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Two Distinct Pathways of the Streptokinase-Mediated Activation of Highly Purified Human Plasminogen†

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ABSTRACT: Two human plasminogens (Pg I and Pg II) were isolated in high yield and in pure form from pooled human plasma fraction $III_{2,3}$ by affinity column adsorption and elution on lysine-agarose beads followed by carboxymethylcellulose ion exchange chromatography, 4°, pH 5.0. These plasminogens both have molecular weights of about 85,000 and glutamic acid as their amino acid N-terminals. Following activation to plasmins, by streptokinase, active site equivalent weights equal to determined molecular weights were obtained. Proteolytic activities of both plasmins were about 40 CTA units/mg of protein. Plasminogen activation by catalytic streptokinase (SK) or urokinase (UK) as well as with stoichiometric quantities of streptokinase was investigated by stopping the reactions at the desired times with the active site titrant *p*-nitrophenyl-*p'*-guanidinobenzoate (NphOGdnBz). The reaction mixtures were reduced in sodium dodecyl sulfate (SDS) and the reaction products separated and quantitatively analyzed by SDS acrylamide gel electrophoresis. Native plasminogens Pg I and Pg II are single polypeptide chains with molecular weights of about 85,000. Conversion to plasmin (P) using either catalytic streptokinase or urokinase results in the cleavage of two characteristic peptide bonds yielding plasmin molecules containing three peptide chains ($P_{60,20,5}$), the order of cleavage being exclusively $Pg_{85} \rightarrow Pg_{80,5} \rightarrow P^a_{60,20,5}$ (where superscript a denotes the presence of a *p*-nitrophenyl-*p'*-guanidinobenzoate titratable active site and the subscripts indicate the approximate molecular weight in thousands of peptide chains involved). Interaction of Pg with stoichiometric amounts of streptokinase (SK) leads to the plasminogen activator complex $SK_{37,6,4} \cdot P^a_{60,20,5}$. Bond cleavages of Pg within this complex occur entirely by the reactions $Pg^a_{85} \rightarrow Pg^a_{65,20} \rightarrow P^a_{60,20,5}$. This order is the reverse of that found in catalytic activations. The SK peptide bond splits within the complex occur in the order $SK_{47} \rightarrow SK_{43,4} \rightarrow SK_{37,6,4}$. End group studies of isolated peptides from the NphOGdnBz treated $SK_{37,6,4} \cdot P^a_{60,20,5}$ complex showed the peptide orders (N)-SK_6-SK_{37}-SK_4-(C) for SK_{47} and (N)-P_5-P_{60}-P_{20}-(C) for Pg_{85} (the (N) and (C) designating the NH_2 and COOH termini, respectively).

Plasminogen (Pg)[1] activation by streptokinase (SK)[2] because of its practical importance in medicine and its unique nature has been the subject of many investigations. It is generally accepted that SK combines directly with either plasmin (P) or Pg to form a stoichiometric complex (Pg activator) capable of rapid conversion of Pg to P. A summary of data leading to these concepts can be found in a recent review by Amery and Claeys (1970). However, the direct role played by SK in the conversion of the active center of P, with little or no Pg activator activity, into an efficient enzyme for this action is not well understood.

In previous communications (McClintock and Bell, 1971a,b) we demonstrated that human plasminogen is able to combine directly with streptokinase in a 1:1 molar ratio to yield a complex. This complex then undergoes a time and temperature dependent unimolecular reaction which generates an active site. The results showed clearly that the for-

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[1] Unless otherwise stated, the terms Pg and P refer to plasminogens and plasmins of human origin.

[2] Abbreviations used are: ACTH, β-corticotropin; CM, carboxymethyl; CTA, Committee on Thrombolytic Agents, National Heart Institute; Dip-F, diisopropyl phosphorofluoridate; Dns-Cl, 1-dimethylaminonaphthalene-5-sulfonyl chloride; EACA, ε-aminocaproic acid; HSEtOH, 2-mercaptoethanol; Nph, *p*-nitrophenol; NphOGdnBz, *p*-nitrophenyl-*p'*-guanidinobenzoate; SDS, sodium dodecyl sulfate; SBTI, soybean trypsin inhibitor; SK, streptokinase; UK, urokinase.

mation of this active site does not require prior cleavage of the plasminogen molecule into the two characteristic polypeptide plasmin chains as described by Robbins *et al.* (1967). Reddy and Markus (1972) have also independently arrived at the conclusion that active site appears prior to peptide bond cleavage in this moiety. The appearance of active site on the SK · Pg complex was interpreted as being due to an SK induced conformational rearrangement in the Pg moiety which makes available the proteolytic potential of the zymogen. That this was indeed the case was demonstrated by the fact that if *p*-nitrophenyl-*p'*-guanidinobenzoate (NphOGdnBz) was not present, the addition of SK to Pg resulted in the rapid disappearance of both intact proteins with the concomitant appearance of a number of split products which included the characteristic polypeptide chains of plasmin. In order to obtain a better understanding of the mechanism of SK mediated activation of Pg a more complete study of this process was undertaken. The results of these studies, along with the brief description of improved methods of preparation of human plasminogens, are reported in this paper.

Experimental Section

Materials

Streptokinase (SK) was prepared as described by De Renzo *et al.* (1967) and stored as the lyophilized powder. The two preparations used assayed 110,000 and 113,000 units/mg of protein, respectively (SK units as described by Christensen, 1947).

Porcine calcitonin was the PC-2 described by Bell *et al.* (1970). The β-corticotropin was that reported by Shepherd *et al.* (1956). Human serum albumin was prepared by the method of Taylor *et al.* (1956).

Ribonuclease A was purchased from Pharmacia Fine Chemicals Co., Piscataway, N.J.

A sample of high purity urokinase (UK) was the generous gift of Dr. G. Barlow, Abbott Laboratories, North Chicago, Ill. Using a molecular weight of 53,000 this sample was estimated by the NphOGdnBz burst assay to be 31% by weight active UK protein (UK is a trypsin-like protease and reacts stoichiometrically with NphOGdnBz in the active site assay described below).

The soybean trypsin inhibitor (SBTI) was purchased from Worthington Biochemicals Corporation, Freehold, N.J. (Crystalline, Code SI, Lot 91A) and used without further purification. This material had a weight average molecular weight of 18,000 by equilibrium centrifugation, appeared to be homogeneous in reduced SDS gel electrophoresis, but gave a molecular weight of 15,000 in the reduced SDS acrylamide gel system when compared to other proteins.[3]

The ε-aminocaproic acid (EACA) was obtained from Eastman Kodak Co.

Other chemicals not mentioned specifically in the Methods section were of reagent grade.

Plasminogen Assays. Plasminogen was in all cases assayed as plasmin following activation with streptokinase (SK). Activation was achieved by reaction at 25° for 15 min at pH 7–8 using a Pg/SK molar ratio of approximately 100:1 (*ca.* 550 SK units/mg of high purity Pg). A concentration of 0.05–0.1 M in lysine was maintained in all activation solutions to assure adequate solubility of the SK · P activator complex.

Proteolytic activities of the resultant plasmins were determined by the hydrolysis of azocasein prepared by the method of Charney and Tomarelli (1947). Reagents and conditions of activation were as described by Hummel *et al.* (1965). The assay design followed the caseinolytic method recommended by the National Heart Institute Committee on Thrombolytic Agents (CTA) as reported by Johnson *et al.* (1969). Comparisons with the primary plasmin standard supplied by this committee made it possible to express these data in terms of CTA units.

The spontaneous plasmin in plasminogen preparations was estimated directly by either the azocasein or active site titration methods by the elimination of the SK activation step in the plasminogen assays.

