Strong, P. N., & Kelly, R. B. (1977) *Biochim. Biophys. Acta 469*, 231–235.
Strong, P. N., Goerke, J., Oberg, S. G., & Kelly, R. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 178–182.
Thesleff, S. (1977) *Naunyn-Schmiedeberg's Arch. Pharmacol. 297*, 5–7.
Wernicke, J. F., Oberjat, T., & Howard, B. D. (1974) *J. Neurochem. 22*, 781–788.
Wernicke, J. F., Vanker, A. D., & Howard, B. D. (1975) *J. Neurochem. 25*, 483–496.
Wheeler, K. P., Walker, J. A., & Barker, D. M. (1975) *Biochem. J. 146*, 713–722.
Wolwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446–1454.

Human Plasma Prekallikrein. Studies of Its Activation by Activated Factor XII and of Its Inactivation by Diisopropyl Phosphofluoridate†

Bonno N. Bouma,‡ Lindsey A. Miles, Gregory Beretta, and John H. Griffin*

ABSTRACT: Human plasma prekallikrein was purified from normal plasma. The purified prekallikrein appeared homogeneous on polyacrylamide gels in the presence of sodium dodecyl sulfate and mercaptoethanol and gave two protein bands with approximate M_r 85 000. Proteolytic activation of prekallikrein by purified human β-factor XII_a (M_r 28 000 form) resulted in the formation of kallikrein. The apparent molecular weight of kallikrein determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the absence of mercaptoethanol was identical with that of prekallikrein; reduction of kallikrein yielded a heavy chain of M_r 52 000 and two light chains of M_r 42 000 and 37 000. The appearance of kallikrein activity was directly correlated with the limited proteolysis due to β-factor XII_a. Kinetic and immunologic studies demonstrated that plasma prekallikrein is a factor XII dependent plasminogen proactivator. The rate constant for the inactivation of prekallikrein by diisopropyl phosphofluoridate was similar to that previously reported for trypsinogen. This observation raises the possibility that low intrinsic catalytic activity of prekallikrein may play a role in the initiation of the intrinsic blood coagulation pathway.

Kallikrein is a serine protease that effects the release of vasodepressor peptides or kinins (including bradykinin) from kininogens. These kinins can also increase vascular permeability, contract smooth muscles, produce pain, and influence the migration of leukocytes (Erdos, 1966). Kallikrein can be derived from tissue or from plasma (Kraut et al., 1928), and the plasma enzyme circulates as an inactive zymogen, prekallikrein.

Fletcher trait plasma (Hathaway et al., 1965; Hathaway & Alsever, 1970) which possessed abnormalities in the intrinsic coagulation pathway and in the generation of kinins and of kaolin-activated fibrinolytic activity (Saito et al., 1974; Weiss et al., 1974) was shown to be deficient in prekallikrein (Wuepper, 1973).

Plasma prekallikrein participates in the initiation of the intrinsic pathway of blood coagulation. After exposure of plasma to a negatively charged activating surface such as kaolin or glass, surface-bound factor XII (Hageman factor) undergoes a conformational change resulting in a structure that is highly susceptible to proteolytic cleavage, and this proteolytic cleavage is mainly responsible for the activation of factor XII (Griffin & Cochrane, 1976a; Revak et al., 1977; Griffin, 1978). Surface-bound factor XII_a in the presence of high molecular weight kininogen is a potent activator of factor XI and of prekallikrein (Griffin & Cochrane, 1976a; Revak et al., 1978). The newly formed kallikrein readily dissociates from the surface (Wiggins et al., 1977; Cochrane & Revak, 1979) and reciprocally activates more surface-bound factor XII, thereby augmenting the amount of factor XII_a and consequently the activation of factor XII and prekallikrein. Prekallikrein (Mandle et al., 1976; Donaldson et al., 1977) and factor XI (Thompson et al., 1977) exist in normal plasma complexed with high molecular weight kininogen. This high molecular weight kininogen links both factor XI and prekallikrein to a negatively charged surface where they are activated by surface-bound α-factor XII_a[1] (Wiggins et al., 1977). Once activated, the factor XI_a molecules remain localized at the site of activation, in contrast to the kallikrein molecules which dissociate into the surrounding space (Wiggins et al., 1977; Cochrane & Revak, 1979).

The initial events in triggering the reciprocal proteolytic activations of factor XII and prekallikrein are still unclear. However, on the basis of the observations that trypsinogen and chymotrypsinogen possess weak intrinsic activity in reactions with diisopropyl phosphofluoridate as well as with ester and

† From the Department of Immunopathology, Research Institute of Scripps Clinic, and the Department of Biology, University of California at San Diego, La Jolla, California 92037. *Received September 10, 1979.* This work was supported in part by research grants from the National Institutes of Health, NHLB-21544, NIAID-07007, and NHLI-16411. B.N.B. received support from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). J.H.G. is a recipient of a National Institutes of Health Research Career Development Award, NHLB-00192. L.A.M. received support from a National Institutes of Health Training Grant to the University of California, San Diego. Financial support was provided in part by the Division of Scientific Affairs of NATO. A preliminary report of part of this work was presented at the 6th International Congress on Thrombosis and Haemostasis, Philadelphia, PA, June 1977.

‡ Permanent address: Department of Haematology, University Hospital, Utrecht, The Netherlands.

[1] Abbreviations used: factor XI_a, the active procoagulant form of factor XI; α-factor XII_a, the active, two-chain M_r 80 000 form of factor XII; $NaDodSO_4$, sodium dodecyl sulfate; β-factor XII_a, the active M_r 28 000 form of factor XII; DFP, diisopropyl phosphofluoridate.

0006-2960/80/0419-1151$01.00/0 © 1980 American Chemical Society

protein substrates (Morgan et al., 1972; Kassell & Kay, 1973; Gertler et al., 1974; Lonsdale-Eccles et al., 1978), it was suggested that prekallikrein or factor XII might function as a weakly active zymogen in triggering contact activation reactions (Griffin, 1977, 1978; Griffin & Beretta, 1979). For example, when brought in close proximity by binding to a negative surface, activation of factor XII by the active zymogen prekallikrein could take place and thereby trigger reciprocal proteolysis. Studies of the inactivation of prekallikrein by DFP as described here are consistent with this possibility. Studies of the activation of prekallikrein by purified human β-factor XII_a are also reported here.

Efforts to purify human plasma prekallikrein have been reported by several groups (Wuepper & Cochrane, 1972; Kaplan et al., 1972; Wendel et al., 1972; Laake & Vennerod, 1974; Colman & Bagdasarian, 1977; Mandle & Kaplan, 1977; Gallimore et al., 1978). In this paper we describe a new purification method for human plasma prekallikrein. Evidence based on kinetic and immunologic studies is also presented here which demonstrates that prekallikrein can exhibit both prekallikrein and plasminogen proactivator activities. The presence of a factor XII dependent plasminogen proactivator in plasma has been postulated (Niewiarowski & Prou-Wartell, 1959; Iatridis & Ferguson, 1961; Ratnoff & Davie, 1962; Schoenmakers et al., 1963). This plasminogen proactivator was first reported to be a protein distinct from prekallikrein (Iatridis & Ferguson, 1961; Kaplan et al., 1973), but other investigators were unable to confirm this (Laake & Vennerod, 1974; Wendel et al., 1972). Especially the total absence of detectable factor XII dependent plasminogen proactivator activity in the γ-globulin fraction of prekallikrein-deficient (Fletcher trait) plasma supports the latter observation (Vennerod & Laake, 1976; Bouma & Griffin, 1978).

