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Recent Advances in the Chemistry of the Fibrinolytic System

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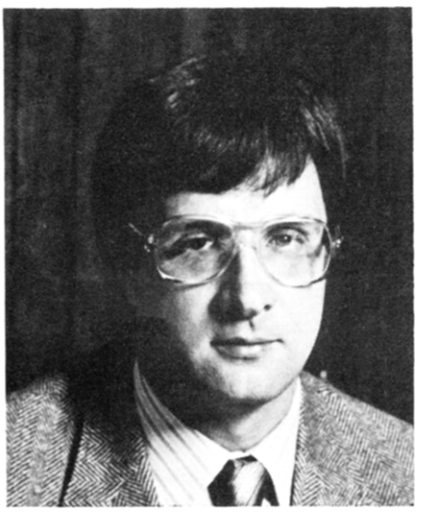

Francis J. Castellino was born in Pittston, PA. He received a B.S. in Chemistry from the University of Scranton in 1964, an M.S. in Biochemistry from the University of Iowa in 1966, and a Ph.D. in Biochemistry from the same university in 1968. At Iowa, his work, with Robert Barker, centered around the physical biochemistry of protein structure, particularly muscle aldolase. Following this, he studied for 2 years as a Research Fellow at Duke University, with Robert L. Hill, investigating the protein chemistry of the lactose synthetase system. In 1970, he joined the faculty of the Department of Chemistry at the University of Notre Dame, where he presently serves as Professor of Chemistry and Dean of the College of Science. His research interests include physical and chemical studies on the components of the blood coagulation and fibrinolytic systems.

I. Introduction

Fibrinolysis refers to the dissolution of the fibrin blood clot by an enzyme system present in blood of all mammalian species so far examined. The fibrinolytic system consists of the plasma zymogen, plasminogen; its activated product, the proteolytic enzyme, plasmin; activators of plasminogen; inhibitors of both plasmin and plasminogen activators; and fibrinogen and fibrin. During the last decade, many significant advances have been made concerning the molecular properties of the fibrinolytic system. As a result of this progress, this very important system can now be described to the general chemical and biochemical audience, and it is the purpose of this review to do so. Much of the early work in this field has been presented in previous reviews of this subject and will not be repeated here in detail. The present review will essentially cover the work performed during the decade of the 1970s and will be strictly concerned with molecular and kinetic aspects of the fibrinolytic system. Of particular interest to this review are (a) molecular aspects of the structure of plasminogen and plasmin, (b) plasmin kinetics, (c) molecular mechanism of activation of plasminogen, (d) the role of antifibrinolytic amino acids in fibrinolysis, and (e) interaction of plasminogen and plasmin with fibrinogen, fibrin, and the very important plasma inhibitor of plasmin, α_2-antiplasmin.

II. Molecular Properties of Plasminogen

Perhaps the single most important experiment, which stimulated many of the molecular advances in the field, was performed by Deutsch and Mertz in 1970.[1] These investigators found that human plasminogen was adsorbed from plasma to a column of L-lysine, which was insolubilized to a Sepharose matrix, and removed in a

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CHART I

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                1                  10                  20                  30                  40                  50
NH2-E-P-L-D-D-Y-V-N-T-Q-G-A-S-L-F-S-V-T-K-K-Q-L-G-A-G-S-I-E-E-C-A-A-K-C-E-E-D-E-E-F-T-C-R-A-F-Q-Y-H-S-K-E-Q-E-C-
       60                  70                  80                  90                 100                 110
V-I-M-A-E-N-R-K-S-S-I-I-R-M-R-D-V-V-L-F-E-K-K-V-Y-L-S-E-C-K-T-G-D-G-K-N-Y-R-G-T-M-S-K-T-K-N-G-I-T-C-Q-K-W-S-S-T-
              120                 130                 140                 150                 160
S-P-H-R-P-R-F-S-P-A-T-H-P-S-E-G-L-E-E-N-Y-C-R-N-P-D-N-D-P-Q-G-P-W-C-Y-T-T-D-P-E-K-R-Y-D-Y-C-D-I-L-E-C-E-E-E-C-M-
       170                 180                 190                 200                 210                 220
H-C-S-G-E-N-Y-D-G-K-I-S-K-T-M-S-G-L-E-C-Q-A-W-D-S-Q-S-P-H-A-H-G-Y-I-P-S-K-F-P-N-K-N-L-K-K-N-Y-C-R-N-P-D-R-E-L-R-
              230                 240                 250                 260                 270
P-W-C-F-T-T-D-P-N-K-R-W-E-L-C-D-I-P-R-C-T-T-P-P-P-S-S-G-P-T-Y-Q-C-L-K-G-T-G-E-N-Y-R-G-N-V-A-V-T-V-S-G-H-T-C-Q-H-
       280                 290                 300                 310                 320                 330
W-S-A-Q-T-P-H-T-H-N-R-T-P-E-N-F-P-C-K-N-L-D-E-N-Y-C-R-N-P-D-G-K-R-A-P-W-C-H-T-T-N-S-Q-V-R-W-E-Y-C-K-I-P-S-C-D-S-
                   |
                  CHO
                340                 350                 360                 370                 380                 390
S-P-V-S-T-E-E-L-A-P-T-A-P-P-E-L-T-P-V-V-Q-D-C-Y-H-G-D-G-Q-S-Y-R-G-T-S-S-T-T-T-T-G-K-K-C-Q-S-W-S-S-M-T-P-H-R-H-Q-
                   |
                  CHO
                400                 410                 420                 430                 440
K-T-P-E-N-Y-P-N-A-G-L-T-M-N-Y-C-R-N-P-D-A-D-K-G-P-W-C-F-T-T-D-P-S-V-R-W-E-Y-C-N-L-K-K-C-S-G-T-E-A-S-V-V-A-P-P-P-
       450                 460                 470                 480                 490                 500
V-V-L-L-P-N-V-E-T-P-S-E-E-D-C-M-F-G-D-G-K-G-Y-R-G-K-R-A-T-T-V-T-G-T-P-C-Q-D-W-A-A-Q-E-P-H-R-H-S-I-F-T-P-E-T-N-P-
              510                 520                 530                 540                 550
R-A-G-L-E-K-N-Y-C-R-N-P-D-G-N-V-G-G-P-W-C-Y-T-T-N-P-R-K-L-Y-D-Y-C-D-V-P-Q-C-A-A-P-S-F-D-C-G-K-P-Q-V-E-P-K-K-C-
       560                 570                 580                 590                 600                 610
P-G-R-V-V-G-G-C-V-A-H-P-H-S-W-P-W-Q-V-S-L-R-T-R-F-G-M-H-F-C-G-G-T-L-I-S-P-E-W-V-L-T-A-A-H-C-L-E-K-S-P-R-P-S-S-Y-
              620                 630                 640                 650                 660                 670
K-V-I-L-G-A-H-Q-E-V-N-L-E-P-H-V-Q-E-I-E-V-S-R-L-F-L-E-P-T-R-K-D-I-A-L-L-K-L-S-S-P-A-V-I-T-D-K-V-I-P-A-C-L-P-S-P-N-Y-
              680                 690                 700                 710                 720
V-V-A-D-R-T-E-C-F-I-T-G-W-G-E-T-Q-G-T-F-G-A-G-L-L-K-E-A-Q-L-P-V-I-E-P-N-K-V-C-N-R-Y-E-F-L-N-G-R-V-Q-S-T-E-L-C-A-
       730                 740                 750                 760                 770                 780
G-H*-L-A-G-G-T-D-S-C-Q-G-D-S*-G-G-P-L-V-C-F-E-K-D-K-Y-I-L-Q-G-V-T-S-W-G-L-G-C-A-R-P-N-K-P-G-V-Y-V-R-V-S-R-F-V-
                   790
T-W-I-E-G-V-M-R-N-N-COOH
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purified state by elution with ϵ-aminocaproic acid (ϵ-ACA). This rapid and simple procedure resulted in plasminogen purification in very high yield and allowed sufficient quantities to become readily available to a number of laboratories for structural investigations. Our laboratory found, as a result of modification[2] of the Deutsch and Mertz technique, that at least 95% of the plasminogen in the plasma of several species can be represented by two major forms, separable by affinity chromatography on Sepharose–lysine, as shown in Figure 1. These two major variants of plasminogen differed in several chemical and physical properties. For example, in the human plasminogen system, the first variant (F-1) eluted from Sepharose–lysine possessed a molecular weight 2000–4000 greater than that of the second form (F-2).[3,4] This molecular weight difference could be entirely accounted for by the difference in carbohydrate content of the two variants.[5] Additionally, the charge properties of the variants differed from each other in both the rabbit[6] and human[3] systems. For rabbit plasminogen, F-1 was resolved into at least five subforms by isoelectric focusing techniques,[6] with p*I* values 6.20–7.78. On the other hand, F-2 was resolved into at least five subforms, with p*I* values 6.95–8.74.[6] Similar results have been obtained for human plasminogen.[3] An additional difference between the two variants of plasminogen resided in their differential binding to antifibrinolytic agents, such as ϵ-ACA, as exemplified by the separation of the two variants on affinity chromatography columns, employing amino acids of this type.[2] Metabolic studies on plasminogen indicated that each affinity variant was independently synthesized, independently degraded, and noninterconvertible to the other variant in plasma.[7]

