Zymogen/Enzyme Discrimination Using Peptide Chloromethyl Ketones

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The mechanisms and control systems involved in the blood coagulation and thrombolytic enzymatic pathways have been subjected to intensive study, and many of the molecular structures and interactions involved have been well characterized (2–5). With few exceptions, the expression of proteolytic activity of these pathways has been shown to occur in complexes composed of a surface, a cofactor or binding protein, and a serine protease (4–6). Many of these components interact with each other and with their substrates through the influence of calcium ions. These complexes convert a precursor zymogen to a catalytically active enzyme which can associate with the components of its respective complex and catalyze the succeeding reaction in the pathway. In coagulation, this culminates in the formation of the insoluble fibrin clot. The thrombolytic system has dual end functions: the cleavage of the fibrin clot into soluble fragments and the deactivation of the enzymes and binding proteins of the coagulation pathway (6–8). The entire, delicately balanced process is further regulated by circulating inhibitors of the enzymes from each system (9,10).

Vascular injury and exposure of the coagulation components to a foreign surface are considered to provide the initiating stimulus for the “extrinsic” and “intrinsic” coagulation pathways (2,3,5). The thrombolytic system is considered to be triggered by the release of plasminogen activators (7,8). However, the initiating events for these pathways are not well characterized and the molecular bases for the initiating activities are not understood. These limitations can be overcome by reagents that would permit the direct study of macromolecular interactions between coagulation components under a variety of conditions and permit the evaluation of the formation of proteolytically active species with a high degree of sensitivity and specificity.

The use of amino acid and peptidyl chloromethyl ketones as irreversible inhibitors of serine proteases was pioneered by Shaw and co-workers (11,12). When an amino acid or peptidyl chloromethyl ketone of proper specificity is reacted with an active serine protease, the histidine of the protein charge transfer complex attacks the α-methylene of the inhibitor, displacing the chloride and leaving the inhibitor attached to the enzyme. The serine protease is thus inactivated by the disabling of the charge transfer complex and by a physical blockage of the active-site region (13). Inhibition of the enzyme is essentially irreversible because the stability of the nitrogen-methylene bond prevents decomposition of the enzyme-inhibitor complex.

Since the enzymes of the blood coagulation and thrombotic pathways are serine proteases of the trypsin family, with specificity for cleavage at the carboxyl end of arginyl and/or lysyl residues, arginine and lysine chloromethyl ketone derivatives have been widely used as inhibitors in these pathways (11,12,14,15). Tosyl-L-lysine chloromethyl ketone is commonly used, as are the more specific peptide derivatives D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPRck) for thrombin and L-isoleucyl-L-glutamylglycyl-L-arginine chloromethyl ketone (BioCap) for thrombin and tissue plasminogen activator (t-PA) (16,17). The abbreviations used are: FPRck, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; BioCap, biotin ε-aminocaproyl; dansyl, 2,6-dimethylaminonaphthalene-1-sulfonyl; EGRck, glutamylglycinyl-L-lysine chloromethyl ketone; PCPS, vesicles composed of 75% phosphatidylcholine, 75% phosphatidylethanolamine, and 25% dioleoyl phosphatidylglycerol; and BioCap, biotin ε-aminocaproyl; dansyl, 2,6-dimethylaminonaphthalene-1-sulfonyl; EGRck, glutamylglycinyl-L-lysine chloromethyl ketone; PCPS, vesicles composed of 75% phosphatidylcholine, 75% phosphatidylethanolamine, and 25% dioleoyl phosphatidylglycerol.
arginine chloromethyl ketone for factor Xa (11, 12, 14, 15). Both of these peptides have been previously demonstrated to react with other proteases and to have general anticoagulant activity (12, 16). Several investigators have used EGRck for specific labeling of proteases by attaching marker groups such as dansyl to the amino terminus of the peptide. Modification of some coagulation components with these fluorescent probes has proved invaluable in the dissection of interactions of the modified protease with other components of the reactive complex such as phospholipid, binding/cofactor protein, substrate, and calcium ions (17–21).

One aspect of the control of coagulation which has generated intense interest is the initiation of the extrinsic pathway (3, 5, 22, 23). In this system, the (zymogen) factor VII is cleaved to proteolytically active factor VIIa and bound to a phospholipid membrane surface through the binding protein tissue factor and Ca²⁺. This complex then can convert the zymogen factors IX and X to their respective enzyme factors IXa and Xa (24, 25). Both factors IXa and Xa are capable of rapidly activating factor VIIa to VIIa, so a rapid feedback loop is established (26). Zur and co-workers (bovine) and Broze and Majerus (human) have demonstrated that factor VII, unlike other zymogen forms, can react with diisopropyl fluorophosphate (DFP), a potent inhibitor of serine proteases (27, 28).

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Similarly, the generally high reactivity of the preparations toward proteases suggests that the chloromethyl ketone groups are substantially unhydrolyzed. Chloromethyl ketones, which are hydrolyzed by treatment with strong base, lose their ability to inhibit proteases. We have noted a tendency for dansyl derivatives to lose fluorescence intensity on standing at room temperature for long periods, even if protected from light. The compounds do, however, retain their ability to inhibit proteases. All of the fluorescent inhibitors are sensitive to photobleaching and were protected from light as much as possible.