Materials and Methods

All chemicals obtained from commercial sources were the best grade available. Human factor XII was purified to greater than 95% homogeneity, as judged on $NaDodSO_4$–polyacrylamide gels, according to published procedures (Griffin & Cochrane, 1976b). β-Factor XII_a was prepared by incubation of factor XII with purified human kallikrein or with trypsin, and then β-factor XII_a was separated from kallikrein or trypsin by using DEAE-Sephadex A-50 (Pharmacia) chromatography as described below. β-Factor XII_a had an apparent molecular weight of 28 000 on $NaDodSO_4$–polyacrylamide gel electrophoresis and was identical with the form of factor XII_a previously described as a prekallikrein activator with prealbumin electrophoretic mobility (Kaplan & Austen, 1970). β-Factor XII_a was stored at 4 or −20 °C in storage buffer (0.15 M NaCl, 5 mM sodium acetate, pH 5.0, 0.5 mM EDTA, and 0.02% sodium azide). Human plasminogen was purified to greater than 95% homogeneity by subjecting to affinity chromatography on lysine–Sepharose (Deutsch & Mertz, 1970) the pool of fractions containing plasminogen derived from the first DEAE-Sephadex column which was used as a first step in the purification of factor XII (Griffin & Cochrane, 1976b). Plasminogen at 2 mg/mL in aliquots of 100 μL was stored at −70 °C in storage buffer. Plasminogen was rendered free of plasmin immediately prior to its use for fibrinolytic assays by adsorbing the 100-μL aliquots of plasminogen 3 times with 50 μL of soybean trypsin inhibitor (Sigma) insolubilized on Affigel 10–agarose beads according to the manufacturer's instructions (Bio-Rad, Richmond, CA) (5 mg of inhibitor per mL of beads).

Reference solutions of plasmin for calibration of the plasminogen proactivator assay were prepared essentially according to the method of Wiman & Wallen (1973). The concentration of plasmin was estimated by using the active-site titrant *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (Chase & Shaw, 1970) and, in addition, by comparison with an American National Red Cross plasmin standard (lot no. 8F897, 10 CTA units/mL, Squibb, New Brunswick, NJ) using a caseinolytic assay (Robbins & Summaria, 1970). Plasmin at 0.5 mg/mL, which was stable at −20 °C in storage buffer, was thawed just prior to its use. Plasmin standards were also calibrated by using the tripeptide S-2251 (D-Val-Leu-Lys-*p*-nitroanilide) (Kabi, Sweden) according to manufacturer's instructions.

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma Chemicals) as a reference.

pH and conductivity measurements were performed at room temperature unless noted otherwise.

The clotting activity of prekallikrein (Fletcher factor), factor XI, factor XI_a, factor XII, and high molecular weight kininogen was determined by using partial thromboplastin time assays as previously described (Bouma & Griffin, 1977). Kallikrein amidolytic activity was assayed by using the tripeptide substrate Bz-Pro-Phe-Arg-*p*-nitroanilide (Vega-Fox, AZ) (Amundsen et al., 1974).

Plasminogen proactivator assays were performed as follows. A mixture of test sample (30 μL), factor XII_a or storage buffer (2 μL), and 1.0 M Tris-HCl–0.15 M NaCl, pH 8.2 (3 μL), was incubated for 15 min at 37 °C. Plasminogen (5 μL) was added and the mixture was further incubated for 60 min at 37 °C. Fifteen microliters of the incubation mixture was applied to wells (3-mm diameter) on fibrin plates (Hyland) by double-filling the wells, and the plates were incubated at 37 °C for 7 h. The diameter of the lysed fibrin ring was then measured and converted to an amount of plasmin activity by using a standard curve which related the concentration of the reference plasmin to ring diameter.

Spectrophotometric assays were utilized for comparing the plasminogen activator activity of urokinase to that of kallikrein. A mixture of kallikrein (10 μL) or urokinase (10 μL) (lot no. 702 609, Calbiochem, San Diego, CA), 0.15 M Tris-HCl, pH 7.8, 0.31 M lysine (13 μL), and plasminogen (17 μL) at 1.8 mg/mL in storage buffer was added, and the mixture was incubated at 37 °C. The pH of this mixture was 7.8. Plasmin activity generation was followed by measuring hydrolysis of the tripeptide S-2251 at a concentration of 0.3 mM at 37 °C.

Purification of Prekallikrein. Freshly prepared citrated normal human plasma was the starting material for isolation of prekallikrein. Blood was collected into tubes containing one-sixth volume of acid–citrate–dextrose anticoagulant (ACD; 1 L contains 13.6 g of citric acid, 25 g of sodium citrate, and 20 g of dextrose) and centrifuged at 3000*g* for 20 min at 22 °C, and the plasma obtained was then centrifuged again at 5000*g* for 40 min at 22 °C.

Fresh frozen platelet-poor human plasma that had been stored at −70 °C was also used as starting material for the isolation of prekallikrein. Essentially identical results were obtained.

All purification steps were carried out by using plasticware or siliconized glassware (Siliclad, Clay Adams) at 4 °C except for the initial dialysis of plasma and the first DEAE-Sephadex column which were performed at 22 °C. All buffers contained 1 mM benzamidine hydrochloride, 0.1 mM EDTA, Polybrene at 50 μg/mL, and 0.02% sodium azide. Buffers were prepared fresh for each step. Dialysis tubing and all containers were rinsed with 2 g/L Polybrene solution and then rinsed with H_2O.

Table I: Purification of Human Plasma Prekallikrein

	vol (mL)	total protein (g)	total units of clotting act.	sp clotting act. (units/mg of protein)	recovery (%)	x-fold purifn
plasma	1140	73.0	855	0.012		
DEAE-Sephadex	2600	6.0	364	0.061	43	5
QAE-Sephadex	120	1.62	345	0.21	40	18
SP-Sephadex, pH 8.1	160	0.49	338	0.69	39	58
SP-Sephadex, pH 5.3	260	0.073	114	1.6	13	130
concanavalin A–Sepharose	23	0.0026	48	18.0	5	1500

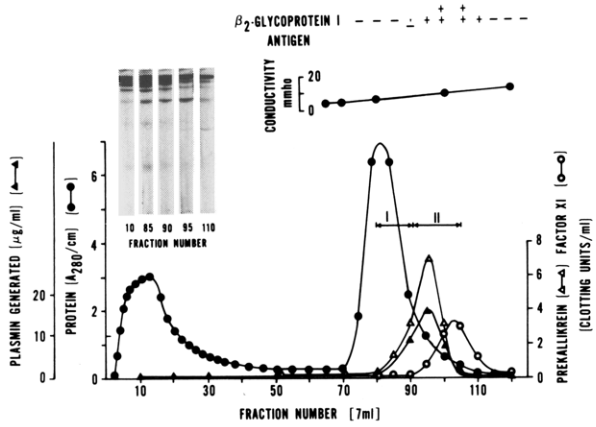

FIGURE 1: Purification of prekallikrein using chromatography on SP-Sephadex at pH 8.1. Elution was effected by using an increasing NaCl gradient as described under Materials and Methods. The insert shows an analysis of several fractions on 7.5% $NaDodSO_4$–polyacrylamide gels in the absence of reducing agents.

The following is a convenient scheme for isolating human prekallikrein from 1.1 L of ACD–plasma (see Table I). Part of the purification procedure is identical with that for the purification of human factor XI (Bouma & Griffin, 1977). Throughout the purification procedure, prekallikrein was detected based on its ability to correct the coagulation deficiency of Fletcher trait plasma (obtained from George King Biomedicals).

Step 1. DEAE-Sephadex Chromatography. Plasma was dialyzed at 22 °C against the starting buffer (40 mM Tris and 10 mM succinic acid, pH 8.34) for chromatography on a 10 × 40 cm column containing 100 g of DEAE-Sephadex A-50 (Pharmacia) as described elsewhere (Bouma & Griffin, 1977, 1978). The protein fraction which did not adhere to the resin contained prekallikrein and factor XI and was used for the next step.

Step 2. QAE-Sephadex Chromatography. A descending pH gradient was used to elute prekallikrein from QAE-Sephadex as described before for factor XI (Bouma & Griffin, 1977). Prekallikrein was assayed in either coagulation or plasminogen proactivator assays. Prekallikrein together with factor XI eluted between pH 9.7 and 9.0 before the bulk of the protein. The fractions containing prekallikrein were pooled for the following step.

Step 3. SP-Sephadex Chromatography. An increasing salt gradient at pH 8.1 was employed to elute prekallikrein from SP-Sephadex C-50 (Pharmacia) by using the same conditions as described for the purification of factor XI (Bouma & Griffin, 1977). As seen in Figure 1, the bulk of the protein did not bind to the column whereas prekallikrein and factor XI were bound. Prekallikrein and plasminogen proactivator activities were eluted between 7 and 10 mmhos conductivity ahead of factor XI which was eluted between 8 and 11 mmhos conductivity (Figure 1). The fractions containing prekallikrein were pooled as indicated in Figure 1. Pool II containing prekallikrein and factor XI was first applied to an SP-Sephadex C-50 column at pH 5.3 at 19 mmhos conductivity as described for the purification of factor XI (Bouma & Griffin, 1977). Prekallikrein and plasminogen proactivator activities did not bind to the resin whereas factor XI activity did adhere. The fractions containing prekallikrein can be combined with pool I from the pH 8.1 SP-Sephadex column or can be worked up separately by using the following final steps.

