Role of the Activation Peptide Domain in Human Factor X Activation by the Extrinsic Xase Complex*

(Received for publication, February 27, 1996)

Robert J. Baugh and Sriram Krishnaswamy‡

From the Division of Hematology/Oncology, Department of Medicine, Emory University, Atlanta, Georgia 30322

The activation of factor X by the extrinsic coagulation system results from the action of an enzyme complex composed of factor VIIa bound to tissue factor on phospholipid membranes in the presence of calcium ions (extrinsic Xase complex). Proteolysis at the Arg52-Ile53 peptide bond in the heavy chain of factor X leads to the formation of the serine protease, factor Xa, and the generation of a heavily glycosylated activation peptide comprising residues 1-52 of the heavy chain. The role of the activation peptide region in mediating substrate recognition and cleavage by the extrinsic Xase complex is unclear. The protease Agkistrodon rhodostoma hydrolase γ (ARHγ), from the venom of the Malayan pit viper, was used to selectively cleave human factor X in the activation peptide region. Three cleavage sites were found within this region and gave products designated Xdes1–34, Xdes1–43, and Xdes1–49. The products were purified to yield Xdes 1–49 and a mixture of Xdes 1–34 and Xdes 1–43. Reversed phase high pressure liquid chromatography analysis indicated that the cleaved portion of the activation peptide was likely removed during purification. All cleaved species were inactive and could not be completely activated to factor Xa by the extrinsic Xase complex or by a purified activator from Russell's viper venom. Steady state kinetic studies using tissue factor reconstituted into membranes yielded essentially equivalent kinetic constants for the activation of intact factor X and the cleaved derivatives under a wide range of conditions. Since Xdes 1–49 lacks all but three residues of the activation peptide and is devoid of the carbohydrate present in this region, the data suggest that the specific recognition of human factor X by the extrinsic Xase complex is not achieved through specific interactions with residues 1–49 of the activation peptide or with carbohydrate structures attached to these residues.

Human coagulation factor X is a serine protease zymogen, which circulates in blood as a two-chain molecule. It is composed of a 303-residue heavy chain, which is covalently linked with residues 1–49 of the activation peptide or with carbohydrate structures attached to these residues.

The abbreviations used are: TF, tissue factor; ARHγ and ARHβ, A. rhodostoma hydrolase γ and β, respectively; CAPS, 3-(cyclohexylamino)propanesulfonic acid; EBL, elderberry bark lectin; MES, 2-(N-morpholino)ethanesulfonic acid; PEG-8000, polyethylene glycol 8000; PCPS, vesicles composed of 75% (w/w) L-dipalmitoylphosphatidylcholine and 25% (w/w) L-α-phosphatidylserine; RVV, factor X activator from Russell's viper venom; S2238, D-phenylalanyl-pipecoyl-arginylglycyl-p-nitroanilide; SpXa, cyclohexylglycyl-glycyl-arginyl-p-nitroanilide; 20E, Tewen 20, polyoxyethyleneboronate monoborate; Xdes1–49, human factor X lacking residues 1–49 of the heavy chain; Xdes1–34, Xdes1–49, 4 a mixture of proteolyzed forms of factor X lacking residues 1–34 and 1–43 of the heavy chain; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; MAL II, Maackia amurensis lectin II.

1 The abbreviations used are: TF, tissue factor; ARHγ and ARHβ, A. rhodostoma hydrolase γ and β, respectively; CAPS, 3-(cyclohexylamino)propanesulfonic acid; EBL, elderberry bark lectin; MES, 2-(N-morpholino)ethanesulfonic acid; PEG-8000, polyethylene glycol 8000; PCPS, vesicles composed of 75% (w/w) L-dipalmitoylphosphatidylcholine and 25% (w/w) L-α-phosphatidylserine; RVV, factor X activator from Russell's viper venom; S2238, D-phenylalanyl-pipecoyl-arginylglycyl-p-nitroanilide; SpXa, cyclohexylglycyl-glycyl-arginyl-p-nitroanilide; 20E, Tewen 20, polyoxyethyleneboronate monoborate; Xdes1–49, human factor X lacking residues 1–49 of the heavy chain; Xdes1–34, Xdes1–49, 4 a mixture of proteolyzed forms of factor X lacking residues 1–34 and 1–43 of the heavy chain; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; MAL II, Maackia amurensis lectin II.

† To whom correspondence should be addressed: Division of Hematology/Oncology, Dept. of Medicine, Drawer AJ, Emory University, Atlanta, GA 30322. Tel.: 404-727-3806; Fax: 404-727-3404.

‡ This work was supported by National Institutes of Health Grants HL-47465 and HL-52883 (to S.K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Since factor X circulates as a two-chain molecule, we prefer to use a residue numbering system that begins at the NH2 terminus of each polypeptide chain. As proteolysis is confined to the heavy chain of factor X, the sites of cleavage are designated relative to the NH2 terminus of the heavy chain. Therefore, the species Xdes1–49 is equivalent to the derivative of porcine factor X designated Xdel143–191 by Duffy and Lollar (1).

2 Nomenclature of Schechter and Berger (2).
Role of Activation Peptide Domain in Human Factor X Activation

Xase complex (16, 17). The second possibility is consistent with the ability of several different proteases to specifically catalyze the same cleavage reaction, including the intrinsic Xase complex of coagulation (18), the factor X-activating enzyme from Russell’s viper venom (RVVX-CP) (10), and trypsin (18). The VIIa-TF complex also catalyzes the activation of factor IX to IXa with kinetic constants comparable with the activation of factor X (19, 20). There are differences between the sequences beyond P1-P3 surrounding the two scissile bonds in factor X and the bond cleaved in factor X (10, 21). The data therefore imply that macromolecular substrate specificity of the extrinsic Xase complex is not completely explained by the recognition of extended sequences surrounding the scissile bond by the VIIa-TF complex.

Recent studies have suggested an important role for carbohydrate structures present in the activation peptide in the recognition and cleavage of factor X by the extrinsic Xase complex (11, 22). Sinha and Wolf (22), observed large effects on the rate and extent of human factor X activation by both the extrinsic and intrinsic Xase complexes following enzymatic removal of the sialic acids. Removal of both sialic acids and O-linked carbohydrates in bovine factor X led to a 5-fold decrease in the catalytic efficiency for activation by the VIIa-TF complex, which could be partially restored by subsequent removal of N-linked sugars (11). In contrast, more recent studies have shown that removal of sialic acids has no detectable effect on factor X activation (23).