Active site titrations of plasmins were carried out by the procedure of Chase and Shaw (1969). The *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride (NphOGdnBz) reagent for these assays was obtained from Cyclo Chemical Company, Los Angeles, Calif. Improved precision in this procedure was achieved by the use of a single tandem mix cell (Tandem Mix Cell No. 238 with Teflon stopper, supplied by Hellma Cells, Inc., Jamaica, N.Y. 11424). Equal volumes of the plasmin solution and the NphOGdnBz substrate in the buffer were placed in the two compartments of the cell, pre- and postburst substrate blank hydrolysis rates being established by the use of a Gilford Model 2000 recording spectrophotometer to monitor the change in absorption at 410 nm. The quantity of plasminogen introduced into the assay, which is required to calculate the active site equivalent weight, was determined from the absorbance at 280 nm using the $E_{cm}(1\%)$ of 17.0 (Robbins *et al.*, 1965). These "burst" data, along with an independently determined molecular weight on the same preparation, were used to estimate purity (*i.e.*, moles active site/mole of protein).

Rapid and sensitive assays of plasmin and streptokinase–plasmin activator complexes were also made by the use of the substrate α-*N*-methyl-α-*N*-tosyl-L-lysine β-naphthol ester as supplied by Nutritional Biochemicals Corporation of Cleveland, Ohio, and the fluorescent procedure of Bell *et al.* (1974).

[3] The molecular weight estimates of SBTI and the P_{20} peptide of this report rest largely on the acceptance of the gel mobility of ribonuclease A used in the standard curve of Figure 5. Ribonuclease was used for this standardization since it had been reported by Weber and Osborn (1969) to respond normally in the 10% acrylamide reduced SDS gel electrophoresis system. It has come to our attention that the observed molecular weight discrepancy between SBTI and RNase may be due to an anomalous response of the RNase [Dunker and Ruechert (1969), using the 10% acrylamide gel system, obtained an apparent molecular weight for RNase which was 21% greater than expected]. If the SBTI gel mobility data, along with its molecular weight of 20,100 [calculated from the structure reported by Koide *et al.* (1972)] is accepted for the standardization curve, the apparent molecular weight of peptide P_{20} could be as high as 25,000, while the apparent molecular weights of the larger peptides would be essentially unchanged. On the basis of disc gel electrophoresis studies in SDS Walther *et al.* (1974) report a light chain plasmin peptide molecular weight of 26,000. Summaria *et al.* (1967) isolated a "highly purified" S-carboxymethyl light chain plasmin peptide which gave a molecular weight of 25,700 by sedimentation equilibrium analysis. This value is probably high since these same authors, in a subsequent paper, state that this light chain derivative has "a strong tendency to aggregate" in the solvent used in their molecular weight studies (Groskopf *et al.*, 1969). Although the molecular weights of P_{20} and the light chain plasmin peptides of Robbins *et al.* (1972) and Walther *et al.* (1974) are not known precisely, it is our belief that these peptides are probably identical since they all are reported to have the same Val-Val N-terminal sequence.

Methods

Plasminogen Purification. Since the mechanism studies of this report were concerned with the kinetics of bond cleavages during the activation of Pg by SK it was essential that both proteins be homogeneous, fully functional, and free of split products or internal bond cleavages. Modifications of affinity and column chromatographic methods, to minimize autoactivation during processing, made it possible to isolate two human plasminogens, Pg I and Pg II, which upon activation gave active site equivalent weights equal to their determined molecular weights, were homogeneous by all physical methods and free of bond breaks due to autodigestion. The source of human plasminogen for these isolations was in every case *fresh, unfrozen* fraction $III_{2,3}$ prepared from *large pools* of human plasma using method 9 of Oncley *et al.* (1949). Initial isolation from this fraction was achieved by the affinity chromatography procedure of Deutsch and Mertz (1970), modified so that it could be operated at 4°. These modifications included use of a citrate salt buffer (9.5 g of NaCl, 4.85 g of citric acid monohydrate, 0.87 g of EDTA, and solid KOH to pH 7.4 and diluted to 1 l.) to resuspend the fraction $III_{2,3}$ (30 g of wet paste/l. of buffer); all phosphate buffers were as potassium salts in order to maintain solubility at 4° and the Pg was eluted from the affinity column with 0.3 M (K^+) phosphate buffer (pH 7.4) containing 0.2 M ϵ-aminocaproic acid (EACA). Recovery of the eluted Pg was achieved by precipitation with ammonium sulfate from the cold solution (310 g of solid $(NH_4)_2SO_4$/l.). The precipitated plasminogen was immediately reconstituted at a concentration of up to 10 optical density units (280 nm) in a suitable storage buffer. For this purpose 0.05 M ammonium acetate–0.02 M lysine buffer (pH 5.0) was routinely used. Plasminogens stored in this buffer at −75° have been completely stable for many months. If complete removal of EACA was desired a second $(NH_4)_2SO_4$ precipitation step was used.

Further purification of the plasminogens prepared by the affinity chromatography procedure described above was achieved by the application of the carboxymethylcellulose column method suggested by Wallén (1962). For these studies the Whatman preswelled CM52 Chromedia microgranular CM-cellulose proved to be the preparation of choice (H. Reeve Angel and Company, Clifton, N.J.). The ion exchange capacity of this CM-cellulose (1.0 mequiv/g) along with its high affinity for plasminogen, at low cation concentration ($R^+ < 0.04$ M), made it suitable for quantitative recovery of Pg from dilute solutions, as well as for column fractionation.

Quantitative recoveries of plasminogens I and II from these chromatography experiments were achieved by (a) dilution of pools with distilled water to a conductance of 7 millimho or less, (b) adsorption of the plasminogen by stirring for 10 min with freshly regenerated (acid form CM52) carboxymethylcellulose [1 g dry or 4 g damp will pick up *ca.* 35–60 mg of PgI and/or Pg II], (c) wash with water on filter and elute with 0.6 M ammonium acetate (pH 5.0), (d) recover by ammonium sulfate precipitation as described for affinity chromatography, (e) store in the frozen state (−75°) in 0.05 M ammonium acetate–0.02 M lysine buffer (pH 5.0) at a concentration of 10 OD_{280} nm/ml.

Starch Gel Electrophoresis. The vertical starch block method of Smithies (1959) as modified by Barg *et al.* (1965) was used to follow the isolation of Pg I and Pg II. The 0.03 M sodium acetate–0.02 M ϵ-aminocaproic acid (EACA) (pH 4.0) buffer described by Wallen (1962) was used for these studies. 6-hr electrophoresis runs at 4° using an applied voltage of 6 V/cm gave satisfactory resolution of the plasminogen bands.

Acrylamide Gel Electrophoresis. A high resolution sliced gel block procedure (Bell *et al.*, 1974) which reduced the effects of thermal distortion on resolution, was used for all acrylamide gel studies. Acrylamide (for electrophoresis), methylenebisacrylamide, and *N,N,N′,N′*-tetramethylenediamine were obtained from Eastman Kodak Company, Rochester, N.Y. Coomassie Brilliant Blue was obtained from Colab Laboratories Inc., Glenwood, Ill.

Molecular Weight Studies. Ultracentrifuge experiments were carried out with a Spinco Model E ultracentrifuge equipped with a phase plate and temperature control unit. Sedimentation velocity runs at 52,640 rpm were performed in double sector cells at three concentrations. Appropriate dialysates were used in the solvent compartment of the cell to establish the buffer base lines. Sedimentation equilibrium runs at 8225 rpm were either with conventional column lengths (3–5 mm in the cell) or the short column method of Yphantis (1960). Plasminogen samples for these studies were prepared by the following steps: (1) dialysis, 4°, 24 hr, *vs.* 0.2 M EACA adjusted to pH 3.1 with HCl, (2) dialysis, 4°, 24 hr, *vs.* 0.001 N HCl, and (3) lyophilization. These desalted samples were then dissolved in 0.1 M NaCl–0.001 N HCl (pH 3.2) for ultracentrifuge studies.

Molecular weight comparisons and homogeneity studies by the SDS acrylamide gel electrophoresis method were made using a sliced gel block method. Buffers, gel concentrations, conditions of reduction, staining and destaining conditions differed from those described by Weber and Osborn (1969) only in that the methylenebisacrylamide was reduced to a final concentration of 0.15% in the gel. 16-hr runs at 25°, using a current density of 24 mA/cm^2, was sufficient to resolve proteins and peptides in the molecular weight range of 5,000–100,000. Plasminogen II, human serum albumin, streptokinase, ribonuclease, β-corticotropin, and porcine calcitonin, following reduction with mercaptoethanol (HSEtOH), were used to standardize this system.

