamide gel electrophoresis was carried out to determine the products.

Trypsin activation of factor XI or its derivatives for clotting assay was carried out by incubating 250 μl of factor XI or a derivative thereof containing 0.1 μg of protein in 0.02 M Tris-HCl, 0.13 M NaCl, pH 7.35, in a total volume of 290 μl. This was incubated for 10 min at 37 °C, then 10 μl of kaolin (2 mg/ml) were added. After 30 min at 37 °C, it was tested for factor XIa activity; at this time, unmodified factor XI was maximally activated. Aliquots of 60 μl were further incubated at room temperature overnight to allow maximum cleavage, then reduced by adding 10 μl of 225 mM dithiothreitol in 18% SDS and incubating for 1 h at 37 °C. SDS-polyacrylamide gel electrophoresis was carried out to determine the products.

Trypsin cleavage of factor XI or its derivatives for autoradiography was carried out by incubating 60 μl containing 0.03 to 0.05 μg of factor XI/125I-factor XI or its derivative in 0.02 M Tris-HCl, 0.13 M NaCl, pH 8.0, with 2 μg of trypsin (2 μl), in the same buffer for 20 min. The reaction was stopped by adding 10 μl of 225 mM dithiothreitol in 18% SDS. This mixture was incubated for 1 h at 37 °C. SDS-polyacrylamide gel electrophoresis was carried out to determine the products.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out on 10% slab gels (140 × 95 × 0.75 cm) at 13 mA for 3 h with tap water cooling, according to the method of Laemmli (26). The slab gels were then either dried on filter paper backing and subjected to autoradiography or sliced into 1.1-mm strips and counted for radioactivity. For estimation of molecular weights, standard proteins were included in each gel and visualized by staining with Coomassie brilliant blue. For autoradiography, the dried gels were taped to a glass plate and on top of the gel were placed a Kodak XRP-1 x-ray film, an intensifying screen (DuPont Cronex Quanta II), and a second glass plate, in that order. This sandwich, which was secured with a clip, was then placed at −70 °C for a variable time, depending on the amount of radioactivity present, and then the film was developed.

RESULTS

Modification of the Free Amino Group of Lysine in Factor XI with Succinic Anhydride—Several indicators of the effect of succinylation of factor XI and of factor XIa are illustrated in Table I. Within 10 min of the addition of succinic anhydride, the factor XI assay activity (column 2) and trypsin-activatable activity (column 3) of factor XI had disappeared and surface binding (column 4) of factor XI was markedly decreased. The clotting activity of factor XIa also disappeared (column 5) and surface binding of factor XIa decreased significantly (column 6).

When factor XI was succinylated for 15 min and then incubated with trypsin, it was cleaved into a heavy chain, a light chain, and another smaller chain (Fig. 1, channel C) as defined by SDS-polyacrylamide gel electrophoresis under reducing conditions. These products, although resembling the tryptic products of untreated factor XI (channel B of Fig. 1) differed from them slightly; having molecular weights appearing to be slightly higher than those from the untreated factor XI. Incubation of succinylated factor XI with factor XII, HMWK, and kaolin did not result in cleavage (Fig. 2, channel E).

TABLE I

<table>
<thead>
<tr>
<th>Modification time (min)</th>
<th>Factor XI</th>
<th>Factor XIa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting activity (%)</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Trypsin-activatable activity (%)</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Surface bound activity (%)</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Surface bound activity (%)</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>0.80 (100)</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>0.28 (35)</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>0.12 (15)</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>0.05 (6)</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>0.00 (0)</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0.00 (0)</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1. Autoradiograph of the SDS-polyacrylamide gel electrophoretic patterns of native and modified factor XI/125I-factor XI, cleaved by trypsin and reduced. A, control—unmodified factor XI not treated with trypsin; B, unmodified factor XI; C, factor XI with amino groups modified; D, factor XI with gliadin groups modified; and E, factor XI with carboxyl groups modified. The samples in Channels B-E were treated with trypsin and then all samples were reduced and subjected to electrophoresis as described under “Materials and Methods.”
XI was both cleaved (Figure 2, channel B) and activated.

Modification of the Guanido Group of Arginine in Factor XI with Phenylglyoxal Hydrate—Table II shows some of the effects of modifying the guanido group of arginine in factor XI and in factor XIa. Only half the clotting activity was lost when factor XI was treated with phenylglyoxal hydrate (column 2). Double the concentrations of phenylglyoxal hydrate did not change this result. Phenylglyoxal hydrate-treated factor XI, which lacked half of the factor XI assay activity, could be activated with trypsin to only about half the original obtainable activity (column 3). The surface binding property of factor XI was not affected by this modification (column 4). Factor XIa also lost half of its original activity when modified with phenylglyoxal hydrate (column 5). The modified factor XIa had the same surface binding as the unmodified factor XIa (column 6).

Trypsin cleaved factor XI with modified guanido groups in a similar manner and to the same extent as it did the unmodified protein (Fig. 1, channels D and B). Incubation of the modified protein with factor XII, HMWK, and kaolin resulted in half the original activity, but the protein was cleaved to the same extent as untreated factor XI as seen in Fig. 2, channels D and B. This was confirmed by slicing the gel into 1.1-mm strips and quantitating the radioactivity in each band.