Evidence for a functional role for the activation peptide domain of factor X has also been developed in studies of Xa formation by the intrinsic Xase complex (IXa-VIIIa-membranes) (1, 24). Duffy and Lollar (1), prepared a proteolytic derivative of factor X by the action of a protease (ARHγ) isolated from the venom of the Malayan pit viper, Agkistrodon rhodostoma (25). This protease cleaves porcine factor X in the activation peptide region three residues NH2-terminal to the activation site, yielding a zymogen that can be completely activated to factor Xa upon subsequent cleavage by RVVX-CP or the intrinsic Xase complex (1). Initial velocity studies by Duffy and Lollar (1) indicated that prior cleavage at this site in the activation peptide reduced the rate of activation of porcine factor X by the VIIa-IXa complex. The reduced catalytic efficiency observed with the porcine factor X derivative lacking most of the activation peptide could be attributed to a decreased kcat compared with native factor X observed in the presence of the cofactor, factor VIIIa. More recent studies have also indicated an important role for the activation peptide in substrate recognition by the human VIIa-IXa complex (24). Product inhibition studies of the VIIa-IXa complex by factor Xa in the presence and absence of the isolated activation peptide have indicated that the activation peptide may mediate interactions between factor X and factor IXa in a carbohydrate-dependent manner within the intrinsic Xase complex (24).

In this study, proteolytic derivatives of human factor X were prepared by cleavage with ARHγ. These derivatives contain an intact activation site but lack various portions of the activation peptide NH2-terminal to this site (Scheme 1). These species were zymogens that could be fully activated to factor Xa by either RVVX-CP or the extrinsic Xase complex. One of these truncated derivatives contained no carbohydrate attachment site, whereas the other two contained one and two sites of N-linked carbohydrate, respectively. Kinetic studies with these purified substrate analogs were used to assess the contributions of the polypeptide sequences and carbohydrate structures in the activation peptide domain to factor X activation by the extrinsic Xase complex.

EXPERIMENTAL PROCEDURES

Materials—Elderberry bark lectin (EBL), jacalin, Maackia amurensis lectin II (MAL II), EBL-agarose, jacalin-agarose, and the Vectastain ABC kit were from Vector Laboratories. Hen egg l-ω-phosphatidylycholine, bovine brain l-ω-phosphatidylserine, 4-chloro-3-naphthol, lactose, melibiose, Heps, Tris, MES, APs, Tween 20, and Russell’s viper venom were from Sigma. The synthetic substrates Spectrozyme Xa (SpXa; cyclohexylglycyl-glycylarginyl-p-nitroanilide) and S2238 (p-phenylalanyl-pipecoyl-arginyl-p-nitroanilide) were obtained from American Diagnostica and Chromogenix, respectively. Lyophilized venom from Malayan pit viper (A. rhodostoma) was obtained from Miami Serpentarium. Fresh frozen plasma was purchased from the Red Cross. Patient plasma obtained by plasmapheresis was donated by the plasmapheresis laboratory of Emory University Hospital. Partially purified human factor X from plasma factor X concentrates was a kind gift of Dr. Charlie Heldebrandt, Alpha Therapeutics (Los Angeles, CA). Recombinant human factor VIIa was purchased from Novo-Nordisk (Gentofte, Denmark). Recombinant human tissue factor agonist (TF) was a kind gift from Genentech (South San Francisco, CA). Small unilamellar phospholipid vesicles composed of 75% (w/w) phosphatidylcholine and 25% (w/w) phosphatidylserine were prepared as described previously (26). Phospholipid concentrations were determined by a colorimetric assay for inorganic phosphate using Malachite Green (27), and the concentrations are expressed as the concentrations of monomeric phospholipid.

Proteins—Human factor X was isolated from fresh frozen plasma, plasmapheresis plasma, and factor X concentrates by barium citrate adsorption, DEAE-Sepharose chromatography, and heparin-Sepharose chromatography essentially as described previously (28). The factor X activator (RVVX-CP) was purified from crude venom of Russell’s viper as described previously (29). Factor Xa was prepared by proteolytic activation of factor X by RVVX-CP, followed by affinity chromatography on benzamidine-Sepharose with minor modifications to the procedures described (30). In this case, reaction mixtures were adjusted to 5 mM EDTA prior to application to the affinity resin. Kinetic titration of Xa using p-nitrophenol-p’-guanidinobenzoate (31), yielded 1.05 mol of active sites/nmol of Xa.

ARHγ was purified from crude venom by a small modification of previously published procedures (1, 25). A mixture of ARHβ and ARHγ proteases was prepared by gel filtration and ion exchange chromatography as described previously. Purification was monitored by measuring the cleavage of S2328 by ARHβ and other contaminants and the ability of ARHγ to cleave the heavy chain of factor X without leading to zymogen activation. Further purification of ARHγ was achieved by applying the protease mixture (3 ml) in 20 mM Heps, 0.15 mM NaCl, pH 7.4, to benzamide-Sepharose (1.5 × 8.5 cm), which binds to and
removes ARH. The unbound fraction was pooled, dialyzed into 20 mM MES, pH 6.0, and applied to S-Sepharose (2.5 x 10 cm) equilibrated in the same buffer. ARH was eluted from the column with a gradient of increasing NaCl in the same buffer (0–0.3 mM NaCl in 4 column volumes). Fractions containing ARH were judged by SDS-PAGE, the ability to cleave factor X, and the lack of activity toward S2238, pooled, exchanged into 20 mM Hepes, 0.15 mM NaCl, pH 7.4, by repeated centrifugal ultrafiltration (Centricon 30, Amicon), and stored at −20°C. Approximately 2.5 mg of pure ARH was obtained from 1 g of crude enzyme.

Tissue factor apoprotein was reconstituted into PCPS vesicles using octylglucopyranoside and exhaustive dialysis as described previously (22). The recovery of TF in the reconstituted mixture was assessed using a 125I-labeled TF labeled as a tracer to monitor the reaction mixture. The final effective concentration of TF following dialysis was considered to be one-half the total concentration due to the two possible orientations of the protein (33–35). Most experiments were also repeated using TF/PCPS preparations that were reconstituted by preincubation of TF with small unilamellar PCPS vesicles in the presence of 0.05% (v/v) Tween 20 as described previously (28, 32). In these experiments, the effective TF concentration was considered equivalent to the total concentration (34).