Amino Acid and Amide Analysis. Plasminogen samples were prepared for amino acid by dialysis *vs.* 0.1 M EACA to displace any free or adsorbed lysine, followed by a second exhaustive dialysis *vs.* 0.001 M HCl. Hydrolysates were prepared by the method of Crestfield *et al.* (1963) using 17–18 hr at 110°. The Technicon amino acid analyzer was used for quantitative analysis following the general procedure of Spackman *et al.* (1958). Hydrolysis corrections of 5% for threonine and 10% for serine as suggested by Moore and Stein (1963) were applied. Amide content was estimated from the ammonia analysis of the acid hydrolysates. No corrections for ammonia generation from amino acids during acid hydrolysis were applied. The total half-cystine content was determined as *S*-aminoethylcysteine following acid hydrolysis of aminoethylated preparations prepared by the procedure of Raftery and Cole (1963). Tryptophan was determined on separate samples by the method of Spies and Chambers (1949).

End Group Studies. The N-terminal amino acid groups of plasminogen preparations were qualitatively identified by the dansylation method described by Gray (1967) and as modified by Gros and Labouesse (1969). The dansyl amino acids were identified by thin-layer chromatography on polyamide sheets (supplied by Brinkmann Instruments Inc., Westbury, N.Y.) using the solvent systems described by

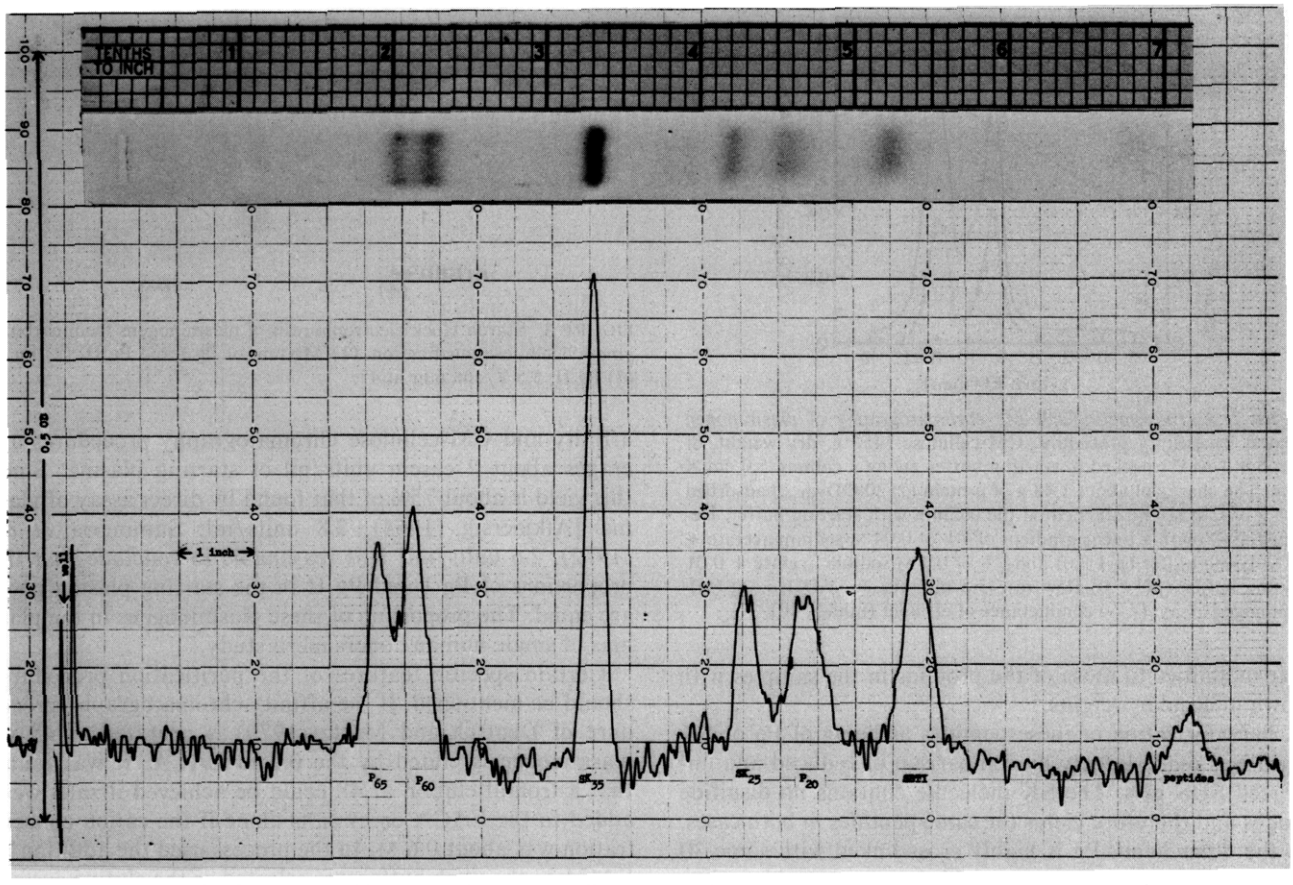


FIGURE 1: Representative SDS acrylamide gel and the corresponding quantitative scan. The sample was a stoichiometric SK · Pg reaction mixture (0.84 mg/ml of Pg I, 0.57 mg/ml of SK at pH 7.4 in 0.05 M phosphate, and 0.1 M lysine) stopped after 25 min at 25° with NphOGdnBz. Each sample was treated with one-tenth its volume of SDS-HSEtOH and 75 μl placed into the gel well.

Woods and Wang (1967). Confirmation of identity of an unknown spot was made by comparison with knowns placed adjacent to the unknown using a third solvent run in the second dimension. For this purpose the solvent consisting of chloroform–amyl alcohol–glacial acetic acid (70:30:3) as recommended by Morse and Horecker (1966) proved most useful. Identification of N-terminal glutamic acid by the dansyl method was confirmed by the application of one stage of the phenylthiohydantoin method as described by Blombäck *et al.* (1966). Peptides for end group studies were isolated from preparative sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis blocks. The desired activation mixtures were treated with excess NphOGdnBz, reduced with 2% mercaptoethanol in 1% SDS, and separated using the block procedure described for the analytical studies. The separated peptides were located by removing and staining a longitudinal gel slice. Recovery of peptides from the unstained portion of the gels was achieved by the procedure of Weiner *et al.* (1972). The identity of the recovered peptides was confirmed by the analytical SDS–acrylamide gel method.

Kinetic Activation Studies. In these experiments the time aliquot, usually 0.25 ml, was removed from the activation reaction and added to a tube containing 5μl of an NphOGdnBz solution (0.01 M, dissolved in dimethylformamide) to stop the enzymatic activation reactions. A 0.2-ml portion of this "stopped" solution was then transferred to a second tube containing a precise amount of internal standard SBTI solution (25 μl of a 5 or 10 mg/ml solution of SBTI protein). Each time aliquot then received 0.1 of the sample volume of a concentrated SDS–HSEtOH solution (10% SDS and 20% HSEtOH in 0.5 M, pH 7.0, phosphate buffer). After reduction overnight at 37° in a closed tube a 75-μl portion was then charged into the wells of the SDS acrylamide gel electrophoresis blocks. Following electrophoresis, slicing, and destaining the amount of each component was estimated by use of a flat-bed linear transport scanner (Bell *et al.*, 1974).