Native human plasminogen is a single chain protein of M_r ~92 000 (F-2)–94 000 (F-1).[4] The amino-terminal amino acid residue of each variant is Glu,[8] and the

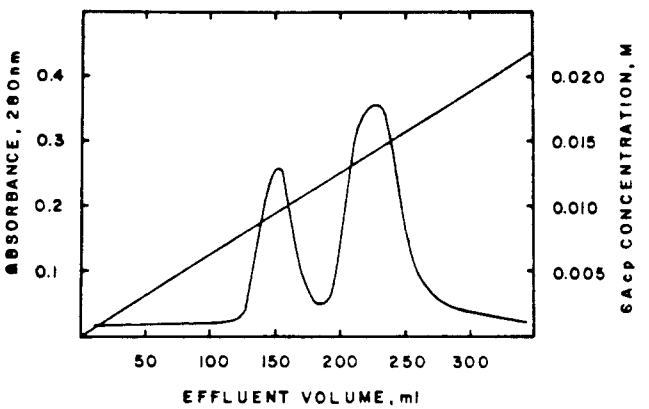
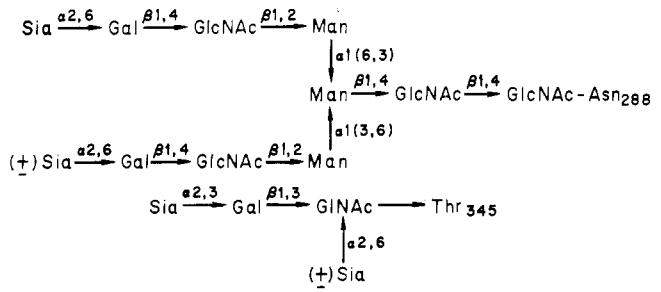


Figure 1. Elution of human Glu_1-Pg variants by affinity chromatography on Sepharose–lysine (taken from ref 2).

carboxyl-terminal amino acid of each variant is Asn.[9] The complete amino acid sequence of native plasminogen is given in Chart I (in the one-letter code for amino acids) and has been compiled from several sources.[10–14] This sequence represents a combination of F-1 and F-2. Therefore, possible existence of subtle sequence differences between the affinity variants cannot be commented upon.

The disulfide pairings are believed[10] (not all have been unambiguously established) to involve the following Cys residues: 30–54, 34–42, 83–161, 104–144, 132–156, 168–296, 165–242, 186–225, 214–237, 255–332, 276–315, 304–327, 357–434, 378–417, 406–429, 461–540, 482–523, 511–535, 557–565, 547–665, 587–603, 679–747, 710–726, and 737–765.

The carbohydrate sequence of human plasminogen has also been established, and differences exist between the two affinity chromatography variants.[15–17] Native human F-1 possesses two sites of glycosylation, one at Asn_{288} and the other at Thr_{345}. The sequences are



On the other hand, native human F-2 only contains the Thr-based oligosaccharide. The residues labeled (±) Sia are not present on all molecules. Some of the human plasminogen subforms owe their existence to incomplete sialylation.[18]

Several very important lower molecular weight forms of human plasminogen have been obtained as a consequence of the proteolytic digestion of the native molecule. The nomenclature employed for these molecules is based upon the sequence position of the amino-terminal amino acid (e.g., native circulating plasminogen is referred to as Glu_1-plasminogen). Exposure of Glu_1-plasminogen (Glu_1-Pg) to the protease, plasmin, for very short times results in cleavage of the amino terminal 76 residues from Glu_1-Pg, yielding a molecule of plasminogen which is of great importance to several properties of the fibrinolytic system, Lys_{77}-plasminogen (Lys_{77}-Pg). Lys_{77}-Pg possesses a molecular weight of approximately 83 000 (F-2)–85 000 (F-1).[4] Quite dramatic conformational differences exist between Glu_1-Pg and Lys_{77}-Pg, in the absence of ϵ-ACA, which are of significance to several functional properties (discussed below) of the molecule. These conformational differences have been monitored by a variety of physical techniques. For example, the $S^0_{20,w}$ value of Glu_1-Pg is approximately 5.7 S whereas the same value for Lys_{77}-Pg is approximately 4.7 S.[19] The difference in the $S^0_{20,w}$ values is much too large to be accounted for by the molecular weight difference in the two molecules. The rotational relaxation time of Glu_1-Pg has been found to be approximately 262 ns and that for Lys_{77}-Pg is approximately 160 ns.[20] Thus, Lys_{77}-Pg appears to be more asymmetric and more internally flexible than Glu_1-Pg.

Other functional low molecular weight plasminogen molecules and large peptide fragments have been isolated upon limited proteolysis of Glu_1-Pg with elastase.[10] After treatment of Glu_1-Pg with pancreatic elastase under appropriate conditions,[21] application of the digest to Sepharose–lysine, and elution with a gradient of ϵ-ACA, the profile shown in Figure 2 is obtained. Peak I contains two fully activatable plasminogens, Val_{442}-plasminogen[10] (Val_{442}-Pg) and Leu_{449}-plasminogen (Leu_{449}-Pg).[22] These two plasminogen forms possess molecular weights of approximately 38 000. They are of remarkable chemical significance, since, here, a plasminogen molecule has been obtained which is completely convertible to plasmin, of molecular weight of only 40% of the native plasminogen molecule. Also, from this chromatograph, at least one other plasminogen can be obtained. From peak IV, Val_{354}-plasminogen (Val_{354}-Pg) is readily isolated.[22] This fully activatable plasminogen molecule possesses a molecular weight of 49 000–53 000, approximately 50% of the native molecule. The existence of these low molecular weight zymogens, all of which are fully capable of activation to plasmin, offers a practically unparalleled opportunity to study structure–function relationships in plasminogen and plasmin. In addition to the above, other important peptide fragments can be isolated from the digestion profile of Figure 2. From peak II, a region of the native plasminogen molecule comprising Tyr_{79}–Val_{337} and Tyr_{79}–Val_{353}[10] (collectively termed K1–3) can be readily isolated,[23] from peak III, a peptide consisting of residues Val_{354}–Ala_{439}[10] can be easily obtained, and from peak V a peptide comprising residues Tyr_{79}-Ala_{439} can be purified.[23]

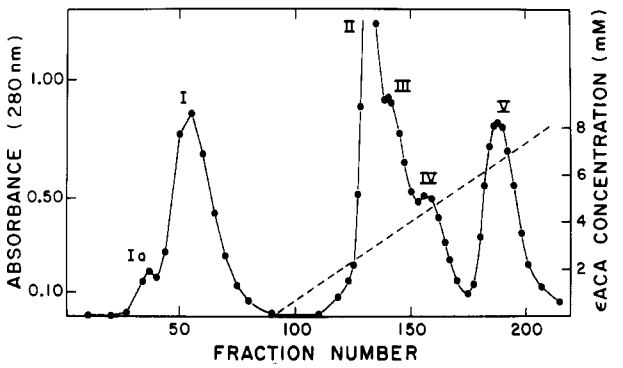


Figure 2. Elution of the limited elastase-digested products of human Glu_1-Pg (affinity variant 2) from Sepharose–lysine. The dashed line refers to the concentration of ϵ-ACA (taken from ref 23).