The purity of all protein preparations was evaluated by SDS-PAGE using the method of Laemmli (36). Protein concentrations were determined using the following molecular weights and extinction coefficients: factor X, 56,000 and 1.16 (37); factor Xa, 45,300 and 1.16 (37); factor Va, 26,000 and 1.19 (25); and RVVX-CP, 93,000 and 1.18 (39): Xdes 1–34, 50,000 and 1.16 (assumed); Xdes 1–43, 45,300 and 1.16 (assumed).

Cleavage of Human Factor X by ARH—The cleavage of human factor X (8.9 µmol, in 20 mM Hpes, 0.15 mM NaCl, 0.1% (w/v) PEG-8000, 5 mM CaCl2, pH 7.4) in Minisorb tubes (Nunc, Roskilde, Denmark) was initiated by the addition of 1 μg ARH in the same buffer. Aliquots of the reaction mixture, held at 37°C, were withdrawn at various times following initiation and quenched with the addition of EDTA (8 mM final concentration). The cleavage of factor X was analyzed by SDS-PAGE with and without disulfide bond reduction with dithiothreitol. The generation of factor Xa was examined in quenched samples by measuring the increase in absorbance at 405 nm, with buffer containing 20 mM EDTA of hydrolysis of the C-terminal sequence (301–304) to SpXa in 20 mM Hepes, 0.15 mM NaCl, 0.1% (w/v) PEG-8000, 10 mM EDTA, pH 7.4 at ambient temperature using a Vmax kinetic plate reader (Molecular Devices). The concentration of factor Xa formed was inferred from the linear dependence of the rate on known concentrations of factor Xa under the same conditions. A small amount of SpXa activity was produced during the reaction (1% or over 3 h), either as a result of factor Xa activation by ARH or by another contaminating protease.

Characterization of the Cleavage Products of Human Factor X—For the lectin binding studies protein samples separated by SDS-PAGE in gels containing 10% acrylamide were electrophoresed to polyvinylidene difluoride membranes (Immoblot PSQ, Millipore Corp.). Electrophoretic transfer was performed in a semidry apparatus (Hoefer Scientific Instruments, San Francisco, CA) using 10 mM CAPS, 50 mM Tris, pH 10. After blotting for 1 h at 250 mA, the membranes were probed with rabbit primary antiseraum directed against the manufacturer’s directions. After washing with 20 mM Hpes, 0.15 mM NaCl, pH 7.4, bands were visualized using 0.025% 4-chloro-1-naphthol and 0.02% (v/v) H2O2 in the same buffer containing 17% (v/v) methanol. The lectin binding properties of the purified cleavage products were assessed by the same procedure following application of 3 µg of purified protein to membranes: 10 cm and 4.5 cm.

For H2-terminal sequence analysis, electrophoresed membranes were air-dried, and protein bands were visualized by brief staining with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol followed by destaining with 50% (v/v) methanol, 10% (v/v) acetic acid. Protein bands were cut from dried membranes and subjected to NH2-terminal sequence analysis using Applied Biosystems Model 470A Protein Sequencer. The sequences of modified forms of factor X were compared with those of factors X and Xa prepared under the same conditions. The repetitive sequence analysis at the Emory University Microchemical facility. Sequences following initiation for a period of 5 min, and initial, steady state measurements of the initial, steady state rate of hydrolysis of SpXa (100 µmol/L) were directly sequenced following drying in a centrifugal evaporator.

Isolation of Cleaved Products of Human Factor X—Reaction conditions were chosen to maximize the yield of cleavage products while minimizing the SpXa activity as well as proteolysis to the β forms of the various species. The preparative reaction mixture containing 20 mg of human factor X was reacted with ARH under the above conditions. Following incubation for 30 min at 37°C, the reaction mixture was quenched with 8 mM EDTA (final), cooled to room temperature, and immediately passed over a benzamidine-Sepharose column equilibrated in the quenching buffer in order to remove any factor Xa that was present. The unbound material was dialyzed versus 20 mM MES, 5 mM CaCl2, 0.15 mM NaCl, pH 7.4, by repeated centrifugal ultrafiltration (Centricon 30, Amicon), and stored at −20°C. Approximately 2.5 mg of pure ARH was obtained from 1 g of crude enzyme.

The modified factor X was adjusted to 0.1 mM CaCl2 and applied to an EBL-agarose column (1.5 x 4.5 cm) equilibrated in 20 mM Hpes, 0.15 mM NaCl, 0.1 mM CaCl2, pH 7.4, which retains Xdes 1–34, Xdes 1–43, but not Xdes 1–49. The column was washed with the same buffer to elute unbound protein, and bound material was eluted with 20 mM Hpes, 0.15 mM NaCl, 0.2 mM lactose, 20 mM EDTA, pH 7.4. Due to heterogeneity in the oligosaccharides (see “Results”), the unbound samples contained some Xdes 1–34 and Xdes 1–43 that did not bind EBL. Pure Xdes 1–43 bound to the column, while the β form did not. Bound protein was eluted with buffer containing 0.1 M sodium citrate. Due to heterogeneity of the oligosaccharide carboxyl-terminal chain, some α-form was not bound to jacalin-agarose.

No attempt was made to separate Xdes 1–34 from Xdes 1–43 in either procedure. The separate samples containing Xdes 1–34, Xdes 1–43, or the α- and β-forms together were concentrated by ultrafiltration in 20 mM Hpes, 0.15 mM NaCl, pH 7.4 and stored frozen at −20°C. These samples were stable for several months under these conditions. Both the conventional and the lectin binding procedures yielded 85% of the unbound fraction using Sephacryl S-200 (see above). For activation by RVVX-CP or with the preassembled extrinsic Xase complex. Reaction progress was assessed discontinuously as described previously (32). Aliquots were withdrawn at various times over a 120-min period, quenched with 2 volumes of 20 mM Hpes, 0.15 mM NaCl, 50 mM EDTA, 0.1% PEG-8000, pH 7.4, and the concentration of factor Xa was inferred from initial velocity measurements of SpXa hydrolysis in the presence of 100 µM SpXa as described previously (32). For activation by RRVX-CP, the final protease concentration was 0.51 nM. For measurements with the extrinsic Xase, the final concentrations were 6.7 nM VIIa and 0.17 nM V, and 0.172 µM PCPS.