The quantitation of the scanned dyed protein spots was achieved by planimetry of the base line corrected peaks from the recorder charts. It was assumed that each Coomassie-stained protein and peptide in this study adsorbs dye in proportion to the amount of that particular protein present in the stained spot. Fishbein (1972) has shown that several proteins exhibit this proportionality. For the studies reported below, however, the emphasis is on the kinetics of appearance and disappearance of the various molecular species. These results may be deduced from the dye scans without a knowledge of specific dye binding information for the component under consideration. Variations in the procedure due to slicing, degree of staining, and sample sizes were largely overcome by introduction of precise amounts of SBTI into each of the time aliquots immediately after the activation reaction was stopped by NphOGdnBz. All band areas were then normalized by comparison to the response of this SBTI internal standard. A typical gel and the corresponding scan are presented in Figure 1.

In addition to following the appearance and disappearance of protein bands, the molecular weights of the various species were estimated directly from the gels by comparing

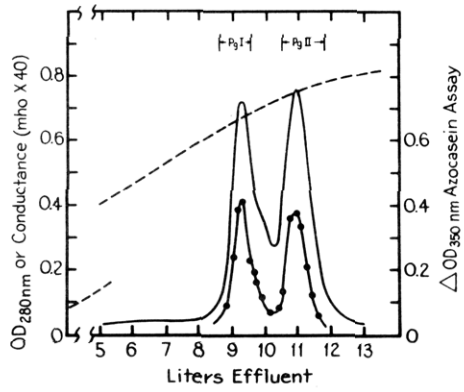


FIGURE 2: Carboxymethylcellulose chromatography of plasminogen prepared by affinity procedure. CM-cellulose (450 g, dry weight, of Whatman CM52) packed in starting buffer giving a column 5.0 cm × 70 cm. The charge of about 1.48 g of protein (2530 OD_{280nm}) adsorbed on 15 g of CM52 and slurried on the column with starting buffer. Developed at 4° with a linear gradient of 7 l. of 0.04 M sodium acetate + 0.01 M lysine buffer (pH 5.0) and 7 l. of 0.4 M sodium acetate + 0.01 M lysine buffer (pH 5.0) flow rate 3.5 ml/min. (—) $OD_{280\,nm}$; (●) plasminogen assay; (- - -) conductance of effluent (mho × 40).

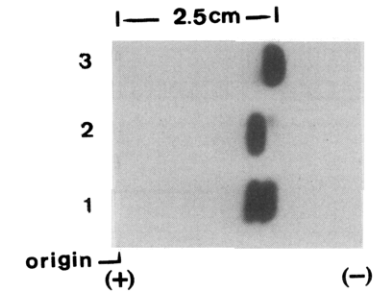


FIGURE 3: Starch block electrophoresis of plasminogens from carboxymethylcellulose purification. (1) Mixture of Pg I and Pg II, (2) Pg I, (3) Pg II, 5.5 V/cm, 6 hr at 4°.

their mobilities to those of the proteins in the samples with known molecular weights.[4]

Characterization of these peptides as being of Pg or SK origin was made largely by comparison of reduced and unreduced SDS gels. The SK molecule contains no disulfide bridges and therefore yields the same peptides in both cases. On the other hand, Pg is highly cross-linked with some 30 disulfide bridges and does not yield the same peptides when reduced and unreduced.

Results

Plasminogen Purification and Characterization. Purified plasminogen was isolated from fraction $III_{2,3}$ in good yield using the cold room affinity chromatography procedure. An average of 529 mg of protein was obtained/100 g of fraction $III_{2,3}$ paste (equivalent to 8 l. of plasma) which gave no detectable plasmin activity. This plasminogen could be clearly resolved into two major components by pH 4.0 starch block electrophoresis. Reduced SDS acrylamide gel electrophoresis showed a single protein band having a mobility corresponding to a molecular weight of about 85,000. Upon activation to plasmin with SK, this material contained 24–36 CTA units/mg and gave an NphOGdnBz active site equivalent weight of 105,000–140,000. Therefore, on the basis of a molecular weight of 85,000 for plasminogen, this material represented 60–80% pure activatable plasminogen.

The two major components obtained by affinity chromatography were resolved and further purified by CM-cellulose chromatography as shown in Figure 2. The plasminogens have been designated as Pg I and Pg II. The complete resolution of these two proteins is clearly demonstrated by the starch block electrophoresis study shown in Figure 3. These studies clearly show the presence of two human plasminogens (Pg I and Pg II), present in approximately equal amounts in fraction $III_{2,3}$ from large pools of human plasma. The overall yield of Pg I and Pg II isolated by these 4° affinity and CM-cellulose chromatography procedures averages about 2 casein units/ml of starting plasma. Since this yield is about 75% of that found by direct assay of plasma [Alkjaersig (1964), 2.8 units/ml; Summaria *et al.* (1972), 2.6 units/ml] it is reasonable to conclude that the proportions of Pg I and Pg II in the starting plasma pools are equal. The proportion of these plasminogens in the plasmas of single human donors merit study.

Certain specific features of the purification procedures should be mentioned. If the affinity chromatography procedure of Deutsch and Mertz (1970) is operated at 4° no plasminogen is eluted by the 0.2 M EACA. It was found that a frontal elution at 4° could be achieved if salts were added to the EACA or by salts alone if the cation concentration was about 0.6 M. In the process used the addition of EACA to the wash buffer was selected as the eluting system only as a matter of convenience. Lysine agarose beads have anionic properties which make it possible for them to function in much the same manner as the CM-cellulose in the second column step. In a human plasminogen isolation by lysine–agarose chromatography Brockway and Castellino (1972) demonstrated the separation of two plasminogens by a gradient elution with EACA. It would seem likely that this method of elution could be used to separate human Pg I and Pg II in a one-step procedure. In the CM-cellulose procedure of Figure 2 purification was achieved without any evidence of inactive material being separated. The impurities in the plasminogens prepared by the 4° affinity chromatography procedure are composed of about 10% with low affinity for CM-cellulose and 10–20% with very strong binding properties. Selective pickup of the charge by CM-cellulose removed essentially all of the low affinity impurities. The high binding impurities were not removed from the column under the conditions which completely eluted the plasminogens.

The CM-cellulose column procedure, as described, cannot be expected to completely separate performed plasmin of the charge from Pg I. When plasminogen prepared by the DEAE-Sephadex method of Robbins *et al.* (1965), containing about 5% plasmin, was applied to a CM-cellulose column the Pg I and Pg II peaks were resolved; however, the plasmin of the charge was found by assay to be only slightly ahead of and not completely resolved from the Pg I peak. The instability of Pg I to dialysis at pH 7.4, seen in the molecular weight studies described below, may have been due to a trace of plasmin not removed by the CM-cellulose column.

Active site titrations of the preparations of Pg I and Pg II (isolated by the affinity CM-cellulose column methods) recovered from the experiment of Figure 2 gave site equivalent weights of 85,000 and 83,000. Subsequent preparations

[4] In the figures and discussion, subscript number designations are used to indicate the approximate molecular weight, in thousands, of the peptide chain or chains in the molecular species under consideration. A superscript *a* denotes the presence of an active enzyme center in this species.

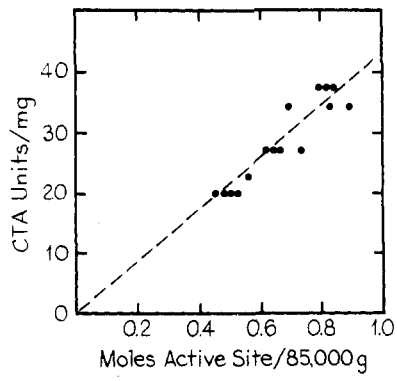


FIGURE 4: Plasminogen activities by azocasein and active site titrations. Azocasein comparisons were derived from 10 or more dilutions covering the linear response ranges of both unknown and the standard. Individual active site data for each plasmin are shown.

using these methods gave site equivalent weights all in the 85,000 range. Proteolytic activity comparisons of these pure plasminogens were not made against the CTA standard. However, assay comparisons by azocasein and active site methods of five plasminogens covering a range of purities are shown in Figure 4. These data suggest that the proteolytic activities of plasmins derived from pure Pg I and Pg II must be about 40 CTA/mg of Pg protein. Plasminogens I and II after activation with streptokinase gave equivalent assay responses in the α-*N*-methyl-α-*N*-tosyl-L-lysine β-naphthol ester fluorescence assay at a substrate concentration of 1×10^{-4} M, 25°, pH 7.0; hydrolysis rates of 6.3×10^{-8} mol/min per mg of Pg I and 6.0×10^{-8} mol/min per mg of Pg II were obtained.