It became of considerable importance to ascertain whether the isolated fragments existed in a manner similar to that in the intact protein, i.e., whether these fragments exist as independent domains in the intact protein. We addressed this problem by a thermodynamic method, differential scanning calorimetry (DSC).[23] Figure 3 shows the thermal denaturation profiles of Lys_{77}-Pg, K1–3, Val_{442}-Pg (actually a mixture of Val_{442}-Pg and Leu_{449}-Pg), and K4. The latter three peptides represent fragments of the entire structure of Lys_{77}-Pg. In Lys_{77}-Pg (Figure 3A), at least two thermal denaturing transitions are seen, one with a temperature midpoint (T_m) at 53.5 °C and another at a T_m of 61.2 °C. The total ΔH of the transitions is 480 kcal mol^{-1}. Figure 3, B–D, respectively, illustrates DSC thermograms of K1–3, Val_{442}-Pg, and K4. All thermal transitions found in Lys_{77}-Pg can be located in one or more of the three component peptides, and the total ΔH value of these peptides, when summed, is very close to the value for Lys_{77}-Pg. This strongly supports the argument that these particular peptides exist as independent domains in the intact Lys_{77}-Pg structure. Similar conclusions are reached when the thermograms of Val_{354}-Pg and K1–3 (two components of Lys_{77}-Pg which represent its entire structure) are treated in this manner.

On the basis of amino acid sequence homology of certain regions of human plasminogen with "kringle" structures of prothrombin,[24] Sottrup-Jensen et al.[10] have proposed that a portion of plasminogen (from Tyr_{79} to Arg_{560}) can be represented by five kringle structures with interconnecting peptide strands. A depiction of this model is shown in Figure 4. From our DSC data, we feel that these "kringle" structures likely exist as independent domains and can be isolated as such. These putative "kringle" structures are very important for some of the functional properties of plasminogen

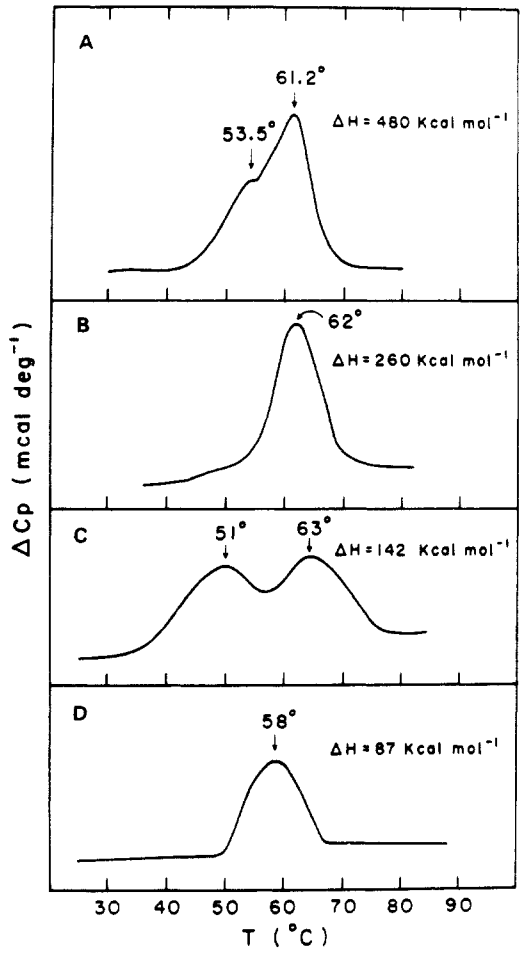


Figure 3. Thermal denaturation profiles of human Lys_{77}-Pg (A) and the elastase-produced fragments K1–3 (B), Val_{442}-Pg (C), and K4 (D) in the absence of ϵ-ACA. The T_m positions and the ΔH values are indicated (taken from ref 23).

and plasmin, and this aspect will be developed during the course of this review.

III. Molecular and Kinetic Properties of Plasmin

A. Physical and Chemical Properties

Human plasmin is a two-chain structure, stabilized by two disulfide bonds. Native plasmin is formed as a consequence of cleavage of at least two peptide bonds in the plasminogen molecule. One cleavage at Arg_{560}–Val_{561} is essential for the catalytic activity to be expressed.[25] Here, no peptides are released, since disulfide bond linkages at Cys_{557}–Cys_{565} and Cys_{547}–Cys_{665} stabilize the plasmin molecule. As a consequence of plasmin formation, autolysis of Lys_{76}–Lys_{77} occurs, with covalent liberation of the amino-terminal 76 residues, yielding the final plasmin.[4] This plasmin is termed Lys_{77}-plasmin (Lys_{77}-Pm). Native plasmin thus consists of a heavy chain, M_r ~58 000–63 000[4] disulfide linked to a light chain, M_r 23 000–26 000.[4,25]. Plasmin nomenclature is based upon the sequence position of the amino terminus of the heavy chain, or heavy chain fragment, since the light chain of all plasmin molecules so far isolated possesses the same amino-terminal residue, Val_{561}. Correspondingly, native plasmin is termed Lys_{77}-plasmin (Lys_{77}-Pm). Several other plasmins have been isolated, each based upon the particular plasminogen molecule undergoing activation. Activation of either Glu_1-Pg or Lys_{77}-Pg yields Lys_{77}-Pm. However, Glu_1-Pm can be prepared when Glu_1-Pg is activated under conditions which result in immediate inactivation of the plasmin formed (since plasmin, by autolysis, will cleave the Lys_{76}–Lys_{77} peptide bond in Glu_1-Pm, yielding Lys_{77}-Pm). In the presence of the Kunitz basic pancreatic trypsin inhibitor (PTI), Glu_1-Pm can be prepared from either rabbit Glu_1-Pg[26,27] or human Glu_1-Pg.[4,28] However, the resulting Glu_1-Pm will not be active, since the inhibitor will be bound to the active center. Val_{442}-Pm is readily obtained upon activation of Val_{442}-Pg.[21,29] This plasmin possesses the same light chain as Lys_{77}-Pm but contains a fragment of the Lys_{77}-Pm heavy chain consisting of residues Val_{442}-Arg_{560}, of approximate molecular weight 14 000. Additionally, Val_{354}-Pm can be obtained upon activation of Val_{354}-Pg.[22] Again, this plasmin contains the identical light chain of Lys_{77}-Pm, but only a fragment of the Lys_{77}-Pm heavy chain (Val_{354}–Arg_{560}), of approximate molecular weight 28 000.

The physical properties of plasmin have not been extensively studied. The interaction of Lys_{77}-Pm with columns of Sepharose–lysine is virtually identical with that of Lys_{χ}-Pg,[30] indicating that the antifibrinolytic amino acid binding sites of Lys_{77}-Pg are conserved in Lys_{77}-Pm. A similar finding applies to Glu_1-Pm. The $S^0_{20,w}$ value of human Lys_{77}-Pm is 4.80 S, a value approximately the same as that found for Lys_{77}-Pg.[19] The $S^0_{20,w}$ value found for human Glu_1-Pm (complexed to the above plasmin inhibitor) is 5.68 S, again very close to that of Glu_1-Pg.[19] Thus, the conformational differences observed between Glu_1-Pg and Lys_{77}-Pg are also found between Glu_1-Pm and Lys_{77}-Pm.