Steady state kinetic constants for the activation of factor X or its derivatives were determined by varying the concentration of native or ARH-modified factor X between 0 and 2.0 µM. The concentration of RRVX-CP, was 0.34 nM. The extrinsic Xase complex was preassembled to yield final concentrations of 6.7 nM VIIa and 0.17 nM TF reconstituted with PCPS, 0.4% Erythro E. coli membrane phosphatidylcholine, 410 µM PCPS. The extrinsic Xase complex formation was determined discontinuously in samples quenched at 15–30-s intervals following initiation for a period of 5 min, and initial, steady state measurement rates of factor Xa formation were determined by linear regression analysis. Steady state kinetic constants were determined by nonlinear least squares regression analysis according to the Michaelis-Menten equation using the Marquardt algorithm (40). For measurements with the extrinsic Xase complex, the kcat was calculated assuming a 1:1 stoichiometry for the VIIa-TF interaction, that the velocity contribution of free VIIa was negligible, and that 6.7 nM VIIa was sufficient to

16128 Role of Activation Peptide Domain in Human Factor X Activation
role of activation peptide domain in human factor X activation

Cleavage of human factor X by ARHγ—SDS-PAGE analysis of the cleavage of factor X by ARHγ was conducted as described under "Experimental Procedures." Samples (5 μg of protein) were analyzed following disulfide bond reduction with dithiothreitol and visualized following staining with Coomassie Brilliant Blue. Lane 1, human factor X; lanes 2–8, factor X incubated with ARHγ and sampled at 1, 3, 5, 8, 10, 15, and 20 min following initiation. Lane 9, factor X treated with RVVx,Cp, to yield Xa. The apparent molecular weights of the standards are indicated on the right.

Separation of Cleavage Products by Reversed Phase HPLC—Analytical reversed phase HPLC analysis of human factor X and its cleavage products was achieved using a 30.0 x 0.46-cm Aquepore Butyl Brownlee cartridge (Applied Biosystems) and a Beckman System Gold chromatography apparatus. Samples (50–100 μg in 0.2-0.5 ml) were loaded in aqueous buffer, and proteins were eluted at a flow rate of 1 ml/min with a linear gradient of increasing acetonitrile (0–60% (v/v) CH3CN in 0.05% (v/v) trifluoroacetic acid) over a 40-min period. The absorbance of the effluent was continuously monitored at 215 nm. Peak areas were calculated by integration following subtraction of the baseline chromatogram.

RESULTS

Cleavage of Human Factor X by ARHγ—The cleavage of porcine factor X by ARHγ has been previously documented, and the single product obtained has been characterized with respect to cleavage by RVVx,Cp as well as by the intrinsic Xase complex (1). The cleavage pattern of human factor X by ARHγ is more complex. Analysis by SDS-PAGE indicates cleavage of the factor X heavy chain at several sites (Fig. 1) with no degradation of the light chain. As has been observed with porcine factor X (1), the cleavage products resulting from the action of ARHγ on human factor X are inactive but can be fully activated by RVVx,Cp in both the clotting and chromogenic assays (below).

The products of the cleavage of human factor X were identified by NH2-terminal sequence analysis of the bands separated by SDS-PAGE following electrophoretic transfer to polyvinylidene difluoride membranes. The determined sequences (underlined in Scheme 1) are illustrated in the context of the published human factor X sequence (43). The band(s) represented by a smear in the Mγ = 38,000 range gave only two unique NH2-terminal sequences, indicating ARHγ cleavage sites after residues 34 and 43 in the heavy chain. The indistinct appearance of this band may result from carbohydrate microheterogeneity as well as from some cleavage in the COOH-terminal β-peptide region. Based on the cleavage sites in the heavy chain, this species was designated a mixture of X'Hc des 1–34 and X'Hc des 1–43. Two other heavy chain bands appeared at longer incubation times, which essentially co-migrated with the two bands observed following treatment of human factor X with RVVx,Cp (Fig. 1). Identical NH2-terminal sequences were obtained for either band, indicating cleavage after residue 49 in the heavy chain of the zymogen. This form was designated X'Hc des 1–49 and is equivalent to the single product generated following cleavage of porcine factor X by ARHγ described by Duffy and Lollar (1). This species contains three residues NH2-terminal to the cleavage site for Xa formation and therefore retains the P1 through P3 recognition sites necessary for zymogen activation. As has been reported in the porcine system, the light chain band seemed unaffected by ARHγ and always yielded the same NH2-terminal sequence, which was coincident with the published sequence of the intact factor X light chain (43). As discussed previously (25), the identified sites failed to suggest any obvious sequence preference for cleavage by ARHγ. It is possible that the cleavage sites for this protease are at least partly determined by neighboring carbohydrate structures or by other structural elements in addition to primary sequence.

The cleavage products were further characterized by their ability to bind lectins in the dot-blot method or following electrophoretic transfer from SDS-PAGE to polyvinylidene difluoride membranes. Jacalin, MAL II, and EBL were the lectins chosen for this analysis, as they have been shown to recognize carbohydrate structures on factor X (22, 44). MAL II and EBL bind oligosaccharide structures in the activation peptide, whereas jacalin selectively binds oligosaccharides present in the β-region at the COOH terminus of the heavy chain (see Scheme 1).

A qualitative evaluation of the reactivity of the various heavy chain species with these lectins is presented in Table I. Only factor X could be detected with MAL II. Factor X heavy chain as well as the bands designated X'Hc des 1–34, X'Hc des 1–43 reacted with EBL, whereas the X'Hc des 1–49 bands and factor Xa were not detected by this lectin. In contrast, all of the heavy chain species with the exception of the lower bands of X'Hc des 1–49 or factor Xa could be visualized following the reaction with biotinylated jacalin. Thus, the sugars detected by MAL II are present in the heavy chain of factor X but are not detected in the heavy chains of the proteolyzed species. N-linked carbohydrates recognized by EBL are present only in factor X and X'Hc des 1–34, X'Hc des 1–43 but not in X'Hc des 1–49 or factor Xa. These conclusions are consistent with the sites of glycosylation and the determined NH2-terminal sequence for each of these bands (Scheme 1). The differential reactivity of the two X'Hc des 1–49 bands toward jacalin also permits the tentative conclusion that the two bands identified as X'Hc des 1–49 arise from the partial proteolytic removal of the β-peptide. Consequently, the upper band is designated X'Hc des 1–49α and the lower band X'Hc des 1–49β by analogy to the α- and β-forms of factor Xa (12). Isolation of ARHγ Cleavage Products of Human Factor X—Purification of the cleaved derivatives was attempted using the differential lectin binding properties of these forms (Table I). Quantitative separation of X'Hc des 1–34, X'Hc des 1–43 from X'Hc des 1–49
could not be achieved by simply using a combination of lectin affinity chromatography steps with EBL-agarose and jacalin-agarose as was suggested by the data in Table I. Pilot experiments with several different preparations of human factor X indicated that only 40–60% of the total applied factor X bound to either EBL-agarose or jacalin-agarose. In each case, the unbound fraction did not bind upon repassage through fresh resin, and the bound fraction could be quantitatively rebound to the matrix following elution and reapplication to the same column. Only a fraction of the unbound or bound fractions obtained from chromatography on EBL-agarose could be bound by jacalin-agarose. These distinct fractions differed marginally from each other and from the starting material in two-stage dotting assays as well as in chromogenic assays of Xa formation following treatment with RVVX-CP. Indistinguishable reclotting assays as well as in chromogenic assays of Xa formation from each other and from the starting material in two-stage dotting assays as well as in chromogenic assays of Xa formation following treatment with RVVX-CP. Indistinguishable results were also obtained when activation of the bound and unbound fractions was assessed by SDS-PAGE. The results suggest that human factor X is heterogeneous with respect to carbohydrate and are in agreement with observations made with bovine factor X (44). While it is unknown whether this heterogeneity results from subtle or large differences in oligosaccharide structure, the different forms of factor X do not seem to exhibit obvious differences in biological activity.