Sedimentation velocity and equilibrium ultracentrifuge studies of Pg I and Pg II were carried out in 0.1 M phosphate + 0.1 M EACA (pH 7.4) and in 0.1 M NaCl + 0.001 M HCl (pH 3.2) buffers. The plasminogens were dialyzed *vs.* these buffers for 24 hr at 4° prior to examination. Sedimentation equilibrium studies showed that Pg I, pH 3.2; Pg II, pH 7.4; and Pg II, pH 3.2, were essentially homogeneous. However, Pg I when dialyzed *vs.* pH 7.4 buffer showed about 10% of lower molecular weight material, indicative of proteolytic action during dialysis. Using a partial specific volume of 0.71 (Davies and Englert, 1960 and Barlow *et al.*, (1969) the weight average molecular weights calculated from equilibrium centrifugation data were: Pg I (pH 3.2) = 77,200; Pg II (pH 3.2) = 79,500; Pg II (pH 7.4) = 83,600. Sedimentation velocity studies in the concentration range of 0.2–0.5% gave linear regression lines of the form $s_{20,w}^0 + bC$. Where C is expressed as g/100 ml of plasminogen Pg I (pH 3.2) $s_{20,w}^0 = 5.4$, $b = -1.3$; Pg II (pH 3.2) $s_{20,w}^0 = 5.3$, $b = -1.8$; Pg II (pH 7.4) $s_{20,w}^0 = 5.1$, $b = -2.6$.

Molecular weight estimates for undialyzed preparations of reduced Pg I and Pg II by the pH 7.4 SDS acrylamide gel electrophoresis method of Weber and Osborn (1969) gave values of about 85,000 for both materials. Extended runs comparing Pg I, Pg II, and a mixture of Pg I and Pg II in this reduced SDS acrylamide gel electrophoresis system failed to show molecular weight differences between the two plasminogens. Standardization of this molecular weight method was made using the sliced gel block method which gave the data shown in Figure 5.[3] All data of this figure were obtained on one block except those shown for β-corticotropin (ACTH) and porcine calcitonin-2 (PC). The mobilities of these standards were normalized to the standard block by comparisons with SK_{47} and ribonuclease (RNase) in the second block.

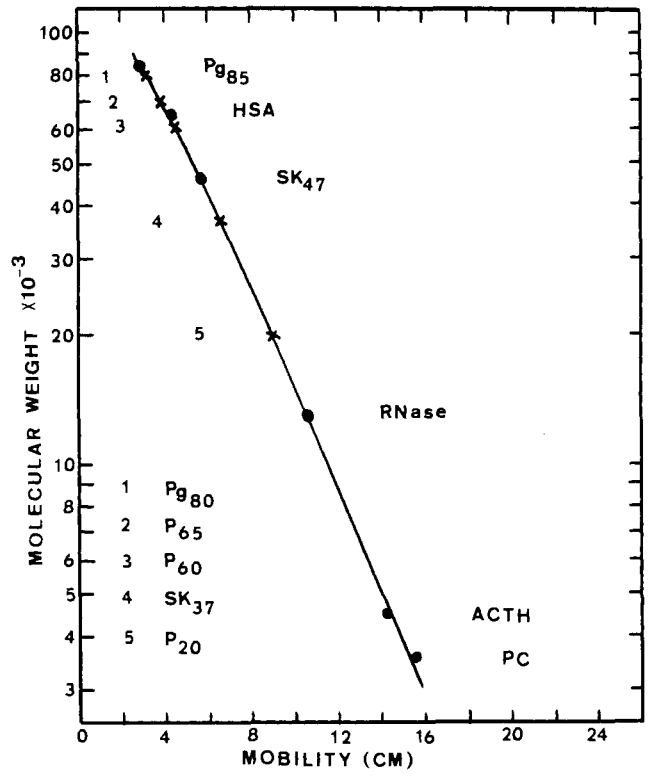


FIGURE 5: Acrylamide gel electrophoresis molecular weight standardization. (●) Standards; PC, porcine calcitonin; ACTH, β-corticotropin; RNase, ribonuclease; SK_{47}, streptokinase; HSA, human serum albumin; Pg_{85}, Pg II whose molecular weight was determined to be 85,000 by centrifuge methods. (X) Products of Pg activation by SK.

Amino acid composition data as given in Table I do not show significant differences between Pg I and Pg II. Glutamic acid was the only N-terminal amino acid residue found for Pg I and Pg II by the dansylation and phenylthiohydantoin methods. No trace of glutamine was observed in the latter method.

The C-terminal studies with Dip-F treated carboxypeptidases A and B were inconclusive for both Pg I and Pg II, as well as their reduced and carboxymethylated, reduced and aminoethylated, citraconylated, and performic acid oxidized forms. These enzymes released amino acids slowly from these preparations; however, substantial amounts of lysine and arginine were among those released. A study of reduced enzyme digests and the corresponding enzyme controls in the SDS acrylamide gel electrophoresis system showed the generation of several lower molecular weight components in the 3- and 24 hr digests. Appearance of these components was indicative of sufficient tryptic activity in the Dip-F treated enzymes to invalidate the amino acid release data.

Isoelectric focusing studies of NphOGdnBz-treated Pg II and Pg I + Pg II in the presence of 5 M urea, and carried out by the method of Summaria *et al.* (1972) also indicated that the total net charges of the two Pg's were very similar. (NphOGdnBz treatment was used to inactivate any traces of plasmin present which might have generated isoelectric forms.) Both Pg II and the mixture of Pg I + Pg II focused in a narrow band with all the protein being found in the pH 6.4–6.7 region.

On to the basis of N-terminal data, Pg I and/or Pg II could correspond to the plasminogens isolated by Rickli and

TABLE I: Amino Acid Composition of Pg I and Pg II.

Amino Acid	μmol/mg[a]		Residues/85,000 g		Residues/mole
	Pg I	Pg II	Pg I	Pg II	Pg I and Pg II
Aspartic acid	0.855	0.844	72.7	71.8	72
Threonine	0.760	0.759	64.7	64.6	65
Serine	0.660	0.653	56.2	55.6	56
Glutamic acid	1.05	1.05	88.2	88.2	88
Proline	0.643	0.635	54.6	54.0	54
Glycine	0.687	0.695	58.4	59.2	59
Alanine	0.446	0.433	37.9	36.8	37
Valine	0.492	0.468	41.7	39.8	41
1/2-Cystine[b]	0.468	0.473	39.8	40.4	40
Methionine	0.114	0.108	9.7	9.2	9 or 10
Isoleucine	0.213	0.210	18.1	17.9	18
Leucine	0.480	0.482	40.8	41.0	41
Tyrosine	0.330	0.337	28.1	28.6	28
Phenylalanine	0.206	0.221	17.5	18.8	18
Ammonia	0.810	0.783	68.9	66.6	66–69
Lysine	0.527	0.544	44.8	46.4	45 or 46
Histidine	0.266	0.262	22.6	22.3	22
Arginine	0.469	0.473	39.9	40.2	40
Tryptophan[c]	0.252	0.268	21.4	22.8	21 or 22

[a] Average of two analyses. [b] Determined as aminoethylcysteine. [c] Determined by method of Spies and Chambers (1949).

Cuendet (1971) and the plasminogen A of Wallén and Wiman (1972). These data clearly indicate that neither Pg I nor Pg II are the same as the N-terminal lysine plasminogen isolated and extensively studied by Robbins *et al.* (1965, 1972) or plasminogen B of Wallén and Wiman (1972). The isoelectric points of Pg I and Pg II fall in the range reported by Wallén and Wiman (1972) and do not contain the multiple forms with p*I* values in the 7.2–8.3 range reported by Summaria *et al.* (1972). The proteolytic activities of plasmins generated from Pg I and Pg II appear to be 30–40% greater than any so far reported in the literature. Active site equivalent weight studies have not been reported; therefore comparisons by this more reliable method are not possible at this time.