B. Kinetic Properties with Synthetic Substrates

Plasmin is a serine protease and is sensitive to inhibition by both diisopropyl fluorophosphate (DFP)[31] and tosyllysine chloromethyl ketone (TLCK).[32] The active-site serine and histidine residues are found in the plasmin light chain, serine at sequence position 741[33] and histidine at sequence position 602.[34] Although plasmin functions in fibrinolysis and fibrinogenolysis,[35] plasmin has been shown to be active in other systems. For example, plasmin is capable of digesting factor XIIa into factor XIIa fragments[36] as well as activating several complement zymogens, e.g., C1,[37] C3,[38] and C5.[39] Also, plasmin is capable of inactivation of C1 inhibitor.[40] Additionally, plasmin has been shown to substitute for factor D in the alternate complement pathway.[41] Plasmin has also been implicated in digesting proaccelerin,[42] antihemophilic factor,[42] ACTH,[43] glucagon,[43] and γ-globulin.[44]

Regarding nonphysiologic substrates, plamin possesses tryptic-like specificity. This enzyme hydrolyzes proteins and peptides at lysyl and arginyl bonds.[45] Plasmin possesses esterolytic activity toward basic amino acid esters[46] and amidolytic activity toward basic amino acid amides.[47] Several *p*-nitrophenolate esters are also good plasmin substrates. Some recently determined kinetic constants for various forms of human plasmin toward synthetic substrates are listed in Table I.

We have recently undertaken a study[48] aimed at comparing the steady- and pre-steady-state kinetic parameters of several forms of human plasmin toward synthetic substrates under conditions which are well

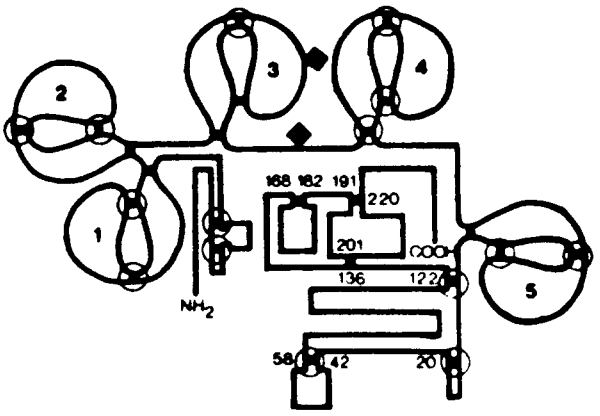


Figure 4. Proposed structure of human plasminogen, drawn to emphasize the kringle structures (taken from ref 10).

TABLE I. Steady-State Esterase and Amidase Kinetic Constants for Human Plasmin

enzyme	substrate	K_m, M	k_{cat}, s^{-1}
Glu_1-Pm[a]	Cbz-L-lysine-*p*-nitrophenyl ester	1.4×10^{-5}	22.3
Lys_{77}-Pm[a]	Cbz-L-lysine-*p*-nitrophenyl ester	1.5×10^{-5}	22.3
Lys_{77}-Pm[b]	α-*N*-tosyl-L-arginine methyl ester	7.7×10^{-4}	23.0
Val_{442}-Pm[c]	α-*N*-tosyl-L-arginine methyl ester	4.2×10^{-3}	45.0
Lys_{77}-Pm[d]	α-*N*-benzoyl-L-arginine ethyl ester	1.9×10^{-4}	12.0
Val_{442}-Pm[d]	α-*N*-benzoyl-L-arginine ethyl ester	1.8×10^{-4}	12.6
Lys_{77}-Pm[d]	D-Val-Leu-Lys-*p*-nitroanilide	1.2×10^{-4}	11.7
Val_{442}-Pm[d]	D-Val-Leu-Lys-*p*-nitroanilide	1.3×10^{-4}	10.4
Lys_{77}-Pm[d]	Bz-Phe-Val-Arg-*p*-nitroanilide	9×10^{-4}	10.0
Val_{442}-Pm[d]	Bz-Phe-Val-Arg-*p*-nitroanilide	1.2×10^{-3}	12.8

[a] Reference 113. The temperature was 30 °C, and the pH was 6.0. [b] Sodetz, J. M.; Castellino, F. J. *Biochemistry* **1970**, *11*, 3167. The temperature was 22 °C, and the pH was 8.0. [c] Reference 21. The temperature was 30 °C, and the pH was 8.0. [d] Reference 29. The temperature was 25 °C, and the pH was 7.8.

defined and with plasmins which are well characterized. The rationale behind the study revolved around an evaluation of the role of the heavy chain in the kinetics of plasmin. Since we had the above-described plasmins, with various sizes of the heavy chain, at our disposal, the above-stated goal of the study was relatively simple to address. For steady-state experiments, we employed the substrate NH_2-D-Val-Leu-Lys-*p*-nitroanilide (S-2251). The K_m and k_c values obtained are listed in Table II, along with K_I values for the competitive inhibitor, benzamidine hydrochloride. No dramatic differences are noted in these parameters for any of the plasmins employed, suggesting that the heavy chain of plasmin is not important in the steady-state kinetics of this enzyme toward synthetic substrates.

Plasmin is typical of a variety of serine proteases in which the mechanism of action involves an acyl-enzyme intermediate. Kinetic expressions for this class of enzymes have been developed.[49,50] The catalytic sequence is

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_2} E{\cdot}S' \xrightarrow{k_3} E + P_2$$
$$+$$
$$P_1$$

TABLE II. Steady-State Kinetic Values of the Various Plasmins toward the Peptide Substrate, S-2251, at 37 °C, pH 7.4

enzyme	K_m, mM	k_{cat}, s^{-1}	K_I, mM[a]
Lys_{77}-Pm	0.40 ± 0.05	19.8 ± 1.0	0.41 ± 0.05
Val_{354}-Pm	0.40 ± 0.05	25.3 ± 1.2	0.29 ± 0.05
Val_{442}-Pm	0.36 ± 0.05	25.8 ± 1.2	0.25 ± 0.04

[a] Data for benzamidine hydrochloride.

TABLE III. Pre-Steady-State Kinetic Parameters of Various Plasmins with the Substrate *p*-Nitrophenyl *p'*-Guanidinobenzoate, at 30 °C, pH 8.3

enzyme	K_s, μM	k_2, s^{-1}
Lys_{77}-Pm	5.6 ± 0.7	0.26 ± 0.05
Val_{354}-Pm	6.7 ± 0.7	0.28 ± 0.05
Val_{442}-Pm	11.1 ± 1.0	0.60 ± 0.08

where E is the enzyme, S is the substrate (ester), E·S is the enzyme–substrate complex, P_1 is the first product released (alcohol), E·S' is the acyl-enzyme intermediate, and P_2 is the second product released (acid). In this scheme, K_s represents the binding constant of substrate to enzyme, k_2 refers to the rate constant for acylation, and k_3 is the deacylation rate constant. We have measured K_s and k_2 for the substrate, *p*-nitrophenyl *p*-guanidinobenzoate, for each of the above plasmins (k_3 is essentially zero). The data obtained are listed in Table III. K_s and k_2 values for Lys_{77}-Pm and Val_{354}-Pm are nearly identical. However, the K_s for Val_{442}-Pm is approximately twice as high as the other plasmins, indicating reduced strength of interaction of the substrate with this enzyme. On the other hand, the higher k_2 value for Val_{442}-Pm than for either Lys_{77}-Pm or Val_{354}-Pm suggests that acylation of this form of plasmin is more rapid than for the two larger plasmins. Thus, some subtle differences in the active site of this low molecular weight form of the enzyme are indicated by these studies.