To determine the range of activity of the various fluorophore-peptide combinations, proteases and their corresponding zymogens were reacted with an excess of each reagent. In all cases, the extent of reaction was monitored by gel electrophoresis. Fluorescent bands were photographed and marked for electrophoresis. Fluorescent bands were photographed and marked for electrophoresis.

We report here the preparation of a variety of fluorescent chloromethyl ketone reagents and their application to the question of activity in factor VII.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

The chemical properties and spectral characteristic of the fluorescent labeled chloromethyl ketones are shown in Table I. All were free of unlabeled peptide and free fluorophore and appeared stable on long-term storage in 10 mM HCl at −20 °C. Contamination by small quantities of hydroxymethyl ketone could not be ruled out absolutely, since the methods used for analysis could be expected to produce small amounts of hydrolysis. The generally high reactivity of the preparations toward proteases suggests that the chloromethyl ketone groups are substantially unhydrolyzed. Chloromethyl ketones, which are hydrolyzed by treatment with strong base, lose their ability to inhibit proteases. We have noted a tendency for dansyl derivatives to lose fluorescence intensity on standing at room temperature for long periods, even if protected from light. The compounds do, however, retain their ability to inhibit proteases. All of the fluorescent inhibitors are sensitive to photobleaching and were protected from light as much as possible.

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**FIG. 1. Electrophoretic analysis of zymogens and proteases treated with fluorescein-EGRck.** The proteins were reacted with fluorescein-EGRck (50 μM) in Hapes/saline for 2 h at room temperature. Aliquots containing 10–20 μg of protein were removed, prepared for electrophoresis in 62.5 mM Tris-HCl at pH 6.8, 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and analyzed by SDS-PAGE using 5–10% acrylamide gels. The fluorescent bands were visualized by excitation with long wavelength UV light and photographed through a cut-off filter (top). Subsequently, the total protein was visualized on the gel following staining with Coomassie Brilliant Blue (bottom). Lanes 1 and 15, molecular weight standards; lanes 2, prothrombin; lane 3, α-thrombin; lane 4, factor VII/VIIa; lane 5, factor IX; lane 6, factor IXa; lane 7, factor X; lane 8, factor Xa; lane 9, protein C; lane 10, activated protein C; lane 11, plasminogen; lane 12, plasmin; lane 13, tPA; and lane 14, urokinase. All proteins used were of human origin, with the exception of bovine protein C and bovine activated protein C.

The reaction of urokinase with [β-FPRck, [α-EGRck, and [α-YGRck (where [α] represents any one of the many fluorescent probes used). The reaction of urokinase with [β]-FPRck derivatives was relatively inefficient, but all other combinations tested gave essentially quantitative label incorporation at molar excesses.

Portions of this paper (including “Experimental Procedures,” Tables I and II describing the spectral properties and reactivities of the fluorescent chloromethyl ketones, and Figs. 2 and 3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
of chloromethyl ketone below 5:1. In contrast, none of the zymogen forms could be induced to incorporate label, even at molar ratios in excess of 50:1. When a small amount of label was observed to incorporate into a zymogen preparation, SDS-PAGE analysis of the product showed this to be due to traces of activated protease which contaminated the preparation.

The incorporation of label could be monitored directly in those cases where a chromogenic substrate assay was available. Results of typical experiments are shown in Fig. 2. In these studies each aliquot of inhibitor was allowed to react until the rate of incorporation was very slow before a subsequent aliquot was added. The slow hydrolysis rate of the chloromethyl ketone precluded waiting until all previously added inhibitor was consumed. Incremental additions of reagent were continued until only base-line hydrolysis of the chromogenic substrate could be observed. For a given protease, the total amount of inhibitor required depended both on its structure and on the rate of addition of the chloromethyl ketone. When [\(\phi\)]-EGRck derivatives were used to inhibit factor Xa, 2–5 M eq were required to block activity. [\(\phi\)]-FPRck and [\(\phi\)]-YGRck derivatives were generally more efficient in inhibition of both factor IIa and factor Xa, typically giving complete inhibition with two or less molar equivalents.

Significant inhibition of factor X activation was observed when factor VIIa was pretreated with fluoresceinyl-FPRck (1.0 eq). However, detailed evaluation of the efficiency of inhibition of factor VIIa by the chloromethyl ketones was not feasible since excess inhibitor present in the initial incubation step interfered with the coupled assay required to evaluate factor Xa generation. In all cases requiring coupled assays, reactivity with the inhibitors prepared in the present study was determined only qualitatively by the incorporation of label into enzyme observed after SDS-PAGE. In these instances, quantitation of rate was not attempted.

The only combination of active protease and chloromethyl ketone which failed to incorporate substantial fluorescence was two-chain urokinase reacted with [\(\phi\)]-FPRck derivatives. This result is illustrated by the ability of urokinase to convert plasminogen to plasin in the presence of lissamine-rhodamine-FPRck, an inhibitor which is readily incorporated into plasin produced during the reaction. This observation suggests the availability of a useful technique to study directly the activation of plasminogen catalyzed by urokinase.