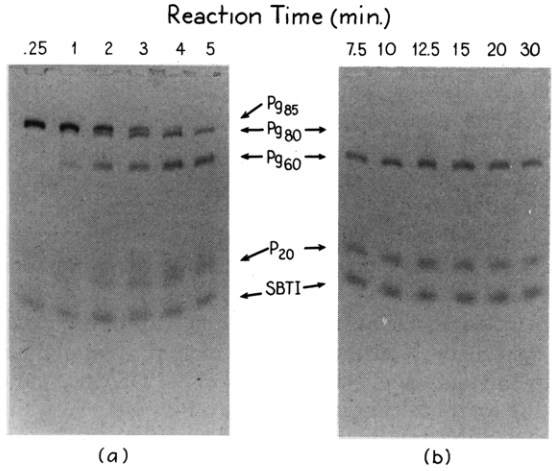


FIGURE 6: SDS acrylamide gel separation of plasminogen activation products (catalytic SK). Pg I at 0.8 mg/ml in 0.05 M phosphate buffer–0.1 M lysine at pH 7.4 at 25° was treated with SK at 5 μg/ml (Pg:SK, 90:1 on a molar basis). Samples were removed at the indicated times as described in the Experimental Section. (a) 140–160 mA, 16 hr; (b) 130–145 mA, 16 hr.

Catalytic Activation of Plasminogen with Streptokinase. In all activation kinetic studies, Pg I and Pg II gave equivalent results. Therefore, only the studies with Pg I will be presented here.

Human Pg I was treated with catalytic SK (Pg:SK, 100:1 on a molar basis). Samples were removed from the digestion mixture at the times indicated and treated as described in the Experimental Section. Figure 6 shows the stained acrylamide gels from the early part of the time study. The positions in Figure 6 of the peptides released by reduction determines their approximate molecular weights.[4] The quantitative scan data from this time study are shown in Figure 7.

As can be seen, there is a rapid loss of Pg_{85} and the appearance of P_{60} and P_{20} fragments. These two fragments are probably similar to the heavy and light chains reported by Robbins *et al.* (1967). However, in addition to these fragments there appears to be an early conversion of Pg_{85} into a slightly smaller molecule of molecular weight of about 80,000. The double banding in the 80,000–85,000 re-

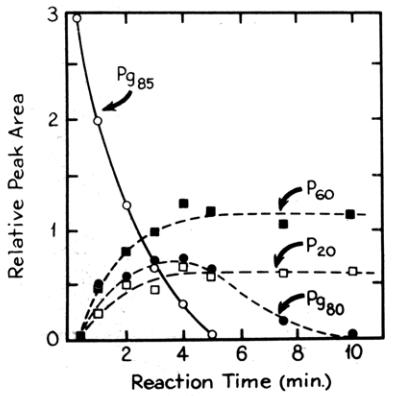


FIGURE 7: Plasminogen activation (catalytic SK) quantitative data obtained by scanning the gels of Figure 6. Peak areas were normalized to the SBTI area as described in the Experimental Section.

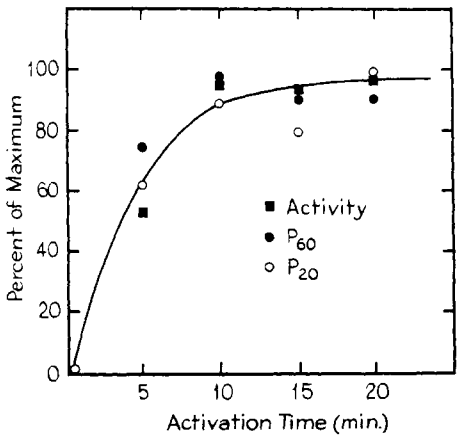


FIGURE 8: Peptides and active site formation as function of time (catalytic SK). Pg I at 0.75 mg/ml in 0.05 M phosphate–0.02 M lysine at pH 7.4 and 25° was treated with SK at 4.3 μg/ml (Pg:SK, 100:1 on a molar basis). Samples were removed at the times indicated and burst data obtained (*cf.* footnote 5). The burst samples were treated with SDS–HSEtOH and applied to acrylamide gels as before. (●) P_{60}; (○) P_{20}; (■) active site by NphOGdnBz burst.

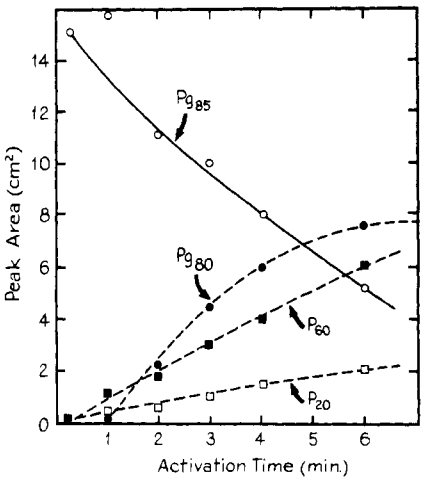
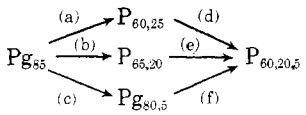


FIGURE 9: Plasminogen activation (catalytic UK) quantitative SDS gel scan data. Pg I at 0.8 mg/ml in 0.05 M phosphate–0.1 M lysine at pH 7.5 and 25° was treated with UK (3.75 μg/ml of the UK preparation represents Pg:UK, 440:1 on a molar basis). Aliquots were removed, treated as before, and applied to the SDS gel system. (○) Pg_{85}; (●) P_{80}; (■) P_{60}; (□) P_{20}.

gion of the gels is indicative of this conversion and can readily be seen in the 1–5-min samples of Figure 6. These data suggest that a peptide of about 5000 molecular weight is generated from plasminogen by one or more of the following sequential reactions.



Absence of double banding in the P_{60} and P_{20} regions of the gels of Figure 6 completely rules out the (a), (d), and (b), (e) pathways. This fact also makes it clear that the P_5 peptide cannot be between P_{60} and P_{20} in Pg_{85}. The data of these studies do not give information as to which end of the Pg molecule the 5000 molecular weight peptide is attached nor if P_5 required reduction for its release. When the samples were charged on the gel at much higher concentrations, direct evidence for the P_5 peptide was obtained. The extensive destaining of the gels of Figure 6 reduced the staining of this peptide and perhaps the concentration of the peptide itself to a level below the quantitative sensitivity range of the scanning procedure. This P_5 peptide may be the same as the 6000 molecular weight peptide isolated by Taylor and Botts (1968) from an unreduced Pg mixture following its catalytic activation with SK.

In a separate experiment the time-dependent appearance of P_{20} and P_{60} chains from plasminogen, using a catalytic amount of streptokinase, was compared with the formation of active site. The time course of active site formation was followed using the active site titrant NphOGdnBz. The results of this experiment are shown in Figure 8. As can be seen, the formation of active site correlates well with the formation of P_{60} and P_{20}. It should be noted that in this experiment no Pg_{80} was detected in the SDS gels after 5 min of reaction. Although all of the Pg_{85} had presumably passed through the Pg_{80} intermediate form, full enzyme site activity had not been generated in this time interval. Activity generation appeared to follow the formation of the final product $P_{60,20,5}$ suggesting that the intermediate $Pg_{80,5}$ does not contain an active enzyme center. This result is in agreement with the conclusions independently deduced from UK activation studies by Wiman and Wallén (1973), Rickli and Otavsky (1973), and Walther *et al.* (1974).