The ARHγ cleavage products isolated using lectin affinity chromatography were therefore homogeneous for EBL and/or jacalin binding carbohydrate. Since the difference in carbohydrate structure might influence the kinetics of activation (22), the cleavage products were also isolated by more conventional methods as described under “Experimental Procedures.” Either procedure led to the purification of Xdes 1–49γ to apparent homogeneity and also yielded a mixture of Xdes 1–34γ and Xdes 1–43γ, that was apparently free of other proteins. The result obtained following SDS-PAGE analysis of the purified fractions is illustrated in Fig. 2. Both procedures yielded equivalent products as judged by NH2-terminal sequence analysis as well as by SDS-PAGE.

Kinetics of Activation by RVVX-CP and the Extrinsic Xase Complex—Both Xdes 1–49γ and the Xdes 1–34γ–Xdes 1–43γ mixture could be activated by RVVX-CP to yield Xa as judged by coagulation assays and by initial velocity measurements of SPXa hydrolysis. The products visible following SDS-PAGE and visualization with Coomassie Brilliant Blue were equivalent to purified human factor Xa. Complete progress curves for the activation of these species either by RVVX-CP or by the VIIa-TF-PCPS complex were compared with the progress curves obtained using the same concentration of human factor X (data not shown). The limiting amount of factor Xa formed by the activation of factor Xdes 1–49γ or the Xdes 1–34γ–Xdes 1–43γ mixture was between 80 and 90% of the starting zymogen concentration or that seen with human factor X by either catalyst. These data suggest that loss of part or most of the activation peptide following the cleavage of factor X by ARHγ yields a truncated zymogen that can be completely activated by further proteolysis at the Arg52-Ile53 site in the heavy chain. Similar results have been documented in the porcine system (1). Prolonged incubation of the reaction mixtures with the VIIa-TF-PCPS complex led to a slow decrease in active product formed. This may be a result of further cleavage of the heavy chain of Xa to yield the inactive γXa species previously described (12).

Initial velocity measurements of the activation of factor X, Xdes 1–49γ, and the intermediate mixture of Xdes 1–34γ–Xdes 1–43γ were conducted using either RVVX-CP or the extrinsic Xase complex. Representative data from experiments with several different preparations of human factor X and its derivatives are illustrated in Fig. 3. The initial velocity data of factor Xa formation from factor X and its derivatives by either RVVX-CP or the extrinsic Xase complex could be adequately described by the Henri-Michaelis-Menten equation, and the determined kinetic constants are listed in Table II.

Measurements with RVVX-CP yielded kinetic constants for factor Xdes 1–49γ that were within a factor of 2 of those obtained for factor X. As this difference is considered minor, the results indicate that residues 1–49 in the activation peptide domain of the heavy chain contribute undetectably to substrate recognition or cleavage by RVVX-CP. Although the catalytic efficiency for the cleavage of human factor X by RVVX-CP is comparable with the values reported for bovine and porcine factor X, the K M and the k cat values are 10–20-fold lower than the constants observed in the other systems (1, 45). Similar values were obtained with six different preparations of human factor X isolated from fresh frozen plasma, patient plasma obtained following plasmapheresis, and from factor X concentrates. In addition, parallel experiments conducted with bovine or porcine factor X yielded approximately 15-fold higher values of K M and k cat, in agreement with previous studies (1, 45). On the basis of these observations, it was concluded that the observed kinetic constants reflect an intrinsic property of human factor X.

Kinetic measurements for the cleavage of factor X and its truncated derivatives by the extrinsic Xase complex also yielded similar results (Table II). K M and k cat values for the activation of Xdes 1–49γ or a mixture of Xdes 1–34γ and Xdes 1–43γ were within a factor of 1.5 of the apparent constants obtained for factor X under equivalent conditions. These data suggest that the NH2-terminal 49 residues in the activation peptide

![Fig. 2. Analysis of the isolated factor X derivatives by SDS-PAGE.](image)
domain also do not contribute significantly to the recognition of human factor X by the VIIa-TF complex. Comparable results were obtained with several different preparations of human factor X and the proteolytic derivatives prepared from different sources as well as with material purified by the two different techniques. In addition, experiments were also repeated using TF reconstituted by dialysis with increasing concentrations of PCPS, TF reconstituted with preformed vesicles in the presence of Tween (28, 32), and the extrinsic Xase complex assembled with limiting concentrations of TF and saturating concentrations of VIIa. In each of these cases, although the apparent steady state kinetic constants were different, the values determined for the activation of human factor X differed very slightly from those observed for the activation of Xdes 1–49 or the mixture of Xdes 1–34 and Xdes 1–43 (data not shown). Therefore, the near equivalence of the kinetic constants for these three substrates (Table II) is unlikely to be a fortuitous consequence of a specific set of conditions chosen for this analysis.

The observations are consistent with the initial conclusion that structures present in the first 49 residues of the activation peptide domain in the heavy chain do not significantly contribute to the binding of substrate or its cleavage by either RVVXCP or the extrinsic Xase complex. It therefore follows that the highly specific proteolytic cleavage of factor X by the membrane-assembled VIIa-TF complex, which leads to factor Xa formation, probably does not involve specific binding interactions with polypeptide or carbohydrate structures present in this 49-residue portion of the activation domain of the substrate.