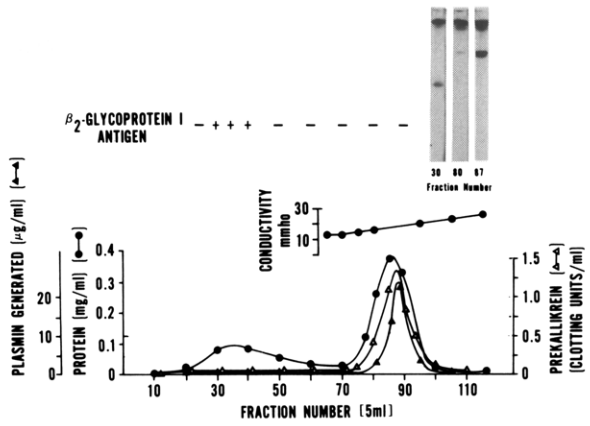

FIGURE 2: Purification of prekallikrein using chromatography on SP-Sephadex at pH 5.3. Elution was effected by using an increasing NaCl gradient as described under Materials and Methods. The insert shows an analysis of several fractions on 7.5% $NaDodSO_4$–polyacrylamide gels in the absence of reducing agents. On the gel of fraction 30, β_2-glycoprotein I is seen in the middle of the gel. Immunological analysis also indicated the presence of this protein in fraction 30 and its absence beyond fraction 60.

Step 4. SP-Sephadex Chromatography. A second SP-Sephadex C-50 column (2.6 × 7.5 cm) involving a salt gradient at pH 5.3 was employed. The resin (2 g) was washed with 2 bed volumes of starting buffer (0.1 M sodium acetate and 0.08 M NaCl, pH 5.3, 14 mmhos conductivity at 22 °C), and the sample, which had been dialyzed for 18 h at 4 °C against the starting buffer, was applied. The column was then washed with 500 mL (10 bed volumes) of starting buffer. This exhaustive wash with starting buffer is essential for the complete elution of β_2-glycoprotein I before the gradient is applied. Gradient elution was effected with 150 mL of starting buffer in the stirred proximal chamber and 150 mL of 0.1 M sodium acetate and 0.28 M NaCl, pH 5.3, in the distal chamber. The flow rate was 25 mL/h, and 5-mL fractions were collected. Prekallikrein and plasminogen proactivator activities were eluted between 15 and 22 mmhos conductivity. Figure 2 shows the results when "pool II" from the SP-Sephadex pH 8.1 column was subjected to this step. This column achieved complete separation of prekallikrein from β_2-glycoprotein I which has previously been frequently observed as a major contaminant of prekallikrein preparations.

Step 5. Concanavalin A–Sepharose Affinity Chromatography. Affinity chromatography on concanavalin A–Sepharose (Pharmacia) at 4 °C was employed as described previously for factor XI (Bouma & Griffin, 1977) to remove IgG from the prekallikrein preparations. Prekallikrein adheres to the concanavalin A–Sepharose, whereas IgG does not. Prekallikrein was eluted with the starting buffer (0.1 M sodium phosphate, pH 7.4, 0.4 M NaCl, 0.02% sodium azide, and 1 mM benzamidine) containing 0.5 M α-D-methyl glucoside (Sigma Chemicals). Polybrene and EDTA were absent from

this buffer. The fractions containing prekallikrein were combined and dialyzed at 4 °C overnight against storage buffer.

Step 6. Sucrose Density Gradients. In some preparations of prekallikrein, traces (i.e., less than 10% of total protein) of high molecular weight protein, presumably γ-globulins, were observed on nonreduced $NaDodSO_4$ gels after the concanavalin A–Sepharose column. These traces were removed by the use of sucrose density gradients (5–20% w/v). Typically, a 0.5-mL sample of the prekallikrein preparation was layered on top of a 10.4-mL sucrose gradient and centrifuged for 16 h at 4 °C at 100000*g* in a Beckman SW 50-1 rotor.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) was carried out either on 7.5% gels according to the general method of Weber et al. (1972) or by using 10% gels according to Laemmli (1970). The gels were stained for protein with Coomassie blue G-250.

Amino Acid Analysis. Fifteen-microgram samples in duplicate from two different preparations were hydrolyzed in vacuo with 6 N HCl at 105 °C for 24, 48, and 72 h and analyzed on a Beckman 121 M amino acid analyzer. Values for threonine, serine, and tyrosine were corrected for destruction during hydrolysis. Values for valine, leucine, and isoleucine were obtained from the 72-h analyses. Half-cystine was determined as cysteic acid following performic acid oxidation (Hirs, 1967). Tryptophan was analyzed in separate alkaline hydrolysates (Hugli & Moore, 1972).

Kinetics of Appearance of Prekallikrein and Plasminogen Proactivator Activities. Experiments reported in Figure 5 were performed in the following manner. The following mixtures were incubated for varying times at 37 °C: 4 μL of Tris buffer (1 M Tris-HCl and 0.15 M NaCl, pH 8.2), 1 μL (0.6 ng) of β-factor XII_a, and 20 μL (8 μg) of prekallikrein. The protein reagents were in storage buffer. At various incubation times, 5 μL of the γ-globulin fraction of goat antihuman factor XII antiserum was added and the mixture was put on ice. This amount of antiserum γ-globulin was shown in control experiments to inhibit completely the ability of β-factor XII_a (0.6 ng) to activate prekallikrein without affecting either the spectrophotometric assay of kallikrein or the plasminogen activator assay described above. Five microliters of this reaction mixture was tested for kallikrein amidolytic activity. Fifteen microliters of the reaction mixture was mixed with 2 μL of plasminogen (2 mg/mL) in a conical centrifuge tube to test for plasminogen activator activity. After 5 s of centrifugation, the sample was incubated for 1 h at 37 °C. Then 15 μL of this reaction mixture was applied to a fibrin plate, and the amount of plasmin generated was assayed as described above. The extent of activation was expressed as the percent of the maximum observed activity of kallikrein and plasminogen activator. In some experiments a 10–20-μL aliquot of the prekallikrein activation reaction mixture was used for $NaDodSO_4$–polyacrylamide gel electrophoresis before addition of the antifactor XII antiserum. In this case, the aliquot was placed into a plastic tube containing 20 μL of 3% $NaDodSO_4$ with or without 3% β-mercaptoethanol and immediately put into a boiling water bath for 5 min.

For the study reported in Figure 7, reaction mixtures were composed of 5 μL of buffer (0.5 M Tris-HCl, pH 8.1), 15 μL of prekallikrein (14 μg), and 10 μL of β-factor XII_a (7 ng). After varying times (0–60 min) at 37 °C, 20 μL was withdrawn for $NaDodSO_4$ gel analysis. Five microliters of goat antihuman factor XII γ-globulin was added to the remaining 10 μL of the reaction mixture. Following mixing and centrifugation of this tube, 5 μL was withdrawn and assayed for kallikrein amidolytic activity. Cleavage of prekallikrein was assessed by analyzing the absorbance profiles of stained $NaDodSO_4$–polyacrylamide gels using a Gilford spectrophotometer equipped with a gel scanning attachment.

For the prekallikrein activation study reported in Figure 8, prekallikrein was labeled with ^{125}I or ^{131}I by using the chloramine-T method (McConahey & Dixon, 1966) or the Bolton–Hunter reagent (Bolton & Hunter, 1973). In other studies not presented here, the insolubilized-lactoperoxidase method was used to radiolabel prekallikrein (David & Reisfeld, 1974). Reaction mixtures contained 10 μL Tris-HCl (0.5 M, pH 8.1), 100 μL of prekallikrein (45 μg), 5 μL of [^{125}I]prekallikrein (Bolton–Hunter labeled) (1 μg, 1 μCi), 5 μL of [^{131}I]prekallikrein (chloramine-T labeled) (1 μg, 1 μCi), and 2 μL of β-factor XII_a (70 ng). After varying times at 22 °C, 8-μL aliquots were withdrawn and subjected to $NaDodSO_4$ gel analysis. At the same time, a 5-μL aliquot was withdrawn and mixed with 5 μL of antifactor XII γ-globulin; then 5 μL of this 10-μL sample was assayed for kallikrein amidolytic activity. Cleavage of radiolabeled prekallikrein was assessed by analyzing the radioactivity profiles of sliced reduced $NaDodSO_4$ gels (1.2-mm slices).