Catalytic Plasminogen Activation with Urokinase. Since plasminogen can also be activated to plasmin by urokinase, a well-characterized activator obtained from urine, it was desirable to examine the mechanism of UK mediated Pg activation and to compare the results to those obtained using SK. The design of this experiment was similar to that used for the catalytic SK experiments. Pg was activated with catalytic UK (Pg:UK, 440:1 on a molar basis, the molarity of UK being determined by active site titration with NphOGdnBz as described in the Experimental Section). Aliquots were removed from the activation mixture as a function of time, treated as before, and examined by SDS acrylamide gel electrophoresis. Quantitative analysis of the stained gel shows the time-dependent disappearance of intact Pg_{85} and the concomitant appearance of smaller peptide chains. These quantitative evaluations of the gels are shown in Figure 9. (The catalytic quantity of UK used was not visible in the gel.) The catalytic activation of Pg by UK also shows the appearance of a Pg_{80} component as well as P_{60} and P_{20} peptides. These results are in agreement with the conclusions of Wiman and Wallén (1973) and Walther *et al.* (1974) and indicate that the UK activation process goes by a sequential mechanism which is probably identical with that seen in the catalytic SK experiments. Side by side SDS gel electrophoresis comparisons (not shown) of UK, catalytic SK, and stoichiometric SK activations of Pg showed that the molecular weights of the P_{60} peptides generated by the three procedures were the same. The same conclusion could also be made for the P_{20} peptides generated in these reactions.

The kinetics of the formation of P_{60} (or P_{20}) and the disappearance of Pg_{85} in the catalytic UK and SK activation processes makes it appear that UK and the SK · P activator complex can carry out both bond splitting steps of the catalytic process with nearly equal effectiveness (on a molar basis).

Stoichiometric Plasminogen Activation with SK.

[5] The time necessary to establish the preburst NphOGdnBz hydrolysis rate made it impossible to obtain "burst" data in less than 5 min.

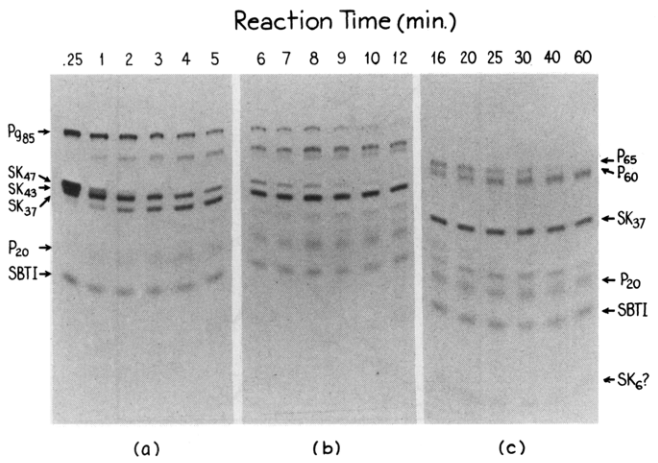


FIGURE 10: SDS acrylamide gel separation of Pg–SK activation products (stoichiometric SK). Pg I at 0.84 mg/ml in 0.05 M phosphate–0.1 M lysine at pH 7.4 and 25° was treated with SK at 0.57 mg/ml (Pg:SK, 1:1.2 on a molar basis). Samples were removed at the indicated times and treated as before (a) 130–155 mA, 16 hr; (b) 136–160 mA, 16 hr; (c) 150–180 mA, 16.5 hr.

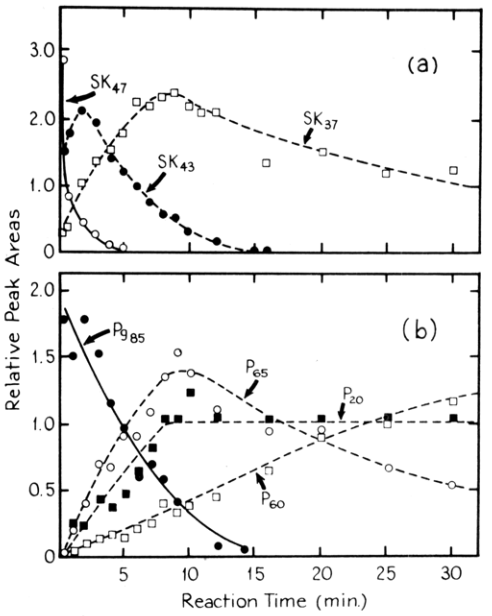


FIGURE 11: Plasminogen activation with stoichiometric SK. Quantitative data obtained by scanning the gels of Figure 10. Peak areas have been normalized to the SBTI area as described in the Experimental Section. (a) SK and breakdown products; (b) Pg and breakdown products.

Human plasminogen was mixed with stoichiometric amounts of streptokinase. Samples were removed at the indicated times and treated as before. Figure 10 shows, by SDS acrylamide gel electrophoresis, the time-dependent appearance of breakdown fragments. As can be seen, the patterns are much more complicated than when catalytic quantities of SK were used. (SDS acrylamide gel electrophoresis of control Pg shows the single Pg_{85} band while the SK sample used in these experiments already shows 15–20% conversion of $SK_{47} \rightarrow SK_{43}$. For this reason, 20% excess SK has been included in the stoichiometric studies.) The quantitative evaluation of these breakdown products is shown in Figure 11. These results describe the kinetics of the peptide bond splits in SK as well as Pg. A most notable feature is the very rapid conversion within the first 3 min of all the SK_{47} to SK_{43}. Following this there appears to be a somewhat slower conversion of SK_{43} to SK_{37}. SK_{37} is subject to further degradation to at least two peptides in the 25,000–30,000 molecular weight range.

In contrast to the catalytic process, the bond splits occurring in Pg during the stoichiometric reactions proceed *via* a different intermediate. No P_{80} is observed at any time in these stoichiometric reactions. Instead, a P_{65} intermediate form is observed. Initially the rate of formation of P_{65} and P_{20} corresponded to the rate of disappearance of Pg_{85}. At later times the rate of appearance of P_{60} paralleled the loss of P_{65}.

The bond splitting steps ($Pg^{a}_{85} \rightarrow P^{a}_{65,20} \rightarrow P^{a}_{60,20,5}$) which occur during the stoichiometric activation of Pg by SK have half-lives at 25° of 3–5 min. Earlier studies from our laboratories (McClintock and Bell, 1971b) demonstrated that the active enzyme center was generated by a unimolecular rearrangement in the stoichiometric SK · Pg complex prior to any bond splits. An Arrhenius plot of this rearrangement rate data showed this rearrangement to have an activation energy of about 21 kcal/mol of Pg and a half-life of about 15–20 sec at 25°. This is indeed much faster than any of the subsequent peptide bond splitting reactions.

Peptide Order in Native Pg_{85} and SK_{47}. In order to determine the peptide order in both SK and Pg the peptides were isolated from preparative sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis blocks and their N-terminal amino acid residues determined. Activation conditions were selected to maximize the yields of the desired peptides. The results of N-terminal analysis of these peptides along with the parent Pg and SK are shown in Table II.

The larger protein charges used in these preparative SDS acrylamide blocks along with the necessity to extract the entire block thickness made it impossible to cleanly resolve the P_{65} and P_{60} peptides by this technique. Therefore, the whole P_{65}–P_{60} region of the gel was taken for analysis. The SK_{47} sample of Table II contained an estimated 10–15% of SK_{43}. In kinetic studies with this sample the SK_{43} appeared to be functional and converted to SK_{37} in the normal man-

TABLE II: N-Terminal Amino Acid Summary.

Preparation		N-Terminal Amino Acid Data	
Sample	Activation Method	N-Terminal	Method
Pg_{85}		Glu	Dns, PTH[e]
$P_{65} + P_{60}$	*a*	Glu + Lys	Dns
P_{60}	*a*	Lys	Dns
P_{60}	*b*	Lys	Dns
P_{20}	*a*	Val + Val-Val[c]	Dns
P_{20}	*b*	Val + Val-Val[c]	Dns
P_5[d]	*b*	Glu	Dns
$SK_{47} + SK_{43}$		Ile	Dns
SK_{37}	*a*	Leu + Phe	Dns

[a] Peptides from stoichiometric SK · Pg activation. [b] Peptides from catalytic SK · Pg activation. [c] Confirmed by authentic Dns-Val-Val. [d] P_5 recovered from block by electrodialysis. [e] PTH, phenylthiohydantoin.

ner. The SK activity of this preparation, by the Christensen (1947) assay procedure, was 110,000 units/mg of protein. The recovered SK_{37} of Table II when tested by the bovine fibrin plate method (Alkjaersig *et al.*, 1959) for its ability to combine with plasmin to form activator or its ability to activate Pg directly had less than 1% of the activities of the parent SK_{47}.