The fluorophore/protein ratios determined for several proteins which were modified with a variety of chloromethyl ketones and then isolated free of excess inhibitor are listed in Table II. Direct quantitation of fluorophore/protein ratios in the isolated labeled proteins by absorbance measurements generally gave values close to 1:1, but these measurements may be complicated by changes in the molar extinction coefficient of the chromophores which could accompany their incorporation into the protein.

Hydrolysis Rates for Peptide Chloromethyl Ketones—Hydrolysis of chloromethyl ketones to the corresponding hydroxymethyl compounds, while significant, is not so rapid at physiological pH as to remove inhibitory activity reliably. As measured by the ability to inhibit factor Xa, the apparent half-life of [\(\phi\)] peptide chloromethyl ketone at neutral pH in Hpes/saline at 20 °C is 2–5 h, and significant amounts (5–20%) of the inhibitory activity remains after 24 h (Fig. 3). At 37 °C, the reaction is somewhat more rapid, but 2–5% activity remains after 6 h. The rate of hydrolysis under these conditions is faster with [\(\phi\)]-EGRck derivatives than with [\(\phi\)]-FPRck (Fig. 3). Increasing the pH greatly enhances the hydrolysis rate. At pH 11.0 (1.0 mM NaOH) a 5-min incubation results in complete loss of inhibitory activity, suggesting complete hydrolysis.

Fluorescence Properties of the Probes and Probe-Protein Adducts—The fluorescence behavior of the probes is generally not greatly affected by the nature of the peptide or chloromethyl ketone to which it is attached. However, dramatic changes may be observed upon protein incorporation. For protein incorporated probes, wavelength shifts in excitation and emission maxima (relative to the unattached inhibitor) are generally small, typically from 0 to 5 nm. Some increase generally occurs in excitation at 280 nm. This is attributable to energy transfer between the protein and the probe. The increase is especially pronounced when the probe has an excitation band in the 320–400 nm range. Otherwise, intensity changes in excitation and emission bands are dependent both on the probe and the protein used and may be observed also on binding of the protease to other components of the proteolytic complex (17–19).

Active Site Enzyme Blotting with Biotin Caproyl Peptide Chloromethyl Ketones—Factor Xa and factor VIIa were analyzed for chloromethyl ketone probe incorporation using transfer blotting techniques. In these experiments, factor VIIa and factor Xa were treated with BioCap-FPRck for 2 h, and the products were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose as described under “Experimental Procedures.” The proteins were analyzed in the reduced form in the case of factor Xa and both with disulfide bonds intact and reduced in the case of factor VIIa. Electroblotted proteins were probed with avidin coupled to horseradish peroxidase followed by visualization of the reactive bands by the addition of 4-chloronaphthol (Fig. 4). As analyzed by this technique, only the heavy chains of factor Xa (A) and factor VIIa (B and C) are labeled with BioCap-FPRck, with no incorporation of probe into the respective light chains. This result is completely consistent with the specific modification of the active-site histidine present in the heavy chains of these proteases.

The technology presented in Fig. 4 was further extended by preparing mixtures of factor VII/factor VIIa and factor X/factor Xa for evaluation. The zymogen/enzyme mixtures were

![Fig. 4. Western blot analysis of active-site biotinylated factor VIIa and factor Xa. A, BioCap-FPRck modified factor Xa with 5-, 10-, and 20-µg samples applied to the electrophoresis gel. B (intact disulfide bonds) and C (disulfide bonds reduced), BioCap-FPRck modified factor VIIa with 5-, 10-, and 20-µg samples applied to the electrophoresis gel. Following electrophoretic transfer, the biotin-containing bands were visualized using avidin-peroxidase as described under "Experimental Procedures."](image-url)
treated with BioCap-FPRck and subjected to SDS-PAGE. The gels were evaluated by protein staining, trans-blotting with the biotin-containing polypeptides detected by avidin-peroxidase. Subsequently, gold staining of the transflected nitrocellulose sheet was performed to ensure transfer of the relevant protein species present had been accomplished. Fig. 5a illustrates the results of this experiment with a synthetic mixture of factor X and factor Xa (A). Note that only the two biotinylated bands corresponding to the factor Xa α and β heavy chains are detected by this procedure (B). Subsequent gold staining of the protein transferred in the experiment (C) illustrates that all peptide materials are transferred, but only the active site chains are labeled (B). When the same experiment was performed with a mixture of factor VII and factor VIIa (in Fig. 5b) an equivalent result is observed. There is no incorporation with any label into factor VII even though the concentration in factor VII in this experiment is relatively high relative to the concentration of factor VIIa.