Immunological Reagents. β_2-Glycoprotein I was detected by double immunodiffusion using anti-β_2-glycoprotein I antiserum (Behringwerke, Germany). The antiserum to human factor XII was prepared in goats, and the unadsorbed antiserum gave one precipitation line against normal plasma which showed a reaction of identity with the single line against purified factor XII when tested in immunodiffusion. No precipitation line was observed between the unadsorbed antiserum and factor XII deficient plasmas from two unrelated patients. Antiserum to human prekallikrein was prepared by four successive weekly injections of 75 μg of protein in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) to a year-old goat in multiple subcutaneous sites. Serum obtained from the immunized goat before the initiation of immunization was used as control serum and is referred to as "preserum". This preserum gave no precipitation line against normal human plasma when analyzed by double immunodiffusion.

Immunological Studies. The inhibition of prekallikrein clotting activity in normal human plasma by antiprekallikrein was tested by mixing equal volumes of plasma with either antiprekallikrein antiserum or the preserum. Prior to use, the preserum and the antiserum were heated for 50 min at 56 °C. The mixtures were incubated for 10 min at 37 °C, and after a 10-fold dilution of the mixture in Tris-buffered saline (0.15 M NaCl and 0.01 M Tris-HCl, pH 7.4) containing 1 mg/mL bovine serum albumin, 50-μL aliquots were used in assays of the procoagulant activity of prekallikrein, factor XI, factor XII, and high molecular weight kininogen. A 100-μL aliquot was used for prothrombin time assays. The prothrombin time assay was performed in triplicate by using rabbit brain thromboplastin (Ortho Diagnostics) according to the manufacturer's instructions.

Inhibition of Kallikrein and Plasminogen Activator Activity by Antiprekallikrein Antibodies. For these experiments, the γ-globulin fractions of the goat preserum and the antiprekallikrein antiserum were prepared by using DEAE-Sephadex A-50 chromatography as described above for the first step in the purification scheme. The starting buffer (40 mM Tris and 10 mM succinic acid, pH 8.34) was slightly modified by using 0.08 M NaCl which gave a conductivity of 11 mmhos. The γ-globulin fractions were then applied to concanavalin A–Sepharose columns as described above in order to remove any goat prekallikrein or factor XI. The portion of each γ-globulin

fraction which did not adhere to the resin was concentrated by precipitation with 50% (v/v) $(NH_4)_2SO_4$. The precipitates were dissolved in 0.01 M sodium phosphate, 0.4 M NaCl, and 0.02% sodium azide, pH 7.4, and were dialyzed against the same buffer overnight at 4 °C. The γ-globulin fractions of the preserum and the antiprekallikrein antiserum were adjusted to the same protein concentration of 10 mg/mL. The simultaneous inhibition of kallikrein activity and plasminogen activator activity was tested by incubating 10 μL (0.5 μg) of prekallikrein (pool II) or 10 μL (1.4 μg) of prekallikrein (pool I) with 20 μL (200 μg) of antiprekallikrein γ-globulin or dilutions thereof for 10 min at 37 °C. Dilutions of antiprekallikrein γ-globulin were made with the γ-globulin fraction of the preserum. Then 2 μL (12 ng) of β-factor XII_a was added. After incubation of this mixture for 10 min at 37 °C, a 10-μL aliquot was used for assaying the kallikrein amidolytic activity. The remaining 22 μL was mixed with 2 μL of plasminogen (2 mg/mL) and incubated for 1 h at 37 °C. The total reaction mixture was then applied to a fibrin plate. For each type of assay, the activity observed using only the γ-globulin fraction of the preserum was defined as 100%.

Kinetics of Inhibition of Prekallikrein by DFP. The following mixtures were incubated in polypropylene tubes for 1 h at 37 °C: 10 μL of phosphate buffer (1 M sodium phosphate, pH 7.4, and 0.5 M NaCl), 10 μL of a 50 mg per mL solution of bovine serum albumin in the same buffer, 5 μL of 30 mM EDTA in the same buffer, 20 μL (18 μg) of unlabeled prekallikrein, and 10 μL (0.25 μCi, 7 μg) of [^{125}I]prekallikrein. This 1-h preincubation was to allow any nonspecific adsorptive loss of prekallikrein to the test-tube wall to reach equilibrium. Then 80 μL of DFP (Calbiochem) solution (0, 8.4, 25.7, or 75 mM in deionized water) was added, and the reaction mixtures were further incubated at 37 °C. At various times, 10-μL aliquots were removed, diluted into 1 mL of Tris-buffered saline (0.01 M Tris-HCl, pH 7.4, and 0.15 M NaCl) containing 1 mg/mL bovine serum albumin, and assayed for prekallikrein procoagulant activity. Each clotting tube was counted for ^{125}I to confirm that the same amount of prekallikrein had been added to each clotting assay. Standard curves using dilutions of pooled normal plasma were determined both at the beginning and at the end of the experiment in order to verify that the reagents used in the clotting assay did not deteriorate over the time span of the experiment. Further control experiments were performed to show that the diluted DFP itself did not affect the clotting assays. In these controls, it was shown also that normal plasma diluted in the presence of DFP at concentrations equivalent to those in the reaction mixtures yielded standard curves similar to those obtained for plasma in the absence of DFP. In other controls, the single chain structure of prekallikrein at the beginning and at the end of the experiments was verified by analyzing the radioactivity profiles of 10-μL aliquots of [^{125}I]prekallikrein on $NaDodSO_4$–polyacrylamide gels under reducing conditions.

Kinetics of Inhibition of Kallikrein by DFP. A series of the following mixtures was incubated in polypropylene tubes at 37 °C: 10 μL of phosphate buffer (0.1 M sodium phosphate, pH 7.4, and 0.1 M NaCl) containing 0.2 mg/mL bovine serum albumin, 5 μL (0.6 μg) of kallikrein or 5 μL (1.2 μg) of trypsin (Worthington bovine trypsin-TPCK), and 10 μL of DFP solution (1.25 mM in normal saline). At various times each reaction mixture was diluted with 0.5 mL of tripeptide substrate S-2302 (H-D-Pro-Phe-Arg-*p*-nitroanilide, Kabi) (0.1 mg/mL in 0.1 M Tris-HCl, pH 8.0, and 0.05 M NaCl containing 1 mg/mL bovine serum albumin) and assayed spectrophotometrically for amidolytic activity at 22 °C. Loss of amidolytic activity as a function of time for both enzymes was linear on a semilog plot, with ~25% of the initial activity remaining after 5 min.