When taken together, the N-terminal data of Table II and the SK activation kinetic data indicate that the peptide order for Pg_{85} is H-P_5-P_{60}-P_{20}-OH. This conclusion is in general agreement with peptide order deduced by others from their UK activation studies [Wiman and Wallén (1973); Rickli and Otavsky (1973); Wiman (1973); and Walther *et al.* (1974)].

As had been reported by Morgan and Henschon (1969), isoleucine was the only N-terminal found for SK_{47}. The observation of a single N-terminal isoleucine in the SK_{47} sample which contained 10–15% SK_{43} suggests that the SK_4 peptide had been split from the carboxyl end of the SK_{47}. The appearance of new N-terminals, leucine and phenylalanine, in SK_{37} suggests that the peptide arrangement in SK_{47} is H-SK_6-SK_{37}-SK_4-OH and indicate some heterogeneity in the SK_{37}.

It should be pointed out that the methods used to deduce the peptide arrangements in Pg_{85} and SK_{47} do not preclude the possibility that smaller peptides have been cleaved from the C-terminal ends of the peptides in question. The consistency of the N-terminal data, with the exception of SK_{37}, makes it unlikely that such small peptides have been cleaved from the N-terminal ends of these peptides.

Discussion

The kinetic data from the previous communication (McClintock and Bell, 1971b) and the results of this study can be used to describe the sequence of peptide bond cleavages which occur during the interaction of plasminogen with catalytic or stoichiometric amounts of streptokinase. The overall scheme of activation steps is shown in Figure 12. In this scheme superscript a denotes the species which contains the active center of plasmin and its activator forms. When Pg and SK are combined in stoichiometric amounts, a simple complex forms which subsequently undergoes a unimolecular rearrangement generating an active site. The initial equilibrium and reaction 1 which generates the active site is much faster than any of the possible bond cleavage reactions (2a–5b) leading to the relatively stable activator complex $SK_{37,6,4} \cdot P^a_{60,20,5}$.

Because of the sequential nature of reactions 2a–5b, it is not possible from the kinetic data shown in Figure 11 to estimate the enzymatic potential of each of the activator forms shown in the dashed square of Figure 12. The information available suggests that the preferred sequence of bond cleavages is *via* reactions 2b, 3c, 4c, and 5b as shown. Qualitatively $SK_{47} \cdot Pg^a_{85}$ being the first form containing the enzyme center must be responsible for reactions 2a and 2b. The initial rates of decay of SK_{47} and Pg_{85} show that reaction 2b is about six times faster than 2a; therefore reactions 2a, 3a, 3b, and 4a are considered to be of minor importance in the overall process. The rate of disappearance of SK_{43} (reactions 3d + 4c measured by either SK_{37} appearance or SK_{43} decay after 5 min) is of the same magnitude ($t_{1/2}$ *ca.* 3 min) as the appearance rate for P_{20} (reactions 3c + 4d). On a kinetic basis alone, it is not possible to distinguish between reactions 3c and 3d as to preferred pathway. However, by examining a later step in the reaction sequence

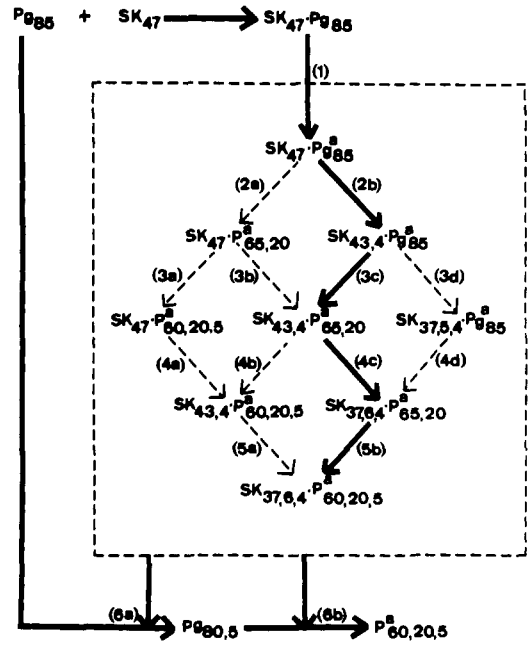


FIGURE 12: Activation of plasminogen by streptokinase. Stoichiometric reactions (1–5b). Catalytic activation of Pg by activator complexes (reactions 6a and b). All possible activator forms are enclosed in dashed square.

it is possible to suggest a preferred pathway for reactions 3 and 4. It is clear that in species $SK_{37,6,4} \cdot P^a_{65,20}$ the P_5–P_{60} bond has now become available for cleavage, *i.e.*, the next step yields $SK_{37,6,4} \cdot P^a_{60,20,5}$. This suggests that both susceptible bonds in SK_{47} must be cleaved in order to make the P_5–P_{60} bond available within the complex. If reaction 3d were operative, yielding $SK_{37,6,4} \cdot P^a_{85}$, the P_5–P_{60} bond would now be available on the Pg^a_{85} moiety and the formation of some $P^a_{80,5}$ would have been expected. Since no P_{80} was ever observed in stoichiometric activation studies, reaction 3d is not considered likely. Reaction 4b is probably less important than 4c since the appearance P_{60} is much slower than the rate of SK_{43} disappearance.

If reactions 3c and 4c are accepted as the preferred pathway for generation of $SK_{37,6,4} \cdot P^a_{65,20}$ the data require that reaction 3c be a rate-limiting step and the rate constant of (4c) be much greater than that of (3c). This must be true because SK_{37}, P_{65}, and P_{20} all appear to be formed simultaneously. This conclusion would suggest that the enzyme center in the $SK_{43,4} \cdot P^a_{65,20}$ species is much more effective than the same center in the $SK_{43,4} \cdot 3Pg^a_{85}$ form.

The catalytic SK activation reactions 6a and b of the scheme of Figure 12 are in general agreement with recent results reported by others for UK activation of Pg. The appearance of H-Val-Val as the only N-terminal sequence in the P_{20} peptide of this report and the light chains described by Robbins *et al.* (1967, 1972) and Walther *et al.* (1974) adds support to the conclusion that the bond cleaved in reaction 6b is unique and the same for SK and UK activation of Pg.

Lack of agreement between Wiman (1973) and Walther *et al.* (1974) as to the new N-terminal groups generated by the initial bond cleavage of reaction 6a suggests that this step may not be uniquely specific and may in part result from spontaneous, proteolytic activity in the Pg preparations as first suggested by Wallén and Wiman (1970).

The demonstration of two unique pathways of activation of Pg by SK by kinetic methods is not sufficient to precisely

describe in the three-dimensional terms the sequence of events involved. The data do show (1) that the covalent attachment of peptide P_5 in P_{85} makes the P_{60}–P_{20} bond unavailable for enzyme cleavage, (2) that association of SK_{47} with Pg_{85} results in the exposure of the enzyme center in the $SK_{47} \cdot Pg_{85}$ complex, blockage of the P_5–P_{60} bond from cleavage and exposure of the P_{60}–P_{20} bond for enzymatic hydrolysis.

The complete explanation of the unique function of SK in the SK · P activator complex and how it confers Pg activator specificity to the enzyme center must await the application of more powerful methods. A complete structure determination of the SK · P or SK · Pg complexes by X-ray analysis should answer these questions. Success with this approach will depend on the preparation of a suitable crystalline complex. For such crystalization attempts, we suggest the formation of the $SK_{47} \cdot Pg^{a}_{85}$ complex, in the presence of an active site acylating agent, under conditions such as described earlier [McClintock and Bell (1971b)].

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