Removal of Active Enzymes from Zymogen Preparations—The biotinylated chloromethyl ketone biotinyl-c-aminocaproyl-YGRck provides for the facile removal of traces of active protease from samples. As with the fluorescent probes, only the active proteases react to incorporate the biotinylated peptide. The biotin is then available for binding to avidin, but only on the (previously) active enzymes. This procedure allows the rapid removal of the blocked, inactivated protease by reaction with avidin linked to a solid matrix. While complete inhibition of protease activity can be obtained with a variety of active site blocking reagents, physical removal of traces of the protease from zymogen preparations has often proven difficult. This is especially true when the activation occurs with no change in molecular size. This method allows for a simple removal of contaminants and is particularly useful in preparing reagents for detection of small amounts of zymogen activation and other applications where a trace of protease is undesirable.

Mixtures of human factors VII and VIIa (5–15% VIIa) were reacted with BioCap-YGRck, using 5-fold excess of chloromethyl ketone (based on total protein). The product was gel-filtered to remove excess inhibitor and reacted with avidin linked to a polycrylate matrix. Gel analysis of the supernatant indicated complete removal of factor VIIa (Fig. 6A). Similar results were obtained when a solution of bovine factor X, doped with an equal amount (w/w) of factor Xa, was treated in a similar fashion. In this instance, the chloromethyl ketone used was BioCap-FPRck and the adsorbent used was avidin-agarose. The results, before and after avidin-agarose chromatography, are illustrated in Fig. 6B.

The spacing provided by the c-aminocaproyl group is essential for this reaction. When biotin was coupled directly to a peptide chloromethyl ketone, reaction with proteases (factors IIa and Xa) proceeded normally, as determined by amidolytic activity measurements. However, no binding of avidin could be detected using avidin-acrylic, fluorescein-avidin, or the chromomethyl ketone. Blotting with the spacer, avidin reacts readily, using any of the above detection methods. The gel filtration step, while not essential, greatly reduces the amount of solid-phase avidin consumed.

Is Single Chain Human Factor VII an Enzyme?—When mixtures of human factors VII and VIIa were reacted with any of the fluorescent or biotinylated chloromethyl ketones, incorporation occurred only into factor VIIa heavy chain as observed on reduced SDS-PAGE gels. The factor VII band showed no label incorporation, even when chloromethyl ketone to protein ratios of 100:1 were used. The initial binding steps for incorporation of peptide chloromethyl inhibitors are much the same as for proteolytic activity (i.e., the binding of the scissile arginine side chain into the P1, subsite). Since the results obtained with factor VII paralleled those found with other zymogens tested, we elected to examine the role of “active zymogen” suggested for the uncleaved factor VII protein (23).

Silverberg et al. (26) have reported that the catalytic activity of bovine factor VIIa toward factor X is enhanced 104-fold when tissue factor/lipid and Ca(II) are present to form the complete catalytic complex (29). We therefore examined the reaction of factor VII/VIIa mixtures with peptide chloromethyl ketones in the presence of tissue factor-phospholipid and Ca2+. The resulting analyses showed rapid and complete labeling of factor VIIa but no incorporation into single-chain factor VII. Each chloromethyl ketone derivative prepared was screened versus mixtures of factors VII and VIIa, and all reacted only with factor VIIa. In more stringent studies, three inhibitors (2,6-dansyl-EGRck, fluorescein-FPRck, and 1,5-dansyl-YGRck) were each reacted at 50:1 molar ratios for 48 h. No incorporation was observed in any case into factor VII. Factor VIIa quantitatively incorporated all inhibitors readily and completely at 5:1 molar ratios and 4-h reaction times. From the complete absence of reactivity for factor VII under these conditions, one must conclude an absence of relative reactivity for the single-chain protein at approximately the 0.001 level.3

To examine the nature of single-chain factor VII further, we prepared samples of human factors VII and X which were

3 Considering a 10-fold higher inhibitor concentration reacted 8 times longer with a 5% minimum detection limit.
Enzyme purging using BioCap-YGRck. A mixture of human factor VII and VIIa (A) or bovine factor X and factor Xa (B) was incubated overnight with BioCap-FPRck to modify the active species present. Excess chloromethyl ketone was removed by gel filtration and samples were prepared for SDS-PAGE analysis before and after the mixtures were adsorbed to solid-phase avidin. SDS-PAGE was conducted on samples with disulfide bonds reduced using 5–15% acrylamide gradients followed by staining with Coomassie Brilliant Blue. In both A and B, lane 1 represents the sample prior to adsorption with solid-phase avidin and lane 2 represents the material that did not adsorb to the avidin matrix. The lanes indicated by S contain molecular weight markers. A, the identified bands correspond to the light chain and the α and β forms of the heavy chains of factor X and Xa. B, the identified bands correspond to single-chain factor VII and the heavy chain of factor VIIa.

exhaustively reacted with biotinylated chloromethyl ketones and then separated from residual inhibitor by gel filtration on Sephadex G-25. For the factor VII preparations, BioCap-YGRck and BioCap-FPRck were used as the inhibitor, and avidin-acrylic and avidin-agarose were used to remove the labeled VIIa. Gel analysis confirmed the complete removal of all factor VIIa (see Fig. 6A). This treatment insured that no activated enzyme remained in the protein preparations. The factor X preparations treated in a similar manner were completely inert toward chromogenic substrate (S2222) even when incubated for 6 h in Hepes/saline with Ca²⁺ and phospholipid (PCPS) vesicles.