C. Plasminolysis of Human Fibrinogen and Fibrin

The molecular mechanism of fibrinogen and fibrin degradation by plasmin has been intensively studied. A summary of the steps involved in the commonly accepted mechanism of the Lys_{77}-Pm-catalyzed digestion of human fibrinogen is shown in Figure 5.[51,52] A slightly but significantly different mechanism has been proposed by Mosesson et al.[53] Native fibrinogen, of total molecular weight 343 000, is composed of three pairs of nonidentical peptide chains ($A\alpha_2$, $B\beta_2$, γ_2), stabilized by disulfide bonds.[54-58] Molecular weights of 64 000, 56 000, and 47 000 have been reported for the Aα, Bβ, and γ chains, respectively.[51] Plasmin begins its digestion of fibrinogen by attack at several locations in the Aα chain[52,59-61] (step 1), leading to release of several Aα chain peptides (fragments A, B, and C) and the first detectable residual fibrinogen product, fragment X_1, of M_r ~240 000–265 000.[62,63] Fragment X_1 consists of a degraded Aα chain (Aα') of M_r 25 000 and intact Bβ and γ chains.[51] Other forms of fragment X (e.g., fragment X_2) exist which have partially degraded Bβ and γ chains.[51,64] The origin of some of these variants is also shown in Figure 5. Fragment X_2 can arise (step 2) from fragment X_1 by symmetrical cleavage at the COOH terminal of the two Bβ chains, leading to a pair of β' chains of individual molecular weight 52 000 (these

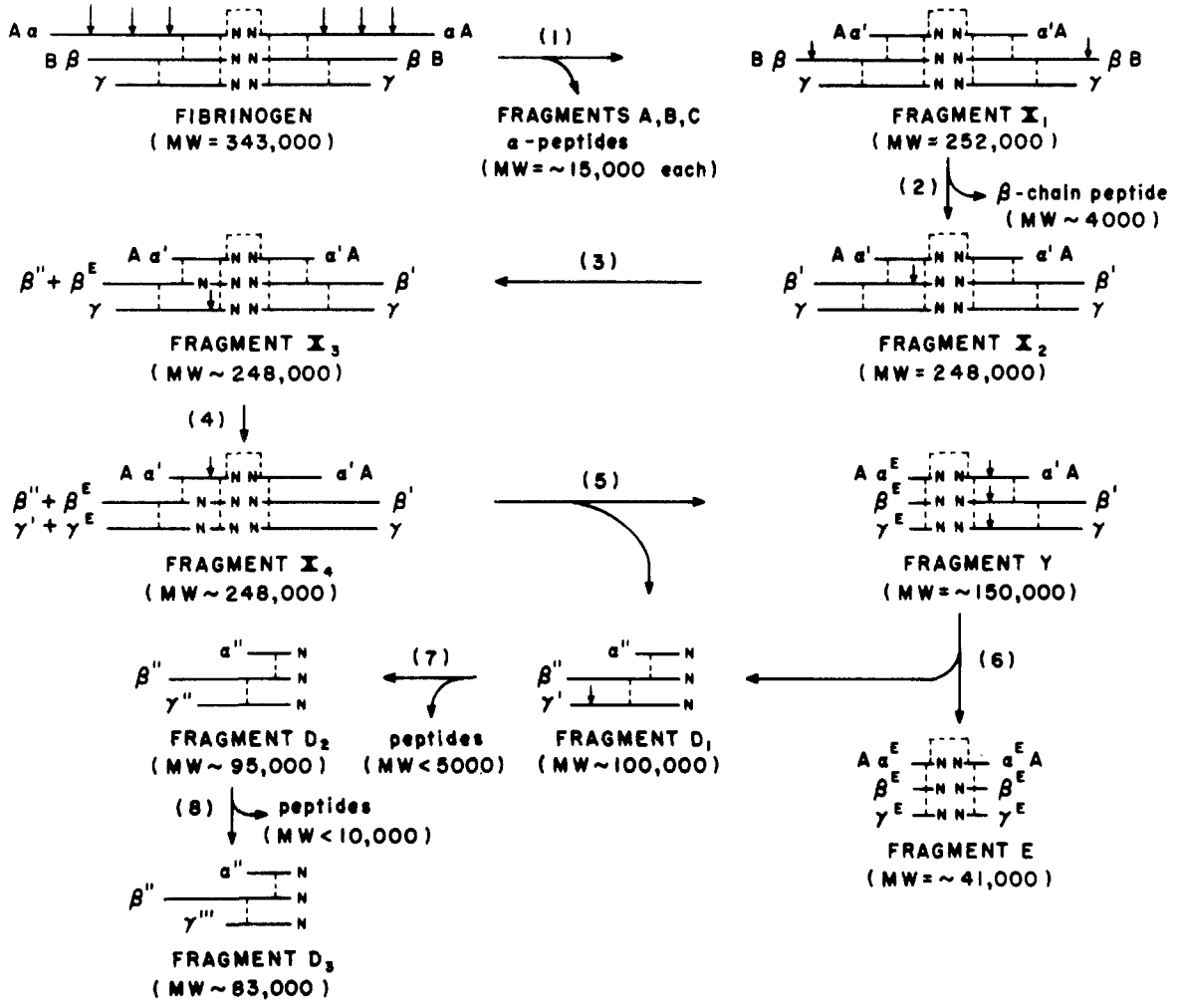


Figure 5. Proposed pathway(s) of the plasminogenolysis of human fibrinogen (taken from ref 51).

chains have also lost fibrinopeptide B). Since fragment D could not arise from fragment X_2 without additional peptide bond cleavages in fragment X_2, other structural variants of fragment X, exemplified by fragment X_3 and fragment X_4, must exist.[51] These latter molecules have not been isolated and characterized. However, these variants would arise by unsymmetrical plasminolysis at the NH_2 terminus of one of the two β' chains and one of the two γ chains, as indicated by steps 3 and 4 in Figure 5. Fragment X (presumably all forms) is clottable by thrombin.[63] From a molecule such as fragment X_4, plasminolysis at the NH_2-terminal portion of the $A\alpha'$ chain leads to release of the well-known fibrinogen fragments D and Y[63] (step 5). Purified fragment D, of M_r ~83 000–100 000,[52,63] consists of a degraded $A\alpha$ chain (α'') of M_r ~15 000, a degraded $B\beta$ chain (B″) of M_r ~44 000, and a series of γ chains, γ' (fragment D_1), γ'' (fragment D_2), and γ''' (fragment D_3), of M_r 42 000, 37 000, and 25 000, respectively.[51,52,65] These different forms of fragment D, which can be isolated,[66] result from plasminolysis of the γ chains, in a fashion depicted by steps 7 and 8 of Figure 5.[51,52] Likely, other such variants of fragment D exist.[65] Degradation of the γ chains, beyond γ', in fragment D can be inhibited by addition of Ca^{2+} to the plasminolysis mixture.[67] Fragment Y[63] (resulting from step 5 of Figure 5) with a total molecular weight of approximately 155 000,[63] is an unsymmetrical molecule consisting of the $A\alpha'$, β', and γ chains, described above, and extensively degraded $A\alpha$ ($A\alpha^E$), $B\beta$ (B^E), and γ (γ^E) chains.[51] The molecular weights of the $A\alpha^E$, β^E, and γ^E chains are approximately 10 000, 8000, and 5000, respectively.[51] Upon cleavages, by plasmin, of fragment Y in the $A\alpha'$, $B\beta'$, and γ' chains (step 6 of Figure 5), a further molecule of fragment D is released, as well as fragment E, M_r ~50,000,[63] consisting of pairs of $A\alpha^E$, β^E, and γ^E ($A\alpha^E{}_2B^E{}_2\gamma^E{}_2$). Fragments Y, D, and E are not clottable by thrombin.[63]