Modification of the Carboxyl Group(s) of Factor XI with Ethylenediamine in the Presence of EDC—Table III illustrates several of the effects of modifying the carboxyl groups of factor XI and of factor XIa. The procoagulant activity decreased gradually to zero in 15 min (column 2). However, the protein treated for 15 min with the reagent (which abolished all activity in the factor XI assay) still could be activated by trypsin to half the original activity (column 3). Prolonged incubation with trypsin did not result in complete activation. The surface binding property of factor XI was not affected by this modification (column 4). Binding of factor XI to surface increased in the presence of EDC alone, but addition of ethylenediamine brought the adsorption back to the original level. Factor XIa lost only about half its original activity on treatment with ethylenediamine in the presence of EDC in 15 minutes (column 5). The surface binding of factor XIa was not affected by the modification of the carboxyl groups (column 6).

When carboxyl group-modified factor XI was incubated with trypsin, it was cleaved into chains similar to the chains from the unmodified protein (Fig. 1, channels E and B). Incubation of the modified protein with factor XII, HMWK, and kaolin neither activated nor cleaved the protein to any significant degree (Fig. 2, channel E). This qualitative judgment was confirmed by slicing the gel and measuring the radioactivity in each slice.

**DISCUSSION**

Three parameters were considered in evaluating the role of charged groups in the expression of factor XI procoagulant activity: adsorption of factor XI to activating surfaces, proteolytic cleavage of factor XI to its activated form, and ability of factor XIa to activate factor IX. Activating surfaces such as glass, kaolin, or diatomaceous earth possess a net negative charge (9) whereas factor XI, with a pI in the range of 9 (27), has a net positive charge at physiologic pH. As illustrated above, modifying the amino group of lysine with succinic anhydride, resulting in conversion of positively charged amino groups to a net negative charge, decreased the adsorption of carboxyl groups to a net negative charge, increased the adsorption of factors XI and XIa to glass significantly. However, simply changing the net surface charge of factor XI, as by modifying the carboxyl groups with ethylenediamine, which results in replacement of carboxyl groups by amino groups had no significant effect on adsorption. Nor was adsorption affected by the addition of a bulky group to the arginine residues (28).
These results suggest a significant and specific contribution of an electrostatic component in surface binding of factor XI to glass, mediated primarily by free ε-amino groups of lysine.

Proteolytic cleavage of factor XI by trypsin occurred in factor XI with modified free amino, guanido, or carboxyl groups. Therefore, some trypsin recognition sites are available in all of these modified proteins. The products of cleavage of factor XI with modified guanido or carboxyl groups had the same apparent size as those of unmodified factor XI, whereas those of the amino group-modified factor XI appeared slightly larger but of the same relative size. When the amino groups are modified with succinyl anhydride, the electrostatic interactions of the negative groups result in unfolding of the protein molecule and a considerable increase in the effective volume occupied by the succinylated protein molecule compared to the untreated protein (23, 29). This likely accounts for the apparent increase in the molecular weight of the succinylated protein and its products of cleavage.

The combination of factor XII and HMWK in the presence of surface cleaved guanido-modified factor XI as did trypsin, indicating that arginine residues, although a known recognition site for trypsin (30), are not involved in this proteolytic cleavage or those arginine residues participating in the activation cleavage reaction catalyzed by either trypsin or factor XII-HMWK are protected from modification. In contrast to trypsin, factor XII-HMWK in the presence of surface did not cleave the amino group-modified or the carboxyl group-modified factor XI. This reaction differed from trypsin activation cleavage in requiring an activating surface, utilizing activated factor XII as the enzyme, and involving another protein, HMWK, to which factor XI is complexed in plasma (31). The absence of cleavage of factor XI containing modified ε-amino groups of lysine by factor XII-HMWK probably reflects the impaired ability of this enzyme to bind to the activating surface. Since free carboxyl groups are not required for adsorption or cleavage by trypsin, their critical role in factor XII-HMWK-catalyzed activation may indicate a role for these residues in the interaction of factor XI with HMWK.

The coagulant activity effected by factor XIa directly indicates the ability of factor Xla to activate factor IX. Succinylation of the amino groups of lysine of factor XIa completely destroyed its procoagulant activity. Furthermore, trypsin cleavage of factor XI with modified free amino groups failed to yield active factor Xla. These results suggest that ε-amino groups of lysine are essential for factor Xla to interact with factor IX, as well as for adsorption to glass.

Modification of guanido groups in factor XI or Xla resulted in loss of half of the coagulant activity, although the modified factor XI was cleaved by both factor XII-HMWK and by trypsin. It appears that whereas the guanido group of arginine is not necessary for adsorption or cleavage of factor XI, it is necessary for interaction of factor Xla with factor IX. This conclusion is supported by earlier experiments by Ratnoff and Saito (32) in which more extreme conditions of modification (2 h at 25 °C with 16 mM phenylglyoxal hydrate) resulted in complete loss of clotting activity.

When the carboxyl groups of factor XI were modified, no activity was detectable in the factor XI assay in which the enzyme is factor XIIa, but on treatment with trypsin half the activity was expressed in the factor Xla assay. This is consistent with the observation that trypsin but not factor Xla can cleave the modified zymogen. When the free carboxyl groups of factor Xla were derivatized, a similar loss of activity occurred. Hence, the free carboxyl groups seem to be important for the direct factor Xla action with factor IX, as well as for cleavage of factor XI by factor XII-HMWK.

These studies indicate that charged groups play an important role in each aspect of expression of factor XI activity. Surface adsorption appears to be primarily a function of the ε-amino groups of lysine. Activation cleavage by factor XII-HMWK appears to involve both free amino and carboxyl groups. All three of the charged groups studied—amino, carboxyl, and guanido—participate in the interaction of factor Xla with factor IX.

REFERENCES