When tissue factor, PCPS, Ca²⁺, and factor VII were added to factor X, factor Xa activity was generated rapidly. Under these conditions, almost complete activation was observed after 2 h (Fig. 7). Electrophoretic analysis indicated that the factor X had been converted completely to factor Xa and the factor VII to VIIa. As controls in these experiments, reaction mixtures were evaluated in which one or two of the protein components of the catalytic complex (tissue factor, factor VII, factor X) were omitted. The controls were evaluated in parallel with the complete system. As seen in Fig. 7 no amidolytic activity was found for any reaction mixtures containing a single protein component or for mixtures containing tissue factor-PCPS/factor VII/Ca²⁺ or factor VII/factor X/Ca²⁺. However, in the reaction in which factor X was incubated with tissue factor-PCPS/Ca²⁺, a slow activation (10% in 2 h) of the factor X was observed by hydrolytic activity (Fig. 7). This result was confirmed by electrophoretic analysis (see below). In contrast to the absence of an active-site histidine, we were able to confirm the earlier reports (29) that single-chain factor VII was able to incorporate diisopropyl phosphorofluoridate. Following treatment with [³H]diisopropyl phosphorofluoridate, radioactivity was detected in single-chain factor VII resolved by SDS-PAGE.

Because of the available feedback system wherein factor Xa activates factor VII rapidly (29), the presence of even trace levels of an endogenous factor X activator cannot be tolerated in this system. We were unable to obtain any tissue factor preparation which did not have a spontaneous capacity to activate factor X to factor Xa, either because of a contaminating protease or because of a mechanism involving factor X and tissue factor itself. We therefore resorted to an indirect

![Fig. 6. Enzyme purging using BioCap-YGRck.](image)

![Fig. 7. Analysis of factor Xa generation using the fully or partially assembled extrinsic factor X activating complex.](image)
test for the activity of uncleaved factor VII toward factor X in a tissue factor-lipid-Ca\(^{2+}\) system. The logic of this experiment is based upon the reaction series "a" through "h" where TF represents tissue factor.

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\begin{align*}
\text{Xa} + [\phi]^\text{-FPRck} & \rightarrow [\phi]^\text{-FPR-Xa} \\
\text{VIIa} + [\phi]^\text{-FPRck} & \rightarrow [\phi]^\text{-FPR-VIIa} \\
\text{X} + [\phi]^\text{-FPRck} & \rightarrow \text{no reaction} \\
\text{VII} + [\phi]^\text{-FPRck} & \rightarrow \text{no reaction} \\
\text{VII, TF} & \rightarrow \text{FPRck, Ca}\(^{2+}\) \\
\text{X} & \rightarrow \text{Xa} \\
\text{PCPS, Ca}\(^{2+}\) & \rightarrow \text{Xa} \\
\text{PCPS, Ca}\(^{2+}\) & \rightarrow \text{Xa} \\
\text{VIIa, TF} & \rightarrow \text{VIIa} \text{ blocked} \\
\text{PCPS, Ca}\(^{2+}\) & \rightarrow \text{Xa} \text{ blocked} \\
\end{align*}
\]

The foregoing segments of this paper clearly illustrate that the reactions listed as a through d are proven, i.e. factor X and factor VII will not react with peptide chloromethyl ketones, whereas factor Xa and factor VIIA readily react and are inactivated by this reaction. The reactions under test are represented by reactions systems e through h. We can be certain at this point that reaction f certainly occurs as illustrated from the data from Fig. 7, i.e. factor X is slowly activated to factor Xa in the presence of tissue factor, PCPS, Ca\(^{2+}\) preparations without addition of factor VII. We can further be certain that factor VII itself is converted to factor VIIA by factor Xa (reaction g), a reaction which is blocked by peptide chloromethyl ketones. In addition, it is certain that reaction h occurs and it is further certain that reaction h can be blocked by peptide chloromethyl ketones since factor VIIA readily incorporates peptide chloromethyl ketone in the presence of tissue factor, PCPS, and Ca\(^{2+}\). The reaction in question thus becomes reaction e which describes the activation of factor X by (single-chain) factor VII, tissue factor, PCPS, and Ca\(^{2+}\) with factor VIIA totally in the uncleaved (zymogen) form. Since uncleaved factor VII cannot incorporate peptide chloromethyl ketone (reaction d) whether or not tissue factor/lipid/Ca\(^{2+}\) is present, the presence of peptide chloromethyl ketone will not have any capacity to inhibit reaction e.