On the basis of the amino acid sequence of the $A\alpha$ chain of human fibrinogen[60,68,69] and the fragments which are observed upon short-term plasminolysis of fibrinogen,[61] some of the plasmin cleavage sites in this chain have been forwarded. Plasminolysis sites likely exist at Ly_{508},[70] Lys_{583},[70] and Ly_{539}[61] to produce known fragments. Another site (or sites) of plasmin digestion may exist of Lys_{413}, Lys_{418}, Lys_{421}, Lys_{427}, or Arg_{424} to produce an $A\alpha$ chain fragment, M_r 48 000.[61]

Molecular aspects of the plasminolysis of human fibrin have also been studied. The conversion of human fibrinogen to fibrin is catalyzed by the serine protease thrombin. To accomplish this conversion, thrombin catalyzes cleavage of fibrinopeptides A (16 residues) and B (14 residues) from the amino terminus of the $A\alpha$ and $B\beta$ chains, respectively, yielding the α and β chains of the fibrin monomer.[71–73] As a result of liberation of these small peptides, the fibrin monomer polymerizes,

through noncovalent interactions, to yield a form of fibrin. Thrombin also catalyzes activation of factor XIII (fibrin-stabilizing factor), which then catalyzes formation of ϵ-(γ-glutamyl)lysine bonds between the monomers of fibrin.[74-76] Two intermolecular cross-link sites exist per γ chain and lead to γ–γ dimers.[77] On each γ chain, the donor Lys residue is located six residues penultimate to the COOH terminus, and the acceptor Gln residue is situated 14 residues penultimate to the COOH residue.[77] Four intermolecular cross-link sites are located in each α chain, leading to formation of α–α multimers.[78] Two Gln residues which act as cross-link acceptors are located at positions 328 and 366 of each α chain.[68] The two Lys cross-link donors are believed to be contributed from among the following five; Lys_{539}, Lys_{556}, Lys_{562}, Lys_{580}, and Lys_{583}.[68]

The mode of action of Lys_{77}-Pm on non-cross-linked fibrin is virtually identical with that of fibrinogen, except that the β and γ chains are degraded at the same rate in non-cross-linked fibrin.[79] Also, in the case of non-cross-linked fibrin, the β' chain (see Figure 5) of M_r 52 000 is not observed as an intermediate of plasminolysis[52,79] as it was in plasminolysis of fibrinogen. Here, degradation of the Bβ chain proceeds directly to the β'' chain (see Figure 5), of M_r 44 500, likely as a result of simultaneous cleavage of the peptide bonds necessary for formation of β''. The rate of this cleavage is approximately equal to the rate of degradation of the γ chain.[79] Similar forms of fragments Y, D, and E are obtained from plasminolysis of non-cross-linked fibrin as were obtained from plasmic digestion of fibrinogen-[51,52,66,79]

The major differences between plasmin digestion of highly cross-linked fibrin vs. fibrinogen and non-cross-linked fibrin result from the fact that different products are observed as a result of covalent cross-linking of the various peptide chains.[66,79] Although fragment E from fibrinogen, non-cross-linked fibrin, and highly cross-linked fibrin appear to be similar physical and chemical entities (the Aα^E and γ^E chains do not contain cross-link sites), differences have been found in immunochemical properties[80] and plasma survival times[81] of fragment E obtained from fibrinogen and highly cross-linked fibrin which may be related to subtle differences in plasmic degradation of each fragment E.[82,83] On the other hand, fragment D, obtained from plasmic digests of highly cross-linked fibrin, exists as a dimer.[66] This dimer is stabilized by two cross-links in the COOH region of the γ' chain (see Figure 5 for reference). Thus, fragment D from highly cross-linked fibrin consists of two α'' chains not possessing cross-link sites, each of M_r ~44 500, and one γ'–γ' cross-linked chain of total M_r ~81 000.[66,79] Further degradation of the γ'–γ' chains to the other γ-chain variants found in various fragment D molecules obtained from fibrinogen and non-cross-linked fibrin does not occur in the fragment D dimer obtained from highly cross-linked fibrin.[79] This suggests that the presence of cross-links in the γ chain prevents further plasmin action of the γ' chain.

The fragment Y species which results from plasminolysis of highly cross-linked fibrin also exists as a dimer, through cross-linking at the γ–γ chains (the cross-linked sites at the Aα^E and Aα' sites have been cleaved from the molecule). This species should contain two Aα^E chains, two Aα' chains, two β^E chains, two β' chains, two γ^E chains, and one γ–γ chain dimer. The total molecular weight of the fragment Y dimer should be approximately 300 000.[79] Various fragment X species likely exist from plasmic digestion of highly cross-linked human fibrin. These are multimeric species, stabilized by cross-links in each α chain of fragment X. No cross-link sites are present in Aα' chains of fragment X.[52] Thus, fragment X contains n + 4 Aα' chains, n + 2 β chains, and n γ–γ dimers, where n is the polymerization number of cross-linked fibrin.[79] In addition to the above, formation of complexes consisting of (a) covalent DD noncovalently linked to E, (b) covalent D-D-E noncovalently linked to covalent D-D-E, and (c) covalent D-X-D noncovalently linked to covalent Y-Y have been shown to exist in plasmic digests of highly cross-linked fibrin.[83]

IV. Mechanism of Activation of Plasminogen

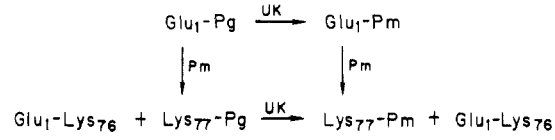
Activators of plasminogen have been found to be present in a variety of sources. The two most widely studied activators have been urokinase and streptokinase.

A. Urokinase

Urokinase (UK) is a serine protease which is inhibited by DFP but not by TLCK.[84] Urokinase has been isolated from urine and from cultures of embryonic kidney cells.[85] Two molecular forms of this enzyme, of M_r 31 300 (S-1) and 54 700 (S-2), have been shown to exist.[86] Available evidence strongly suggests that the S-1 form of UK is a degradation product of the S-2 form.[87,88] No clinical differences for pulmonary emboli dissolution have been reported for the S-1 or S-2 forms.[89] Urokinase and plasmin have similar substrate specificity for small molecules, although the kinetic constants of the two enzymes differ. For the substrate H-Glu-Gly-Arg-*p*-nitroanilide, S-1 UK and S-2 UK possess similar steady-state rate constants, the K_m determined to be ~200 μM and the k_c determined to be ~20 s^{-1}. Regarding possible protein substrates, the only UK substrate is plasminogen.

The molecular mechanism of activation of plasminogen with UK has been studied. At one time it was believed[90-92] that the molecular mechanism of activation of Glu_1-Pg to Lys_{77}-Pm, by UK, involved, first, UK-catalyzed cleavage of the Lys_{76}–Lys_{77} bond in Glu_1-Pg, by UK, yielding Lys_{77}-Pg, and, second, UK-catalyzed cleavage of the Arg_{560}–Val_{561} peptide bond in Lys_{77}-Pg, yielding Lys_{77}-Pm. We initially demonstrated that when activation of rabbit Glu_1-Pg by UK was carried out in the presence of an inhibitor (PTI) which immediately neutralized the plasmin generated as a consequence of activation, only Glu_1-Pm was formed.[26] Subsequently, this was shown to be the case also for activation of human Glu_1-Pg.[4,93] Thus, UK was capable of cleavage of the Arg_{560}–Val_{561} peptide bond without prior cleavage of the Lys_{76}–Lys_{77} peptide bond. Further, we demonstrated for both the human[4] and rabbit systems[27] that plasmin served as an enzyme for cleavage of the Lys_{76}–Lys_{77} peptide bond in the Glu_1-Pm·PTI complex and that UK did not catalyze this bond cleavage at a rate which was appreciable enough to implicate UK in this aspect of the molecular mecha-

nism. The molecular mechanism which we proposed for the human system is summarized as[4]