Results

Preparation of Human Plasma Prekallikrein. The purification steps and the yield for a typical preparation of human prekallikrein are shown in Table I. The first DEAE-Sephadex column is used to separate prekallikrein and factor XI in the γ-globulin fraction from the bulk of plasma proteins. On this column at pH 8.3 at 2 mmhos conductivity, prekallikrein and factor XI did not adhere to the resin. In the following two steps, the QAE-Sephadex column and the SP-Sephadex column at pH 8.1 partially separated prekallikrein from factor XI and other γ-globulins. The insert in Figure 1 shows an $NaDodSO_4$–polyacrylamide gel analysis of several fractions. In fraction 85, prekallikrein appeared as a single band, whereas in fractions eluting later, two protein bands were observed near the molecular weight of prekallikren. In the $NaDodSO_4$ gel of fraction 10 a protein band was observed with an apparent molecular weight very similar to that of prekallikrein. Subsequent analysis of these fractions in double immunodiffusion using the antiprekallikrein antiserum showed no precipitation line, whereas a single precipitation line was obtained with fractions 80–105. As indicated in Figure 1, the fractions were divided into two pools, designated pool I and pool II. Pool II, containing prekallikrein and factor XI, was first applied to an SP-Sephadex C-50 column at pH 5.3 to separate prekallikrein and plasminogen proactivator from factor XI. The factor XI from this step can be further purified as described before (Bouma & Griffin, 1977). The fractions containing prekallikrein can be combined with pool I of the SP-Sephadex pH 8.1 column or can be worked up separately. In either case, the purification procedure is the same; however, the final prekallikrein preparations obtained are different. Prekallikrein derived from pool II shows two protein bands of equal density on $NaDodSO_4$–polyacrylamide gels in the absence of reducing agents, whereas prekallikrein from pool I has one major band (vide infra). On the SP-Sephadex column at pH 5.3, a complete separation of prekallikrein from β_2-glycoprotein I was obtained since the latter protein did not adhere to the resin while prekallikrein did (see insert of Figure 2). The $NaDodSO_4$–polyacrylamide gels in the insert of Figure 2 show that the prekallikrein which eluted early again appeared as a single band, whereas prekallikrein eluting later showed two bands. The prekallikrein preparations were subjected to affinity chromatography on concanavalin A–Sepharose for the removal of IgG. After this step, traces of some high molecular weight proteins were still present and these were removed by using sucrose density gradient ultracentrifugation. An overall purification of 1500-fold was observed. The final solution of prekallikrein contained no measurable factor XI (less than 0.01 unit/mL). On the basis of the observed specific clotting activity of 18 units/mg, the concentration of prekallikrein in normal human plasma is inferred to be 55 μg/mL.

The factor XII dependent plasminogen proactivator cochromatographed with prekallikrein throughout the entire purification procedure.

Polyacrylamide Gel Electrophoresis of Purified Prekallikrein. Two protein bands (Figure 3) were observed on $NaDodSO_4$–polyacrylamide gels in the absence of reducing agents corresponding to apparent molecular weights of 82 000 and 78 000 when prekallikrein derived from pool I material was used. Prekallikrein derived from pool II gave one major band with an apparent molecular weight of 82 000 and a second faint band with an apparent molecular weight of 78 000. After

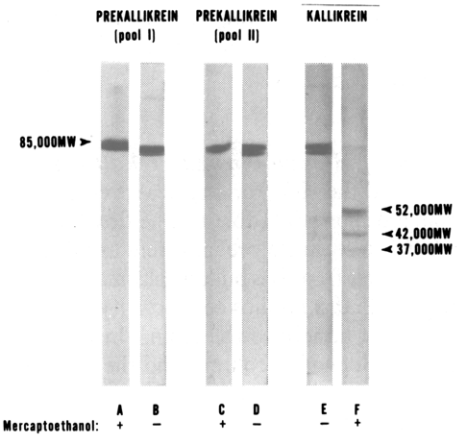

FIGURE 3: Human prekallikrein and kallikrein on 7.5% NaDodSO$_4$–polyacrylamide gels. Gels A, C, and F are run in the presence of reducing agent and gels B, D, and E in the absence. Gels A and B each contain 5 μg of prekallikrein derived from pool I. Gels C and D each contain 5 μg of prekallikrein derived from pool II. Gels E and F each contain 3 μg of kallikrein. Kallikrein was generated by incubation of prekallikrein with β-factor XII$_a$ for 60 min (see Figure 5). For details, see Materials and Methods.

Table II: Amino Acid Composition of Human Plasma Prekallikrein

amino acid	residues/100 residues
lysine	6.49 ± 0.23
histidine	2.45 ± 0.09
arginine	4.01 ± 0.20
aspartic acid	8.40 ± 0.18
threonine	7.77 ± 0.31
serine	9.44 ± 0.43
glutamic acid	10.51 ± 0.32
proline	4.32 ± 0.23
glycine	9.79 ± 0.29
alanine	4.23 ± 0.11
cysteine	4.32 ± 0.05
valine	5.89 ± 0.29
methionine	1.16 ± 0.014
isoleucine	4.55 ± 0.14
leucine	7.13 ± 0.17
tyrosine	3.15 ± 0.23
phenylalanine	3.95 ± 0.22
tryptophan	2.44 ± 0.24

reduction, both prekallikrein preparations gave an apparent single protein band with an apparent molecular weight of 85 000. Thus, human prekallikrein appears to be a single polypeptide chain for which two slightly different forms may exist. The exact chemical or structural differences which give rise to two similar forms remain to be defined.

Amino Acid Composition of Prekallikrein. The amino acid composition of prekallikrein is shown in Table II.

Immunologic Studies. Immunodiffusion experiments using an unabsorbed antiprekallikrein antiserum revealed a single precipitation line against normal plasma, and this line showed a reaction of identity with the single line obtained against purified prekallikrein. No line was observed against prekallikrein-deficient (Fletcher) plasma (Figure 4). The plasma from this patient had no prekallikrein antigen as tested separately by rocket immunoelectrophoresis using the same antiserum.[2] The precipitation lines obtained with the two different prekallikrein preparations showed a reaction of identity with each other and with normal human plasma.

[2] B. N. Bouma, D. M. Kerbiriou, R. A. A. Vlooswijk, and J. H. Griffin, unpublished experiments.

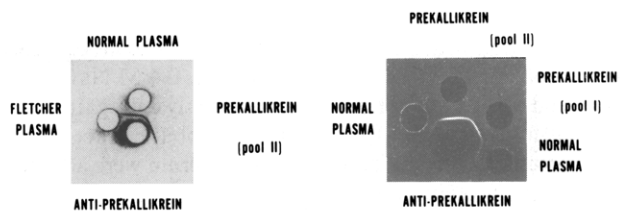

FIGURE 4: Double immunodiffusion of prekallikrein and antiprekallikrein. The wells contained 40 μL of normal plasma and Fletcher plasma (prekallikrein deficient). 30 μL of antiprekallikrein, 10 μL of prekallikrein derived from pool II (7 units/mL), and 20 μL of prekallikrein derived from pool I (1.7 units/mL). The plate on the left was washed and stained.

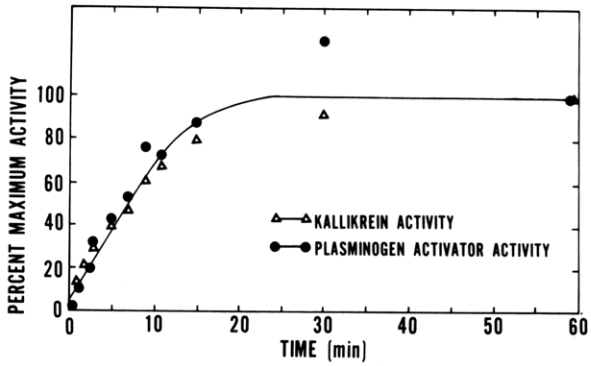

FIGURE 5: Activation of prekallikrein by β-factor XII$_a$. The appearance of kallikrein activity (△) and plasminogen activator activity (●) is expressed as the percent of the maximum activity.

Table III: Characterization of the Specificity of Antiprekallikrein Antiserum on Coagulation Factors of the Intrinsic Pathway[a]

	clotting act. (%)
prekallikrein	<1[b]
factor XI	108
factor XII	100
high molecular weight kininogen	95

[a] Equal volumes of normal plasma and antiprekallikrein antiserum or preserum were incubated for 10 min at 37 °C. The clotting activities were determined after a 10-fold dilution of this mixture into Tris-buffered saline (0.15 M NaCl and 0.01 M Tris-HCl, pH 7.4) containing 1 mg/mL bovine serum albumin. The percent of the remaining clotting activity was determined by comparing the activity observed in the mixtures treated with antiprekallikrein antiserum to the activity observed in the control mixtures which contained preserum. [b] No prekallikrein procoagulant activity was detected under conditions where 1% clotting activity would be measurable.

The antiprekallikrein antiserum inhibited the prekallikrein clotting activity of normal plasma (Table III) as measured in kaolin-activated partial thromboplastin time assays but had no effect on the clotting activity on factor XI, factor XII, or high molecular weight kininogen. The inhibiting effect of antiprekallikrein on the prekallikrein clotting activity was detectable at up to a 100-fold dilution of the antiserum. The antiprekallikrein antiserum did not affect the prothrombin time of normal plasma with an observed clotting time of 15.9 s vs. 17.1 s in the control experiment using preserum. These data suggest that the anticoagulant activity of the antiserum is specific for prekallikrein.