Assessment of the Contribution of Noncovalently Associated Peptides to Factor X Activation—Previous studies have documented a tight, noncovalent interaction between the activation peptide domain and factor Xa following activation of bovine factor X (46). It is therefore possible that the present findings are compromised by the noncovalent association of fragments of the activation peptide domain following cleavage of factor X by ARHγ. This possibility was evaluated by the detection of the activation peptide and its fragments by using reversed phase HPLC following the cleavage of factor X (Fig. 4).

Elution profiles obtained following the application of purified factor X, factor X treated with RVVXCP, and purified factor Xa are illustrated in the upper three panels (Fig. 4, A–C). The application of factor X to the column resulted in the elution of a single major peak with a retention time of approximately 26 min (Fig. 4A). A minor peak was also observed at 28 min that might represent a contaminating protein. Treatment of factor X with RVVXCP did not result in a significant change in the elution position of the main peak but resulted in the appearance of additional peaks eluting between 20 and 24 min (Fig. 4B). Systematic studies conducted by injecting samples removed at various times following the addition of RVVXCP indicated that the kinetics of appearance of this material was coincident with factor Xa formation. SDS-PAGE analysis of fractions indicated that while factors X and Xa eluted at ~26 min, the earlier eluting species did not contain any material that could be visualized with Coomassie Brilliant Blue or by silver staining. This material yielded the expected NH2-terminal sequence of the activation peptide. On the basis of these findings, it was concluded that the activation peptide could be resolved and detected by the HPLC technique.

Factor Xa prepared by cleavage with RVVXCP followed by affinity chromatography on benzamidine-Sepharose in the presence of EDTA showed no evidence of this activation peptide either in the HPLC profiles (Fig. 4C) or by NH2-terminal sequence analysis. Thus, the purification procedure appears to remove the activation peptide from factor Xa.

Similar experiments conducted with human factor X cleaved with ARHγ are illustrated in the lower two panels (Fig. 4, D and E). The crude cleavage mixture yielded several small peaks eluting between 11 and 23 min (Fig. 4D). The appearance of these peaks was correlated with the cleavage of factor X as assessed by SDS-PAGE and was considered to reflect the fragments released from the activation peptide domain following cleavage by ARHγ. This conclusion is consistent with the multiple cleavage sites for this protease in the activation peptide domain. Most of these peaks were absent in chromatograms of the purified Xdes 1–49 derivative (Fig. 4E), suggesting that most, if not all, of the cleaved fragments derived from the activation peptide were removed during the purification procedure. Similar results were obtained with the purified Xdes 1–34 Xdes 1–43 mixture (not shown). Small peaks eluting at 9, 11.5, and 22 min were, however, evident (Fig. 4E), at least some of which did not directly correspond to any peaks in the starting material. While the data are consistent with the removal of most of the peptide material resulting from the cleavages in the activation peptide...
Role of Activation Peptide Domain in Human Factor X Activation

Steady state kinetic constants for the activation of human factor X and truncated derivatives

Table II: Steady state kinetic constants for the activation of human factor X and truncated derivatives

| Enzyme                  | Substrate          | $K_m$ (nM) ± S.D.* | $k_{cat}$ (s$^{-1}$) ± S.D. | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) × 10$^{-6}$ ± S.D. |
|-------------------------|--------------------|---------------------|-----------------------------|------------------------------------------------------|
| VIIa-TF-PCPS            | Factor X           | 238 ± 19            | 7.25 ± 0.22                 | 30.4 ± 3.6                                            |
| VIIa-TF-PCPS            | $X_{des\ 1-49}$   | 247 ± 13            | 5.74 ± 0.11                 | 23.2 ± 2.6                                            |
| VIIa-TF-PCPS            | $X_{des\ 1-43}$   | 161 ± 13            | 9.44 ± 0.25                 | 58.6 ± 6.9                                            |
| RVV$_X$-CP              | Factor X           | 33.4 ± 0.2          | 0.32 ± 0.01                 | 9.08 ± 0.76                                           |
| RVV$_X$-CP              | $X_{des\ 1-49}$   | 17.8 ± 0.2          | 0.17 ± 0.01                 | 9.37 ± 1.37                                           |

* Fitted constants ± 95% confidence limits.

The venom protease from Malayan pit viper, ARH$_Y$, was found to cleave human factor X at multiple sites to yield derivatives of the zymogen that were differentially truncated in the activation peptide domain. These proteolytic derivatives could be completely activated to factor Xa by the action of either RVV$_X$-CP or the VIIa-TF extrinsic Xase complex. Three cleaved species, $X_{des\ 1-34}$, $X_{des\ 1-43}$, and $X_{des\ 1-49}$ (Scheme 1), identified by NH$_2$-terminal sequence analysis, were further purified and used in kinetic studies to assess the possible role of structures present in the activation peptide toward the recognition and cleavage of factor X by the extrinsic Xase complex.

Steady state kinetic studies comparing the activation of human factor X with the proteolytic derivatives by RVV$_X$-CP or by the extrinsic Xase complex yielded equivalent kinetic constants (Table II). Since minor effects on $K_m$ and $k_{cat}$ were observed following cleavage of the activation peptide at residues 34, 43, or 49, the data suggest that the amino acid side chains or carbohydrate structures present in this region do not significantly contribute to the binding of factor X to the extrinsic Xase complex or to the rate-limiting step in catalysis that yields factor Xa. These observations imply a limited role for the NH$_2$-terminal 49 residues in determining the cleavage of factor X by the extrinsic Xase complex.

The highly specific cleavage of factor X by the extrinsic Xase complex implies that the recognition of the scissile bond may be achieved by structures in the substrate that lead to binding interactions at extended sites in factor VIIa or possibly through additional interactions with TF. Mutagenesis experiments with TF have been able to resolve residues that affect the ability of the extrinsic Xase complex to cleave protein substrates such as factor X without affecting the ability of TF to interact with factor VIIa or enhancing its activity toward peptidyl substrates (16, 17). One interpretation of these findings is that TF contains sites for substrate binding. Studies examining the ability of synthetic peptides derived from the heavy chain of factor X to inhibit factor X activation by the VIIa-TF complex have provided evidence for sites on the substrate that are removed from the scissile bond but contribute to substrate recognition by the extrinsic Xase complex (47). More recent work has also implicated oligosaccharide structures present at the four potential glycosylation sites in the activation peptide domain of factor X (Scheme 1) as being important for factor X activation by the extrinsic Xase complex (11, 22).