The initial step in the activation is cleavage of the Arg_{560}–Val_{561} peptide bond in Glu_1-Pg, forming Glu_1-Pm. This form of plasmin, autocatalytically, cleaves the Lys_{76}–Lys_{77} peptide bond in the Glu_1-Pm heavy chain, yielding Lys_{77}-Pm. Either Glu_1-Pm or Lys_{77}-Pm can catalyze cleavage of the Lys_{76}–Lys_{77} peptide bond in any Glu_1-Pg not yet activated, forming Lys_{77}-Pg, which is readily activated[94,95] to Lys_{77}-Pm. The exact pathway for formation of Lys_{77}-Pm, from Glu_1-Pg, is greatly dependent upon the pH, temperature, and buffer components employed.[4]

B. Streptokinase

Streptokinase (SK) is a catabolic byproduct of B-hemolytic streptococcus and can catalyze activation of plasminogen in a species specific fashion. No synthetic substrate has yet been found for SK. The only protein substrates known are plasminogens from human,[96] monkey,[97,98] baboon,[97] chimpanzee,[97] cat,[98] dog,[98] and rabbit[98,99] plasmas. SK is a monomeric protein, of M_r 45 000–50 000,[100,101] containing no carbohydrate or lipid. No cysteine or cystine is present in the molecule.[100,101] Circular dichroism analysis indicates that the helical content of SK is of the order of 10–12%.[101] The particularly fascinating feature of this activator is that is has no proteolytic activity, of itself, and the question arises as to the manner in which it can catalyze cleavage of the Arg_{560}–Val_{561} peptide bond, necessary for activation of plasminogen. Over the years, several laboratories have shown that SK functions by first interacting with another protein (proactivator) in order to form a moiety capable of activation of plasminogen (plasminogen activator). This activator has been shown to be composed of a complex of SK with either plasminogen, plasmin, or both.

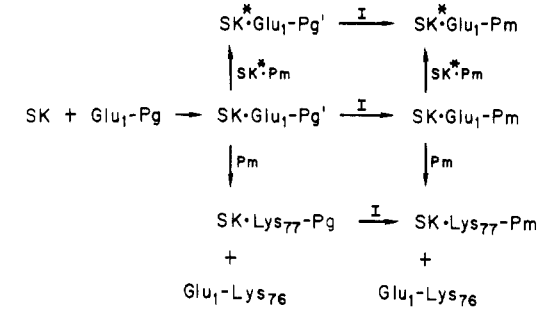
Studies from different laboratories have clearly shown that there are two important steps in the SK-catalyzed activation of plasminogen.[102-106] The first step involves formation of the proteolytic species which serves as the agent which catalyzes the cleavage of the Arg_{560}–Val_{561} peptide bond necessary for activation of plasminogen. The events which occur in the formation of this activator species are generally agreed upon and are summarized as

$$HPg + SK \rightleftharpoons SK{\cdot}HPg \rightarrow SK{\cdot}HPg' \rightarrow SK^*{\cdot}HPm$$

$$SK + HPm \rightarrow SK^*{\cdot}HPm$$

Here, SK and human plasminogen (HPg) bind in an equimolar complex, yielding SK·HPg. As a result of this interaction a conformational alteration occurs in the complex, yielding SK·HPg′, possessing an active site.[104,105] We have directly shown that this active site resides in the plasminogen moiety of the complex.[106] This complex is not stable, and is rapidly altered to a complex (SK*·HPm) of streptokinase and human plasmin (HPm). This is accomplished by an intramolecular cleavage[107,108] of the Arg_{560}–Val_{561} peptide bond

SCHEME I



in the SK·HPg′ complex, catalyzed by the active site in the plasminogen moiety of the complex. Concomitant with formation of HPm, in the complex, SK is proteolytically modified to an altered form (SK*).[101,105,106] The SK*·HPm complex can also be formed from SK and HPm. This altered form of SK possesses a molecular weight of 36 000,[101] suggesting that a peptide(s) of total molecular weight of approximately 9000 has been removed. Our results have shown[101] that this peptide(s) is removed from the amino terminus of native SK. When isolated, SK* appears to possess the same functional properties as does native SK.[101]

We have extended these molecular studies on the formation of plasminogen activator in order to understand the role of the various enzymes which are produced during formation of SK*·HPm. The molecular events which occur are shown in Scheme I.[108] In this scheme, SK and Glu_1-Pg form the SK·Glu_1-Pg′ complex, containing an active site in the plasminogen moiety, which through an intramolecular conversion (I) yielding SK·Glu_1-Pm. In the presence of small amounts of free plasmin (Pm), the SK·Glu_1-Pg′ complex is converted to SK*·Lys_{77}–Pg′, which, through an intramolecular conversion, yields SK*·Lys_{77}-Pm. This latter species can also form from SK·Glu_1-Pm, in the presence of small amounts of free plasmin (Pm). SK·Glu_1-Pg′ can also be converted to SK*·Glu_1-Pg′, by SK*·Pm. The SK*·Glu_1-Pg′ can also, through an intramolecular conversion, be converted to SK*·Glu_1-Pm, which can also be formed from SK·Glu_1-Pm, by SK*·Pm. Most of these species are transiently formed, and the most stable form of plasminogen activator is SK*·Lys_{77}-Pm.

Although neither Glu_1-Pm nor Lys_{77}-Pm is capable of cleavage of the Arg_{560}–Val_{561} peptide bond in Glu_1-Pg or Lys_{77}-Pg, catalytic levels of the SK*·Lys_{77}-Pm equimolar complex[102-106] and/or the SK–plasminogen complex,[105,106,109] containing the active site, are fully capable of catalyzing this activation. This step is accomplished via a proteolytic reaction, using the active site of the plasmin[110,111] (or plasminogen) within the complex. Whether all of the above forms of the activator complex are also capable of activation of plasminogen is not known at this juncture, but is assumed to be the case. Therefore, it appears as though SK* alters the enzyme specificity of Pm when the latter is bound in a complex with SK*. This is substantiated by the data of Figure 6.[106] Here, the activity of Lys_{77}-Pm toward three potential substrates, viz., α-*N*-tosyl-L-arginine methyl ester (TAME), azocasein, and plasminogen, is evaluated, in the presence of increasing levels of SK. Clearly, the TAME activity of Lys_{77}-Pm unaffected by the addition of SK, suggesting that access of small molecules to the plasmin active site is not in-

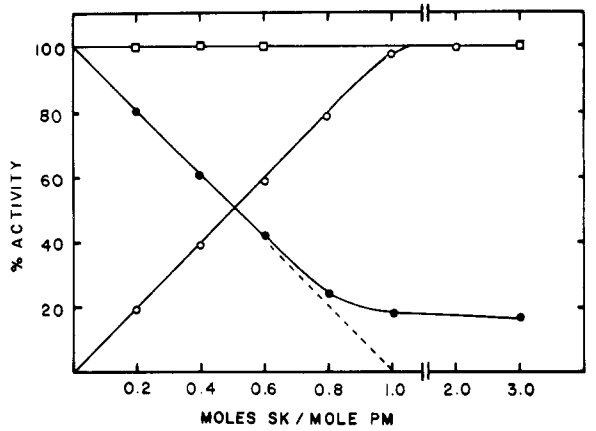


Figure 6. Activity of human Lys_{77}-Pm toward TAME (□), azocasein (●), and bovine plasminogen (○), as a function of added streptokinase (taken from ref 106).