To test the relevance of reaction e, enzyme-free factor VII and factor X were mixed with purified tissue factor, PCPS, and Ca\(^{2+}\) in the presence of an excess of FPRck to ensure complete trapping of any proteases produced so that the only reaction which could occur would be by a factor VII-dependent cleavage of factor X. The control for this experiment was a mixture of factor X, tissue factor, PCPS, and Ca\(^{2+}\) which is, as shown in Fig. 7, slowly activated to factor Xa. The results of this experiment are presented in Fig. 8. Over a 3-h interval the amount of factor Xa produced in the presence of FPRck is negligible whether or not factor VII is present. Since the inhibitor does not interact with factor VII one must conclude that it can have no effect on the reaction rate involving factor VII. The results in Fig. 8 also illustrate that the slow activation of factor X observed in the absence of factor VII (reaction f) is completely abolished by the inhibitor. These observations are consistent with the conclusion that reaction e does not occur, i.e. factor VII is not a catalyst involved in the activation of factor X to factor Xa. The results of the experiment described in Figs. 7 and 8 support the hypothesis that initial factor X activation in this system is provided by a component of the tissue factor preparation, not factor VII. In a noninhibited system, this factor Xa, initially produced, initiates a feedback loop illustrated by reaction h giving rise to factor VIIA. Factor VIIA then participates in reaction g to generate factor Xa at a rapid rate when inhibitor is not present.

FIG. 8. SDS-PAGE analysis of factor X activation in the presence or absence of FPRck. Complete reaction mixtures contained 6.9 \(\mu\)M factor X, 10 nM tissue factor, 60 \(\mu\)M PCPS, and 8 nM factor VII in Hapes/saline containing 5.0 nM Ca\(^{2+}\) at 37 °C. Reactions were conducted using either complete reaction mixtures or reaction mixtures lacking factor VII in the presence or absence of 0.2 mM FPRck. Aliquots from these four reaction mixtures were withdrawn at 15 s, 60 min, and 120 min, quenched as described under "Experimental Procedures," analyzed by SDS-PAGE using gels composed of 12% acrylamide and visualized by Coomassie Blue staining. Lane S, molecular weight markers; lane 1, starting factor X; lanes 2-4, complete reaction mixture in the absence of FPRck; lanes 5-7, complete reaction mixture plus FPRck; lanes 8-10, reaction mixture lacking factor VII in the absence of FPRck; lanes 11-13, reaction mixture lacking factor VII plus FPRck.

**DISCUSSION**

The data presented here can be grouped into three areas of interest: 1) the preparation and characterization of a variety of new fluorescent labeled inhibitors of serine proteases; 2) the development of a specific and quantitative technique for detection and removing traces of activated proteases from zymogen preparations; and 3) new evidence regarding the putative role of single-chain factor VII as the key catalyst in the initiation of the extrinsic pathway.

The most striking aspect of the fluorescent peptide chloromethyl ketones is their general reactivity with active proteases and their absolute failure to react withzymogens. While differences in rate and specificity are observed, the level of incorporation of the probes into the proteases tested is not markedly dependent on the sequences of the peptides used. When 1 eq of fluorescein-FPRck was added to thrombin, factor Xa, and tPA, the observed activity loss after 20-min incubation was 86, 62, and 59%, respectively. Determination of true rate constants is complicated by competing hydrolysis reactions; however, after 20 min, no additional decrease in activity was observed, and the inhibitor can be assumed to have been consumed either by reaction with the protein or by hydrolysis. The relative amounts of activity loss can then be considered a rough estimate of the relative reaction rates. With 2,6-dansyl-EGRck, the observed levels of inhibition were 63, 75, and 48%, respectively, for the three proteases. From these data, it can be inferred that the \([\phi]\)-FPRck derivatives react readily with all three proteases, and that all \([\phi]-\)
EGRck derivatives, while showing some limited specificity for factor Xa, react well with the other proteases. These data are in general agreement with rates of incorporation reported by Kettner and Shaw (12); however, the range of reactivities does not appear to be as large as those reported with the free peptides. This is particularly apparent in the comparison of the levels of inhibition observed with free FPRck versus those seen with the \([\epsilon]-FPRck\) conjugates. These differences presumably arise due to contributions to the protein-peptide interaction by the fluorescent probe itself. This is consistent with the observed weak, nonspecific interactions of many of these fluorescent groups with proteins and with the observation that addition of dansyl groups generally enhances binding (12).

An interesting outcome of these experiments was the observation that an aromatic group in the S3 position substantially enhances reactivity. As reported by Kettner and Shaw (12), unsubstituted FPRck is a good inhibitor of thrombin; and free EGRck is much slower (and somewhat less specific) in its reactions. Addition of the aromatic fluorophore enhances the activity of both EGRck and FPRck with a variety of proteases. Similarly, substitution of the aromatic tyrosyl residue for glutamyl (YGck) gives free peptide more general reactivity.

In addition to their general utility in distinguishing proteases from zymogens, the peptides reported here have proven useful in a wide variety of applications. Pyrene and 2,6-dansyl probes are well suited for fluorescence polarization studies because of their long fluorescent lifetimes and have proven quite useful in measurements of lipid binding (19). The high sensitivity for the detection of these fluorescent labels makes possible determinations of dissociation constants in the nanomolar range and makes them especially useful in stopped flow applications. Preliminary studies have shown the fluorescein and rhodamine probes to be especially useful in detecting small amounts of active protease in zymogen preparations and for monitoring activation processes, either in plasma or in purified systems.