Simultaneous Generation of Kallikrein and Plasminogen Activator Activities by β-Factor XII$_a$. The kinetics of activation of prekallikrein by the M_r 28 000 form of purified human factor XII$_a$ was studied as described under Materials and Methods. As seen in Figure 5, the time course of activation of prekallikrein is identical with that of plasminogen

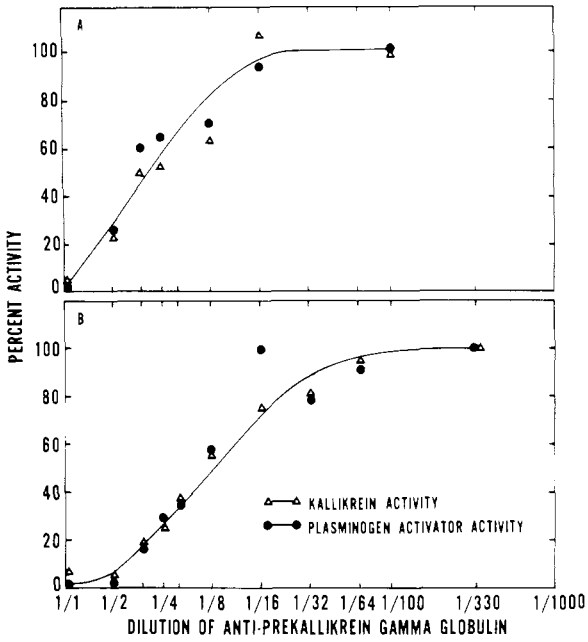

FIGURE 6: Inhibition of kallikrein and plasminogen activator activity by antiprekallikrein antiserum. The remaining kallikrein (△) and plasminogen activator (●) activity is expressed as the percent of the maximal activity observed for controls in which the γ-globulin fraction of the preserum alone was used. (Upper panel) Inhibition profile of prekallikrein derived from pool I. (Lower panel) Inhibition profile of prekallikrein derived from pool II.

proactivator, and both preparations of prekallikrein gave this result. Analysis on $NaDodSO_4$ gels in the presence of reducing agents showed that both prekallikrein bands were cleaved to give three fragments with apparent molecular weights of 52000, 42000, and 37000 (Figure 3). In the absence of reducing agents, the activated kallikrein gave two protein bands with apparent molecular weight values identical with those seen for the two prekallikrein protein bands. This indicates that the prekallikrein is proteolytically cleaved during activation by β-factor XII_a (vide infra) and that the resultant polypeptide fragments of kallikrein are linked by disulfide bonds.

Simultaneous Inhibition of Kallikrein and Plasminogen Activator Activities by Antiprekallikrein Antibodies. The ability of the monospecific antiprekallikrein γ-globulin to inhibit both kallikrein and plasminogen activator activities was studied as described under Materials and Methods in order to test whether prekallikrein and plasminogen proactivator activities arose from two different molecules or from the same molecule. Incubation of prekallikrein derived from pool I (Figure 6, upper graph) or from pool II (Figure 6, lower graph) with the antiprekallikrein resulted in simultaneous and parallel inhibition of kallikrein and plasminogen activator activities. Figure 6 shows the results obtained when the antiserum was added before the activation by β-factor XII_a. Similar results were obtained when the antiserum was added after the activation by β-factor XII_a. These results suggest that the same protein, kallikrein, is responsible for both the kallikrein amidolytic activity and the plasminogen activator activity.

Comparison of Plasminogen Activating Activities of Kallikrein and Urokinase. The relative specific activities of kallikrein and urokinase as plasminogen activators were determined. Glu-plasminogen at a final concentration of 8.5 μM was incubated for 5 min at 37 °C with kallikrein (0.21–0.84 μM) or with urokinase (1.5–7.7 nM), and the amount of plasmin formed was quantitated by using the tripeptide substrate S-2251. Control reaction mixtures containing kallikrein hydrolyzed the substrate, S-2251, and corrections were made

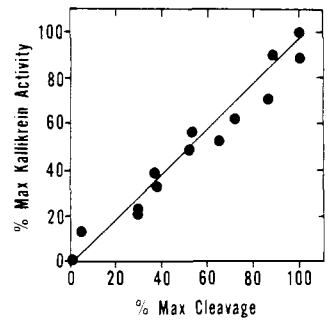

FIGURE 7: Activation of prekallikrein by β-factor XII_a correlated with limited proteolysis. The reaction conditions are described under Materials and Methods. The 100% values were the maximal values observed. Cleavage was determined from analysis of absorbance profiles of scanned stained 7.5% $NaDodSO_4$ gels. Control experiments in which β-factor XII_a was replaced by buffer and incubated for 60 min showed no cleavage of prekallikrein and no kallikrein activity.

for this background activity. Kallikrein generated 0.010 ± 0.003 mol of plasmin per mol of kallikrein per min, and urokinase produced 16.5 ± 1.2 mol of plasmin per mol of urokinase per min. Thus, under these conditions, urokinase appears to be 1650 times more potent on a molar basis than kallikrein in the activation of plasminogen. It is estimated that kallikrein as a plasminogen activator presents on the order of 40 CTA units/mg. In control experiments to assess the effect of autodigestion of plasmin during incubation at 37 °C, plasmin was dialyzed vs. 0.05 M Tris-HCl, pH 7.8, 0.1 M lysine, 0.1 M NaCl, and 0.02% sodium azide. The plasmin was incubated at 37 °C, and samples were withdrawn at various times and assayed by using the tripeptide substrate. Two separate control experiments showed an average drop of 20% in the concentration of active plasmin in 5 min. This effect is not enough to affect the approximate estimate that urokinase is 1650 times more potent than kallikrein under the conditions employed.

Limited Proteolysis as the Mechanism of Activation of Prekallikrein by β-Factor XII_a. Prekallikrein was mixed with β-factor XII_a, and aliquots of the reaction mixture were analyzed at various times for the appearance of kallikrein amidolytic activity and for cleavage on stained reduced $NaDodSO_4$ gels. Cleavage of prekallikrein was determined from absorbance profiles of scanned stained $NaDodSO_4$ gels. The data in Figure 7 indicate that cleavage of the prekallikrein single chain molecule by β-factor XII_a to give disulfide-linked heavy and light chains was directly associated with the appearance of kallikrein activity.

In contrast to the excellent correlation seen in Figure 7 between limited proteolysis and the appearance of kallikrein activity, it was initially and repeatedly found that when β-factor XII_a dependent cleavage was determined by analyzing the $NaDodSO_4$ gel profiles of radiolabeled prekallikrein, the appearance of kallikrein activity preceded the observed cleavage of radiolabeled prekallikrein. Therefore, radiolabeling of prekallikrein was performed by using several different techniques in order to see whether this anomalous result was due to alterations of prekallikrein associated with the labeling process. A reaction mixture containing excess unlabeled prekallikrein and trace amounts of [^{131}I]prekallikrein that was labeled by using the chloramine-T method and of [^{125}I]prekallikrein that was labeled by using the Bolton–Hunter reagent was activated by addition of β-factor XII_a. At various times aliquots were analyzed for kallikrein activity and for cleavage on reduced $NaDodSO_4$ gels. The results shown in Figure 8 indicate that the appearance of kallikrein amidolytic activity representing the bulk of the unlabeled molecules is directly

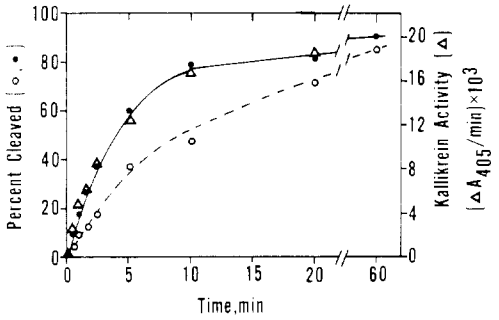

FIGURE 8: Kinetics of β-factor XII_a dependent activation and cleavage of radiolabeled prekallikreins. [^{131}I]Prekallikrein was labeled by using the chloramine-T method (○) and [^{125}I]prekallikrein by using the Bolton–Hunter reagent (●). Kallikrein amidolytic activity was due to activation of the large excess of unlabeled prekallikrein molecules. See Materials and Methods for experimental details.

correlated with the cleavage of [^{125}I]prekallikrein but that the initial rate of cleavage of the [^{131}I]prekallikrein is 50% lower. Radiolabeling prekallikrein using the insolubilized-lactoperoxidase method also gave a molecule that was cleaved more slowly by β-factor XII_a than was the native molecule. Thus, it must be concluded that the method of radiolabeling prekallikrein may alter the functional behavior of the molecule in a subtle but significant manner.