The present kinetic results argue against a significant contribution of the first 49 residues of the activation peptide toward factor X recognition by the extrinsic Xase complex. Thus, specific cleavage at Arg$^{52}$-Ile$^{53}$ in the heavy chain, which results in the conversion of factor X to factor Xa by the extrinsic Xase complex, does not apparently result from substrate-protease or substrate-cofactor interactions that involve heavy chain residues that are NH$_2$-terminal to the P3 position. Therefore, the substrate specificity of this enzyme complex may be

![HPLC analysis of factor X and derivatives](image)

**Fig. 4.** HPLC analysis of factor X and derivatives. Reversed phase HPLC analysis was conducted as described under "Experimental Procedures" for the samples indicated in each panel. The insets illustrate the chromatograms on an expanded scale. The trace in panel D is corrected for the absorbance contribution of ARH$_Y$.

**DISCUSSION**

...
dictated entirely by the P1-P3 residues and/or residues COOH-terminal to the scissile bond in the heavy chain of the molecule or possibly even by structural elements in the light chain of factor X. Studies with synthetic peptidyl substrates have previously implied a role for P' residues for efficient cleavage by the VIIa-TF complex (48).

The venom protease ARHγ has previously been shown to cleave porcine factor X and yield a product equivalent to the Xα-111 derivative identified in the present work (1). Kinetic studies of the activation of this derivative with intact factor X by the porcine intrinsic Xase complex (IXa-VIIIa-PCPS-Ca2+) yielded the same Km but a 100-fold lower kcat, suggesting a significant contribution of the activation peptide region to substrate hydrolysis, possibly through interactions with the protease and/or the cofactor (1). In contrast, experimentally indistinguishable kinetic constants were obtained for the cleavage of human factor X or Xα-111 by the extrinsic Xase complex. Since the activation of factor X by the extrinsic and the intrinsic Xase complexes results from the cleavage of the same peptide bond, one explanation for the results is that the determinants for the recognition and cleavage of the same substrate by the two enzyme complexes are different. Other explanations for these data include the possibility that cleavage(s) in the activation peptide region has a deleterious effect on substrate structure at distant sites that is significant in the case of porcine factor X but kinetically undetectable with human factor X. This possibility is supported by the significant decrease in kcat and in Km for activation by RVVXCP following cleavage of porcine factor X by ARHγ. In contrast, the kinetic constants for human factor X activation by the same enzyme were essentially unaltered following cleavage by ARHγ. This interpretation would suggest that the kinetic consequences of cleavage or removal of the first 49 residues of the activation peptide region are actually related to the indirect effects of this region on structures about the scissile bond or on other domains of factor X rather than the deletion of sites directly involved in binding the protease and/or cofactor in the cognate enzyme complex.

At least two other studies have provided indirect evidence suggesting the importance of the carbohydrates present in the activation peptide domain to factor X activation by the extrinsic Xase complex (11, 22). In these cases, contradictory information regarding the importance of O-linked versus N-linked carbohydrate for factor X activation by the extrinsic Xase complex has been developed following partial deglycosylation of factor X by glycosidases or by the use of specific lectins to inhibit factor X activation (11, 22). More recently, evidence has been presented to show that removal of sialic acids has no effect on factor X cleavage by the extrinsic Xase complex (23).

The proteolytic derivatives of human factor X prepared in the present work (Scheme 1) have provided reagents to assess this possibility more directly. Since the kinetic constants for the activation of these derivatives by the extrinsic Xase complex were equivalent to those obtained for intact factor X, our data are not consistent with these previous suggestions of a specific role for carbohydrates linked to the activation peptide in substrate recognition by this enzyme complex (22). This conclusion is also supported by the observations of heterogeneity in carbohydrate structures present in the zymogen as detected by lectin binding. Although it is possible that this heterogeneity arises from subtle differences in sugars distinct from those involved in substrate recognition, it is difficult to envision how heterogeneity in specific macromolecular recognition elements could yield a substrate preparation that could be adequately characterized by a single set of kinetic constants by initial velocity measurements as well as by analysis of complete progress curves.

Reversible, high affinity interactions have been documented between the fragment 1.2 activation peptide domain of prothrombin and prethrombin 2 (49). This interaction is apparently essential for efficient cleavage of prethrombin 2 by prothrombinase as a result of the ability of the fragment 2 domain to bind the cofactor (50, 51). Similarly, reversible interactions between the activation peptide and factor Xa of high affinity have also been documented following the cleavage of bovine factor X (46). The lack of demonstrable kinetic differences between factor X and the derivatives obtained following cleavage by ARHγ could result from specific binding interactions via the activation peptide domain that are retained through nongenetic interactions between the cleavage products. The HPLC analyses indicate that the complete activation peptide is not present following affinity purification of factor Xa in the presence of EDTA. This is consistent with previous sedimentation velocity studies and the apparent ability of EDTA to disrupt this putative interaction (24, 52). It is not possible to unequivocally rule out the association between trace amounts of peptidyl fragments and the ARHγ-cleaved derivatives of human factor X. The data are, however, consistent with the reasonable conclusion that the fragments of the activation peptide are likely removed during purification of Xα-111-49 and are thus unlikely to mediate interactions between the extrinsic Xase complex and the scissile bond in Xα-111-49.

In summary, the present data suggest that the activation of human factor X by the extrinsic Xase complex is not significantly influenced by the polypeptide sequence or carbohydrates present in the first 49 residues of the activation peptide domain. Thus, residues NH2-terminal to the P3 position do not play a dominant role in the recognition of factor X by the extrinsic Xase complex. It remains to be established whether the macromolecular substrate specificity of the extrinsic Xase is completely determined by the P1-P3 residues or requires additional interactions with P' residues in the substrate.

Acknowledgments—We are grateful to Dr. J an Pohl of the Emory University Microchemical Facility for expert assistance with protein sequencing. We are also grateful to Drs. Pete Lollar and Andreas Betz for reading the manuscript and providing critical comments.