The ability of the biotinylated peptide chloromethyl ketones to serve as specific probes for detection and removal of traces of activated enzymes from zymogen preparations has proven quite useful. In many systems traces of activated protease can distort the interpretation of results; and it often is difficult to distinguish between zymogen forms and active enzyme. Since the biotin probe allows for complete and selective removal of the blocked protease, uncertainty as to residual activity can be eliminated. The high affinity of avidin for biotin, the absolute specificity of the chloromethylketone for the active proteases, and the availability of solid-phase avidin reagents makes these systems very usable. These systems have a number of other potential applications. They can be used to prepare completely homogeneous samples of zymogens for immunochemical studies. The stability of the inhibitor-histidine bond prohibits the use of the system for recovering active proteases.

Our results with factor VII indicate unambiguously that human factor VII does not react with chloromethyl ketones which efficiently label factor VIIa and all other proteolytic enzymes tested and that this lack of reactivity is not altered by the presence of tissue-factor-lipid and Ca\(^{2+}\) in the labeling reaction. While these data demonstrate that factor VII contains <0.001 the reactivity of factor VIIa, they are not absolute proof of lack of protease activity in human factor VII. They do, however, strongly suggest that a functional P1 site or reactive histidine is not available until after cleavage of the zymogen.

We were able to confirm the previously reported reaction of single-chain human factor VII with DFP. However, incorporation of diisopropyl phosphorofluoridate into a protein at an unidentified site is not incontrovertible proof of the presence of a serine 195 (in chymotrypsin) equivalent in a protein. In addition, we have recently reported studies on a congenitally abnormal form of thrombin, Thrombin Quick II, which is altered by a substitution of valine for glycine at position 558 (46). A molecular graphics analysis of the consequence of this substitution suggests a complete blockade of the P1 binding subsite in the Quick II \(\alpha\)-thrombin, that is, the substrate arginine side chain cannot be accommodated into the P1 binding pocket because of steric hinderance. This abnormal thrombin has virtually zero activity toward any arginine-containing substrate but retains complete activity of the active site serine as identified by diisopropyl phosphorofluoridate incorporation studies. Similarly, the data obtained from chemical modification studies with factor VII suggest that while the charge transfer complex may be present in single-chain factor VII, the arginine binding subsite for the reactive histidine is not present.

Using tissue factor generously provided from a variety of sources, we were not able to produce a lipid-reconstituted tissue factor preparation which lacked the ability to convert factor X to Xa directly, albeit at a slow rate. Inspection of data previously published by Rao et al. (30) and by Zur et al. (27) shows similar results in their tissue factor-factor X controls. Since the factor Xa thus produced can rapidly activate factor VII to VIIa, particularly when the factor VII is bound to tissue factor (26), a simple definitive experiment on single-chain factor VII was impossible. We were unable to identify the source of the proteolytic activity in various tissue factor preparations. The proteolytic activity present in tissue factor preparations was completely abolished by incubation with FPRck.

Since the tissue factor preparations were obtained from natural product sources, it is conceivable that they are contaminated with small levels of a direct factor \(X\) activating protease. It is also conceivable that tissue factor itself has the capacity to interact with factor \(X\) to give rise to the expression of an active site much as is observed in the reaction of streptokinase with plasminogen (47). While the latter possibility is a source worthy of study, it is beyond the scope of the present work. However, since factor Xa is rapidly and irreversibly inhibited by peptide chloromethyl ketone while factor VII shows no ability to interact with or be neutralized by these compounds, we could explore the question as to whether factor VII tissue factor reconstituted on phospholipid in the presence of calcium could convert endogenous factor \(X\) to factor Xa. Further, factor VII in the presence of tissue factor and phospholipid does not incorporate chloromethyl ketone. The result of this experiment is that no factor \(X\) activation was observed in the presence of chloromethyl ketone. This experiment indicates that factor VII itself has no proteolytic activity expressed in the tissue factor-factor VII complex toward factor \(X\) as a substrate.

Our data are consistent with reports from Rao et al. (30) and Rao and Rapaport (31) which concluded that human factor VII does not possess catalytic activity to convert factor IX to factor IXa. This conclusion indicates that no satisfactory description of the initiation phase of coagulation presently exists. The significance of the contact pathway of coagulation is significantly muted by the observations that individuals who lack factor XII, prekallakrein or high molecular weight kininogen do not require plasma replacement therapy or appear to suffer from significant hemostatic de-
fects. The elimination of the active zymogen hypothesis which placed factor VII as the initiating serine protease of the extrinsic pathway of coagulation leaves us without a plausible initiating event to start the blood clotting process. It is conceivable that some cellular derived activity, perhaps that present in tissue factor preparations, represents the active component that starts the clotting process.

The reader should be cautioned however that previous conclusions of "active zymogen" capacity in factor VII have been generated with bovine proteins; and there could be present in tissue factor preparations, represents the active component that starts the clotting process.

Our data lead us to the conclusions that human factor VII, as with the other serine protease precursors, is a zymogen; that the apparent activity of factor VII in purified systems is due to traces of activated proteases in the factor VII, factor X, or the intrinsic pathway. The elimination of the active zymogen hypothesis which initiates the extrinsic pathway of coagulation leaves us without a plausible initiating event to start the blood clotting process. It is conceivable that some cellular derived activity, perhaps that present in tissue factor preparations, represents the active component that starts the clotting process.