Inactivation of Prekallikrein and Kallikrein by DFP. The kinetics of the inactivation of the procoagulant activity of prekallikrein by various concentrations of DFP at 37 °C is shown in Figure 9. These semilog plots were linear and dependent on the concentration of DFP. From the slopes of the lines in Figure 9, a second-order rate constant of 0.38 M^{-1} min^{-1} is calculated for the reaction of DFP with prekallikrein. In separate experiments the time course of DFP hydrolysis in the buffer employed for these experiments was determined.[3] Appropriate correction of the data in Figure 9 yielded a corrected second-order rate constant of 0.59 M^{-1} min^{-1} for the inactivation of prekallikrein zymogen by DFP. Similar studies, as described under Materials and Methods, of the inhibition by DFP of the active enzymes kallikrein and trypsin yielded second-order rate constants of 500 and 730 M^{-1} min^{-1}, respectively, for these enzymes.

Discussion

Highly purified human prekallikrein is isolated in its precursor form and consists of two very similar forms with similar apparent molecular weights. Both forms have a single polypeptide chain near M_r 85 000. Human prekallikrein purification as described here results in a 1500-fold purification from plasma. Previous reports described a 1700–3500-fold purification (Gallimore et al., 1978; Laake & Vennerod, 1974). It is difficult to compare further our prekallikrein with that of Laake & Vennerod (1974) or Gallimore et al. (1978) since no polyacrylamide gel patterns were shown. The human prekallikrein of Mandle & Kaplan (1977) was shown to be highly purified on $NaDodSO_4$–polyacrylamide gels and to contain two forms of the molecule similar to data reported here, but no quantitative data were presented to describe the purification procedure or the procoagulant and plasminogen activator specific activities. Bovine (Takahashi et al., 1972a; Heimark et al., 1979) and rabbit (Wuepper & Cochrane, 1972; Johnston et al., 1976) plasma prekallikreins have been highly purified, and the bovine prekallikrein exhibited two very similar forms of the molecule as seen for human prekallikrein (Heimark et al., 1979).

[3] J. H. Griffin and G. Beretta, unpublished experiments.

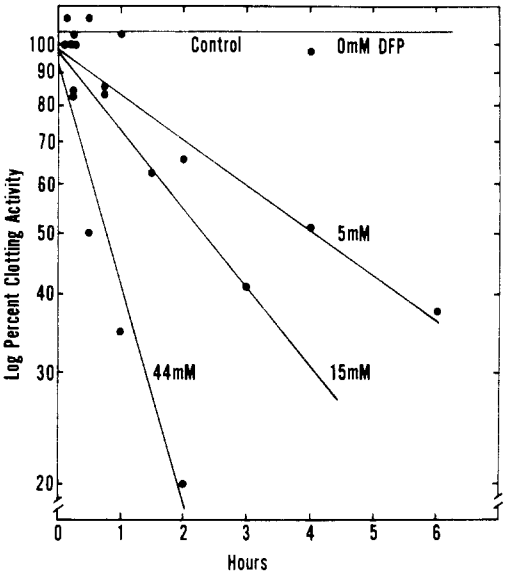

FIGURE 9: Kinetics of inactivation of prekallikrein procoagulant activity by DFP. Prekallikrein was incubated with various concentrations of DFP and then assayed at various times for residual procoagulant activity.

That the two bands seen on the $NaDodSO_4$–polyacrylamide gels both represent prekallikrein is based on the following observations. After activation by β-factor XII_a, both protein bands are cleaved, yielding several fragments, which are held together by disulfide bonds. A goat antiserum raised against purified prekallikrein gave a single line in double immunodiffusion against human plasma and showed a reaction of identity with the single precipitation line of purified prekallikrein. The single precipitation line obtained with two prekallikrein preparations which differed in the ratio of the two proteins also showed a reaction of identity. No precipitation line was observed when the unabsorbed antiserum was tested against Fletcher trait plasma which was deficient in prekallikrein.

The major factor XII dependent plasminogen proactivator present in the γ-globulin fractions of normal human plasma was initially reported to be distinct from prekallikrein. This was based on the observation that this plasminogen proactivator was present in normal amounts in prekallikrein deficient plasma and that $C\bar{1}$ inhibitor inactivated kallikrein but not the plasminogen activator (Kaplan et al., 1972, 1973). However, more recent studies have shown an apparent absence of detectable factor XII dependent plasminogen proactivator activity in the γ-globulin fraction of prekallikrein-deficient plasma (Vennerod & Laake, 1976; Bouma & Griffin, 1978), although this point has been disputed (Mandle & Kaplan, 1977). It is now apparent that $C\bar{1}$ inhibitor does inhibit kallikrein activity as well as factor XII dependent plasminogen activator activity (Laake & Vennerod, 1974; Mandle & Kaplan, 1977). Yet previous studies did not rule out the possibility that prekallikrein preparations were contaminated with trace levels of plasminogen proactivator molecules. The experiments described in this paper suggest that prekallikrein and the major factor XII dependent plasminogen proactivator found in the γ-globulin fraction of plasma are identical. The rate of activation of prekallikrein and of plasminogen proactivator by β-factor XII_a appears to be identical regardless of the ratio of the two similar protein bands in the prekallikrein preparations. Also, a monospecific antiprekallikrein antiserum inhibited the zymogen form of prekallikrein and plasminogen proactivator activities as well as the activated form of each

activity in a simultaneous and parallel manner when different dilutions of antibody were used to inhibit the respective enzymatic activities. Consequently, it is concluded that prekallikrein and plasminogen proactivator activities reside in the same molecule. As a plasminogen activator, kallikrein exhibited ~40 CTA units/mg. This can be compared to estimates of 7.1 and 0.47 CTA unit/mg as calculated from data of other investigators (Laake & Vennerod, 1974; Gallimore et al., 1978). Radcliffe & Heinze (1978) reported that bovine kallikrein displays 21 CTA units/mg. It should be noted that Mandle & Kaplan (1977) have suggested that blood coagulation factor XI is a factor XII dependent plasminogen proactivator. This possibility will require further studies.

Activation of prekallikrein by β-factor XII_a as reported here is directly associated with limited proteolysis producing heavy and light chains linked by disulfide bonds. Mandle & Kaplan (1977) reported a similar polypeptide structure for kallikrein. The polypeptide structures of bovine (Takahaski et al., 1972b; Heimark et al., 1978) and rabbit (Wuepper & Cochrane, 1972; Johnston et al., 1976) prekallikrein and kallikrein are similar to those of the human molecule. Initially it was suggested that the activation of rabbit prekallikrein is associated with the release of an M_r 11 000 fragment (Wuepper & Cochrane, 1972); however, recent studies show that this is not the case and that this fragment appears long after activation of prekallikrein has occurred (Johnston et al., 1976). Mandle & Kaplan (1977) reported kinetic studies of the activation of prekallikrein by β-factor XII_a showing that the appearance of kallikrein activity preceded the observed limited cleavage of [^{125}I]prekallikrein. This result was probably due to the fact that prekallikrein radiolabeled by using the chloramine-T method is cleaved more slowly by β-factor XII_a than is unlabeled prekallikrein or prekallikrein that is radiolabeled by using the Bolton–Hunter reagent.