REFERENCES

1. Duffy, E. J. and Lollar, P. (1992) J. Biol. Chem. 267, 7821–7827
2. Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162
3. Jackson, C. M. (1984) Prog. Hemost. Thromb. 7, 55–109
4. Valcarce, C., Holmgren, A., and Stanfel, J. (1994) J. Biol. Chem. 269, 26011–26016
5. Hertzberg, M. S., Ben Tal, O., Furie, B. and Furie, B. C. (1992) J. Biol. Chem. 267, 14759–14766
6. Rezaie, A. R., Naun sticking-Ward, P. F., Mor rissey, J. H. and Esmon, C. T. (1993) J. Biol. Chem. 268, 8176–8180
7. Niemir, Y. (1988) Blood 71, 1-8
8. Wu, W. and Edgington, T. S. (1994) FASEB J. 8, 385–390
9. Mann, K. G., Je nny, R. J. and Krishnasawamy, S. (1988) Annu. Rev. Biochem. 57, 915–956
10. Di Cicco, R. G., Hermsdorn, M. A. and Davie, E. W. (1977) Biochemistry 16, 5253–5260
11. Inoue, K. and Morita, T. (1993) Eur. J. Biochem. 218, 153–163
12. Mertens, K. and Bertina, R. M. (1980) Biochem. J. 185, 647–658
13. Prydz, E. L. G. and Kessler, G. E. (1994) Blood 84, 194 (abstr.)
14. Stubbins, M. T. and Bode, W. (1995) Trends Biochem. Sci. 20, 23–28
15. Padmanabhan, K., Padmanabhan, K. P., Tulinsky, A., Park, C. H., Bode, W., Huber, R., Blenkinsop, D. T., Cardin, A. D. and Kisiel, W. (1993) J. Mol. Biol. 232, 947–966
16. Rud, W., Milles, D. J., Rehentulla, A. and Edgington, T. S. (1992) J. Biol. Chem. 267, 6375–6381
17. Rud, W., Milles, D. J., Rehentulla, A. and Edgington, T. S. (1992) J. Biol. Chem. 267, 22206–22210
18. Fujikawa, K., Coan, M. H., Legaz, M. E. and Davie, E. W. (1974) Biochemistry 13, 5290–5299
19. Komiyama, Y., Pedersen, A. H. and Kisl, W. (1990) Biochemistry 29, 9418–9425
20. Zur, M. and Niemir, Y. (1980) J. Biol. Chem. 255, 15703–15707
21. Di Cicco, R. G., Kurachi, K. and Davie, E. W. (1978) J. Clin. Invest. 61, 1528–1538
22. Singh, U. and Wolf, D. L. (1993) J. Biol. Chem. 268, 3048–3051
23. Bharadwaj, D., Harris, R. J., Kisl, W., and Smith, K. J. (1995) J. Biol. Chem. 270, 267, 14759–14766
Role of Activation Peptide Domain in Human Factor X Activation

270, 6537–6542
24. Iino, M., Takeya, H., Nishioka, J., Nakagaki, T., Tamura, K., and Suzuki, K. (1994) J. Biochem. (Tokyo) 116, 335–340
25. Lollar, P., Parker, C. G., Kajenski, P. J., Litwiller, R. D., and Fass, D. N. (1987) Biochemistry 26, 7627–7636
26. Higgins, D. L., and Mann, K. G. (1983) J. Biol. Chem. 258, 6503–6508
27. Heron, M. D., and Dunn, J. E. (1975) Anal. Biochem. 63, 607–613
28. Krishnaswamy, S., Field, K. A., Edgington, T. S., Morrissey, J. H., and Mann, K. G. (1992) J. Biol. Chem. 267, 26110–26120
29. Furie, B. C., and Furie, B. (1976) Methods Enzymol. 45, 191–205
30. Krishnaswamy, S., Church, W. R., Nesheim, M. E., and Mann, K. G. (1987) J. Biol. Chem. 262, 3291–3299
31. Chase, T., Jr., and Shaw, E. (1967) Methods Enzymol. 19, 20–27
32. Lawson, J. H., Krishnaswamy, S., Butenas, S., and Mann, K. G. (1993) Methods Enzymol. 222, 177–195
33. Bach, R., Gentry, R., and Nemerson, Y. (1986) Biochemistry 25, 4007–4020
34. Krishnaswamy, S. (1992) J. Biol. Chem. 267, 23696–23706
35. Contino, P. B., Hasselbacher, C. A., Ross, J. B., and Nemerson, Y. (1994) Biophys. J. 67, 1113–1116
36. Lunn, K. (1970) Nature 227, 680–685
37. Di Stefano, R. G., Hermodson, M. A., Yate, S. G., and Davie, E. W. (1977) Biochemistry 16, 698–706
38. Bajaj, S. P., Rapaport, S. I., and Brown, S. F. (1981) J. Biol. Chem. 256, 253–259
39. Gowda, D. C., Jackson, C. M., Hensley, P., and Davidson, E. A. (1994) J. Biol. Chem. 269, 10644–10650
40. Bevington, P. R. (1969) Data Reduction and Error Analysis in the Physical Sciences, pp. 204–246, McGraw-Hill, New York
41. Neuenschwander, P. F., and Morrissey, J. H. (1994) J. Biol. Chem. 269, 8007–8013
42. Waxman, E., Ross, J. B., Laue, T. M., Guha, A., Thiruvikraman, S. V., Lin, T. C., Konigsoerg, W. H., and Nemerson, Y. (1992) Biochemistry 31, 3998–4003
43. Leytus, S. P., Foster, D. C., Kurachi, K., and Davie, E. W. (1986) Biochemistry 25, 5098–5102
44. Horton, G. L., and Trimpe, B. L. (1990) Anal. Biochem. 188, 271–277
45. Kosow, D. P., Furie, B., and Forastieri, H. (1974) Thromb. Res. 4, 219–227
46. Furie, B. C., Furie, B., Gottlieb, A. J., and Williams, W. J. (1974) Biochim. Biophys. Acta 365, 121–132
47. Chattopadhyay, A., and Fair, D. S. (1989) J. Biol. Chem. 264, 11035–11043
48. Butenas, S., Ribarik, N., and Mann, K. G. (1993) Biochemistry 32, 6531–6538
49. Myrmy, K. H., Lundblad, R. L., and Mann, K. G. (1976) Biochemistry 15, 1767–1773
50. Luckow, E. A., Lyons, D. A., Ridgeway, T. M., Eson, C. T., and Laue, T. M. (1989) Biochemistry 28, 2348–2354
51. Eson, C. T., and Jackson, C. M. (1974) J. Biol. Chem. 249, 7791–7797
52. Pryzdial, E. L. G., and Mann, K. G. (1991) J. Biol. Chem. 266, 8969–8977