Acknowledgments—We wish to thank Drs. S. Rapaport, J. Morrissey, and T. Edgington for their generous gifts of tissue factor. We acknowledge the technical assistance of Julie Burnham and the patience and skill of Mary Mahoney who typed the manuscript.

REFERENCES


Continued on next page.
Zymogen/Enzyme Discrimination

Supplemental Procedures

Materials

The fluorescent probe N-hydroxy succinimide esters and sulfonyl chloride were obtained from Molecular Probes, Inc., Eugene, OR, and ultrapure acetonitrile was from Fisher Scientific, Fair Lawn, NJ. N-Hydroxysuccinimide, N-hydroxysuccinimidyl biotin, and sulfonyl chloride were purchased from Pierce Chemical Company, Rockford, IL. N-Hydroxysuccinimidyl acetate was a gift from Dr. James Morrissey, University of California, San Francisco, CA. N-Hydroxysuccinimidyl 2-ethyl acetate was purchased from Pierce Chemical Company. [3H]O-phosphatidylethanolamine was purchased from Amersham Pharmacia Biotech, Piscataway, NJ. [14C]O-phosphatidylethanolamine was purchased from Amersham Pharmacia Biotech, Piscataway, NJ. Phosphatidylcholine vesicles composed of 75 phosphatidylcholine and 25 phosphatidylserine (PC:PS) were prepared as described by Hogeboom and Hogeboom (31). Phosphatidylcholine vesicles were used as vesicles of pure ganglion, and stable, hydrated, further purified by reprecipitation or dialysis.

Methods

Reagents: for all proteins, yields were from each fresh frozen human brain. Human tissue was obtained from the University of Washington tissue procurement program. All proteins were obtained from the National Institutes of Health (NIEHS), Bethesda, MD. All proteins were purchased from Sigma Chemical Co., St. Louis, MO. Phosphatidylcholine vesicles composed of 75 phosphatidylcholine and 25 phosphatidylserine (PC:PS) were prepared as described by Hogeboom and Hogeboom (31). Phosphatidylcholine vesicles were used as vesicles of pure ganglion, and stable, hydrated, further purified by reprecipitation or dialysis.

Results

Seven monoclonal antibodies were used to identify the target antigen. First, we determined the concentration of the target antigen by the ELISA method. The concentration of the target antigen was determined using a standard curve. The concentration of the target antigen was then calculated using the standard curve. The concentration of the target antigen was determined to be 0.5 ng/ml.

Discussion

The fluorescent probe N-hydroxysuccinimide esters and sulfonyl chloride were obtained from Molecular Probes, Inc., Eugene, OR, and ultrapure acetonitrile was from Fisher Scientific, Fair Lawn, NJ. N-Hydroxysuccinimide, N-hydroxysuccinimidyl biotin, and sulfonyl chloride were purchased from Pierce Chemical Company, Rockford, IL. N-Hydroxysuccinimidyl acetate was a gift from Dr. James Morrissey, University of California, San Francisco, CA. N-Hydroxysuccinimidyl 2-ethyl acetate was purchased from Pierce Chemical Company. [3H]O-phosphatidylethanolamine was purchased from Amersham Pharmacia Biotech, Piscataway, NJ. [14C]O-phosphatidylethanolamine was purchased from Amersham Pharmacia Biotech, Piscataway, NJ. Phosphatidylcholine vesicles composed of 75 phosphatidylcholine and 25 phosphatidylserine (PC:PS) were prepared as described by Hogeboom and Hogeboom (31). Phosphatidylcholine vesicles were used as vesicles of pure ganglion, and stable, hydrated, further purified by reprecipitation or dialysis.

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Zymogen/Enzyme Discrimination

Table 1

<table>
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<tr>
<th>Product</th>
<th>Molecular Weight (kDa)</th>
<th>pI</th>
<th>Relative Mobility</th>
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<tbody>
<tr>
<td>1-Aminobenzyl-Fluorochrome</td>
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<td>510/510</td>
<td>4210</td>
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<tr>
<td>1-Phenyl-2-Naphthyl-Fluorochrome</td>
<td>0.44</td>
<td>379/375</td>
<td>3000</td>
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<tr>
<td>1-Aminobenzyl-Fluorochrome</td>
<td>0.49</td>
<td>326/321</td>
<td>5000</td>
</tr>
<tr>
<td>Phenol-Sulfonyl-Fluorochrome</td>
<td>0.33</td>
<td>480/485</td>
<td>75000</td>
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<tr>
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<td>5000</td>
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<td>490/490</td>
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<td>0.30</td>
<td>300/300</td>
<td>42000</td>
</tr>
</tbody>
</table>

* Relative mobility observed on SDS-PAGE as described under EXPERIMENTAL PROCEDURE.

** Excitation maxima (nm), emission maxima (nm) and molecular extinction coefficients are reported for the respective dyes listed in the handbook of Fluorescent Probes, Molecular Probes Inc. (4).