The intrinsic blood coagulation pathway is initiated by exposure of plasma to an activating surface, e.g., glass, kaolin, connective tissue preparations, and a variety of negatively charged materials. Factor XII is central to contact activation. A significant role of human prekallikrein in the intrinsic pathway of coagulation became evident after the discovery of plasmas deficient in prekallikrein (Fletcher trait plasma) that had an abnormality in the intrinsic pathway (Hathaway et al., 1965; Hathaway & Alsever, 1970; Wuepper, 1973). It has been suggested that normal contact activation involves reciprocal proteolytic activation of prekallikrein and surface-bound factor XII (Cochrane et al., 1973; Griffin & Cochrane, 1976a; Meier et al., 1977; Griffin, 1978). The trigger of this reciprocal proteolysis has not yet been defined. It has been shown that trypsinogen exhibits weak but detectable activity against substrates and pseudosubstrates (Morgan et al., 1972; Kassell & Kay, 1973; Gertler et al., 1974; Lonsdale-Eccles et al., 1978). The second-order rate constant for the inactivation of prekallikrein by DFP is higher than that reported for trypsinogen (Morgan et al., 1972) and is ~10^3 lower than that for kallikrein. The DFP reactive site in prekallikrein has not been established in these studies. Nonetheless, intrinsic enzymatic activity of the prekallikrein or factor XII zymogens could provide the trigger for the surface-dependent initiation of the intrinsic coagulation pathway (Griffin, 1977, 1978; Griffin & Beretta, 1979). Further experiments will be required to assess the intriguing hypothesis that negatively charged surfaces convert factor XII into a highly susceptible substrate (Griffin, 1978) for the intrinsic activity of the prekallikrein zymogen.

An adequate definition of the precise physiological and pathophysiological roles for plasma prekallikrein is lacking at this time. It is particularly enigmatic that patients deficient in plasma prekallikrein present asymptomatically in spite of the profound in vitro abnormalities in contact activation reactions. Less than a score of patients have been identified, and there may well emerge in the future correlations of pathologic abnormalities with prekallikrein deficiency. Kallikrein is a relatively weak plasminogen activator when compared to urokinase. Nonetheless, urokinase is found in urine while kallikrein is found in blood. Hence, prekallikrein is the most potent—although apparently weak—plasminogen proactivator that is a normal constituent of the plasma. The plasminogen activator activity of kallikrein may possibly be enhanced by mechanisms involving cofactors, cells, or physiological surfaces that have not yet been described. Thus, further studies will be necessary before the physiological importance of kallikrein as a plasminogen activator can be adequately defined.

Acknowledgments

The support and advice of Dr. Charles G. Cochrane are gratefully acknowledged. The cheerful and skillful technical assistance of Kevin Bickford and Alice J. Kleiss is most appreciated.

References

Amudsen, E., Svendsen, L., Vennerod, A. M., & Laake, K. (1974) *DHEW Publ. (NIH) (U.S.) NIH 76-791.*

Bolton, A. E., & Hunter, W. M. (1973) *Biochem. J. 133*, 529–539.

Bouma, B. N., & Griffin, J. H. (1977) *J. Biol. Chem. 252*, 6432–6437.

Bouma, B. N., & Griffin, J. H. (1978) *J. Lab. Clin. Med. 91*, 148–155.

Chase, T., Jr., & Shaw, E. (1970) *Methods Enzymol. 19*, 20.

Cochrane, C. G., & Revak, S. D. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 1271.

Cochrane, C. G., Revak, S. D., & Wuepper, K. D. (1973) *J. Exp. Med. 138*, 1564–1583.

Colman, R. W., & Bagdasarian, A. (1977) *Methods Enzymol. 45*, 303–322.

David, G. S., & Reisfeld, R. A. (1974) *Biochemistry 13*, 1014–1019.

Deutsch, D. G., & Mertz, E. T. (1970) *Science 170*, 1095.

Donaldson, V. H., Kleniewski, I., Saito, H., & Sayed, I. K. (1977) *J. Clin. Invest. 60*, 571–583.

Erdos, E. G. (1966) *Adv. Pharmacol. 4*, 1.

Gallimore, M. J., Fareid, E., & Stormorken, H. (1978) *Thromb. Res. 12*, 409–420.

Gertler, A., Walsh, K. A., & Neurath, N. (1974) *Biochemistry 13*, 1302–1310.

Griffin, J. H. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 329.

Griffin, J. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1998–2002.

Griffin, J. H., & Cochrane, C. G. (1976a) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2554–2558.

Griffin, J. H., & Cochrane, C. G. (1976b) *Methods Enzymol. 45*, 56–65.

Griffin, J. H., & Beretta, G. (1979) in *Kinins II* (Suzuki, T., & Moriya, H., Eds.) pp 39–51, Plenum Press, New York.

Hathaway, W. E., & Alsever, T. (1970) *Br. J. Haematol. 10*, 161–169.

Hathaway, W. E., Belhasen, L. P., & Hathaway, N. S. (1965) *Blood 26*, 521–532.

Heimark, R. L., Fujikawa, K., & Davie, E. W. (1979) *Biochemistry 18*, 5743–5750.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 197–199.
Hugli, T. E., & Moore, S. (1972) *J. Biol. Chem. 247*, 2828–2834.
Iatridis, S. G., & Ferguson, J. M. (1961) *Thromb. Diath. Haemorrh. 6*, 411.
Johnston, A. R., Ulevitch, R. J., & Ryan, K. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 693.
Kaplan, A. P., & Austen, K. F. (1970) *J. Immunol. 105*, 802–811.
Kaplan, A. P., Kay, A. B., & Austen, K. F. (1972) *J. Exp. Med. 135*, 81–97.
Kaplan, A. P., Goetzl, E. J., & Austen, K. F. (1973) *J. Clin. Invest. 52*, 2591–2595.
Kassell, B., & Kay, J. (1973) *Science 180*, 1022–1027.
Kraut, H., Frey, E. K., & Bauer, E. (1928) *Hoppe-Seyler's Z. Physiol. Chem. 175*, 97.
Laake, K., & Vennerod, A. M. (1974) *Thromb. Res. 4*, 285–302.
Laemmli, L. K. (1970) *Nature (London) 227*, 680.
Lonsdale-Eccles, J. D., Neurath, H., & Walsh, K. A. (1978) *Biochemistry 17*, 2805–2809.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mandle, R., Jr., & Kaplan, A. P. (1977) *J. Biol. Chem. 252*, 6097–6104.
Mandle, R., Jr., Colman, R. W., & Kaplan, A. P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4179–4183.
McConahey, P. J., & Dixon, F. J. (1966) *Int. Arch. Allergy Appl. Immunol. 29*, 185–189.
Meier, H. L., Pierce, J. V., Colman, R. W., & Kaplan, A. P. (1977) *J. Clin. Invest. 60*, 18–31.
Morgan, P. H., Robinson, N. C., Walsh, K. A., & Neurath, H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3312–3316.
Niewiarowski, S., & Prou-Wartell, O. (1959) *Thromb. Diath. Haemorrh. 3*, 593–603.
Radcliffe, R., & Heinze, T. (1978) *Arch. Biochem. Biophys. 189*, 185–194.
Ratnoff, O. D., & Davie, E. W. (1962) *Biochemistry 1*, 967.
Revak, S. D., Cochrane, C. G., & Griffin, J. H. (1977) *J. Clin. Invest. 59*, 1167–1175.
Revak, S. D., Bouma, B. N., Cochrane, C. G., & Griffin, J. H. (1978) *J. Exp. Med. 147*, 719–729.
Robbins, K. C., & Summaria, L. (1970) *Methods Enzymol. 19*, 184.
Saito, H., Ratnoff, O. D., & Donaldson, V. H. (1974) *Circ. Res. 34*, 641–651.
Schoenmakers, J. G. G., Kurstjens, R. M., Haanen, C., & Zilliken, T. (1963) *Thromb. Diath. Haemorrh. 9*, 546.
Takahashi, H., Nagasawa, S., & Suzuki, T. (1972a) *J. Biochem. (Tokyo) 71*, 471.
Takahashi, N., Nagasawa, S., & Suzuki, T. (1972b) *FEBS Lett. 24*, 98–100.
Thompson, R. E., Mandle, R., Jr., & Kaplan, A. P. (1977) *J. Clin. Invest. 60*, 1376–1380.
Vennerod, A. M., & Laake, K. (1976) *Thromb. Res. 8*, 519–522.
Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26*, 3–27.
Weiss, A. S., Gallin, J. I., & Kaplan, A. P. (1974) *J. Clin. Invest. 53*, 622–633.
Wendel, U., Vogt, W., & Seidel, G. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 1591–1606.
Wiggins, R. C., Bouma, B. N., Cochrane, C. G., & Griffin, J. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4636–4640.
Wiman, B., & Wallen, P. (1973) *Eur. J. Biochem. 36*, 25.
Wuepper, K. D. (1973) *J. Exp. Med. 138*, 1345–1355.
Wuepper, K. D., & Cochrane, C. G. (1972) *J. Exp. Med. 135*, 1–20.