TABLE IV. Steady-State Amidase Activity of Various Human Plasmin·SK* Complexes toward S-2251, at 37 °C, pH 7.4

enzyme	K_m, mM	k_{cat}, s^{-1}	K_I, mM[a]
SK*·Lys_{77}-Pm	0.26 ± 0.04	28.8 ± 1.3	0.13 ± 0.02
SK*·Val_{354}-Pm	0.28 ± 0.04	33.5 ± 1.3	0.09 ± 0.02
SK*·Val_{442}-Pm	0.27 ± 0.04	34.5 ± 1.3	0.09 ± 0.02

[a] Data for benzamidine hydrochloride.

fluenced by SK. The general proteolytic activity of plasmin toward azocasein, however, is progressively inhibited by SK addition, and extrapolation of these data to zero proteolytic activity indicates that the equimolar SK*·Lys_{77}-Pm complex would possess no activity of this type. Concomitant with the loss of general proteolytic activity is the progressive increase in plasminogen activation capability of Lys_{77}-Pm upon addition of SK. Maximal activator activity is accomplished at a 1:1 molar ratio of SK and Lys_{77}-Pm. Thus, it appears as though plasminogen is specifically directed into the plasmin active site of the SK*·Lys_{77}-Pm complex, perhaps by interaction with SK*.[112] Similar experiments have been published by Markus and Werkheiser.[109]

The steady-state amidase activity of the SK complexes of Lys_{77}-Pm, Val_{354}-Pm, and Val_{442}-Pm toward S-2251 have been studied in several laboratories.[29,48,113,114] Our data are listed in Table IV.[48] Values for K_m, k_c, and K_I (for benzamidine hydrochloride) for each of these species are virtually identical with each other. This suggests that the plasmin heavy chain has little influence on the steady-state values of SK*·plasmin toward this peptide substrate. However, the K_m and K_I values for the SK*·plasmin complexes are significantly lower than for each corresponding uncomplexed Pm. This indicates that tighter binding of benzamidine hydrochloride, and perhaps S-2251, occurs to plasmin complexed with SK* compared to free plasmin. On the other hand, the k_c values for each plasmin complexed to SK* are slightly higher than for free plasmin, indicating that SK* enhances the catalytic efficiency of plasmin, at least toward the peptide substrate studied.

Pre-steady-state kinetic constants for the SK*·plasmin complexes with *p*-nitrophenyl *p*-guanidinobenzoate have also been determined in our laboratory,[48] and the results are listed in Table V. Values for K_s for each species are approximately equal to each other but lower than for free plasmin, again indicating that binding of substrates (or competitive inhibitors) to SK*·plasmin is tighter than to free plasmin. However, the acylation rate constants (k_2) are not much different than their corresponding values for free plasmin.

TABLE V. Pre-Steady-State Amidase Activity of Various Human Plasmin·SK* Complexes toward the Substrate *p*-Nitrophenyl *p'*-Guanidinobenzoate, at 30 °C, pH 8.3

enzyme	K_s, μM	k_2, s^{-1}
SK*·Lys_{77}-Pm	1.4 ± 0.3	0.31 ± 0.05
SK*·Val_{354}-Pm	1.9 ± 0.3	0.28 ± 0.05
SK*·Val_{442}-Pm	2.3 ± 0.4	0.57 ± 0.08

TABLE VI. Steady-State Activation Parameters for Various Activators toward Human Plasminogen, at pH 7.4, 37 °C[a]

activator	k_{Pg}/K_{Pg}, μM^{-1} min^{-1}, for Glu_1-Pg	for Lys_{77}-Pg	for Val_{442}-Pg
SK·Glu_1-Pg'[b]	74.8	476.3	nd[c]
SK·Val_{442}-Pg'[b]	102.1	81.0	nd
SK·Lys_{77}-Pm[b]	39.0	nd	nd
SK·Val_{442}-Pm[b]	14.8	nd	nd
UK (S1)	23.9	13.6	nd
UD (S2)	27.4	26.5	16.2

[a] Data from ref 114. [b] It has not been determined whether SK actually exits as SK* in these complexes. [c] Not determined.

C. Kinetic Mechanism

The kinetic mechanism of human plasminogen activation has been studied with a variety of activators.[114] It has been found that the following mechanism can accommodate all activator species:

$$\pi + Pg \overset{K_D}{\rightleftharpoons} A$$

$$A + Pg \overset{K_{Pg}}{\rightleftharpoons} A{\cdot}Pg \xrightarrow{k_{Pg}} A + Pm$$

where π is the activator and A the activating enzyme. All other abbreviations have been used previously. This mechanism suggests that an activator (π) must combine with Pg to form the actual enzyme (A) which is responsible for plasminogen activation. The kinetic and molecular mechanisms as applied to SK are in agreement with each other. However, when UK activation is considered, the molecular mechanism does not implicate a UK·Pg' or a UK·Pm complex as the activating enzyme. However, if K_D is large in that case, a UK·Pg or UK·Pm complex would not be present in significant amounts to detect, molecularly. In any event, the discrepancy is minor and can readily be accommodated, if true, in the molecular mechanism. When the above scheme is utilized, steady-state activation parameters for several activators toward different plasminogen forms have been determined.[114] A summary of the data is listed in Table VI. For Glu_1-Pg activation, the most effective activator in this list is SK·Val_{442}-Pg'. The S-1 and S-2 forms of UK were equally effective. For Lys_{77}-Pg activation, the most effective activator is SK·Glu_1-Pg'. These differences in k_{Pg}/K_{Pg} ratios for Lys_{77}-Pg activation are solely due to differences in k_{Pg}.[114] Thus, it appears as though the NH_2-terminal portion of Pg, at least to residue 442, is not involved in the value of the Michaelis constant for activation. Regarding Lys_{77}-Pg activation, it appears as though UK form S-1

TABLE VII. Effect of ε-ACA on the Conformation of Human Plasminogen and Plasmin

	value obtained for				
	$S^0_{20,w}$, S			ρ_h, ns[d]	
protein	N[a]	N + ε-ACA[b]	ΔF,[c] %	N[a]	N + ε-ACA[b]
Glu_1-Pg	5.73	4.80	+7-8	262	156
Lys_{77}-Pg	4.75	4.65	0	160	158
Glu_1-Pm	5.68	4.83	+7-8	258	160
Lys_{77}-Pm	4.80	4.82	0	157	160

[a] pH 7.8 at 25 °C in the absence of ε-ACA. [b] pH 7.8 at 25 °C in the presence of 0.1 M ε-ACA. [c] Represents the intrinsic fluorescence change in the protein after addition of 0.1 M ε-ACA to the buffer system employed. [d] Represents the rotational relaxation time of the fluorescent-labeled proteins.

is less effective than UK form S-2. This difference resides in the value for k_{Pg}.

It was mentioned above that SK exhibited species specificity toward other mammalian plasminogens: sheep plasminogen is refractive to SK activation and rabbit plasminogen is only weakly activated by SK.[98] However, all species of plasminogen are activated equally well by the SK*·human Lys_{77}-Pm complex.[98] This suggests that the basis of species specificity of SK resides in the ability of various plasminogens to form activator complexes with SK. In the rabbit system, we found that SK was rapidly degraded to ineffective forms of the protein upon complex formation with rabbit plasmin.[106] This could contribute to explanation of the reason(s) for the lack of activity of SK toward certain species of plasminogen.

Recent evidence has shown that a protein which potentiates the activity of SK toward human plasminogen activation[115-121] and rabbit plasminogen activation[122] exists in plasma. In both cases, it was found that this same effect could be observed when fibrinogen,[120,121] fibrin,[120] or fibrinogen degradation products[122] were added to the systems of interest. This highly interesting development is being pursued by several laboratories.

V. Interaction of Antifibrinolytic Amino Acids with Plasminogen

A. Chemical and Physical Studies

Amino acids, such as ε-ACA and its analogues, have long been known to produce in vivo antifibrinolytic effects, i.e., inhibit dissolution of the blood clot. These amino acids also induce striking conformational alterations in Glu_1-Pg. It was initially recognized by Alkjaersig[123] that the $S^0_{20,w}$ value of human plasminogen (uncharacterized as to its amino terminus) was dramatically reduced in the presence of ε-ACA. Over the years, we have considerably expanded this finding to the effects of ε-ACA and its analogues on the conformation of various forms of plasminogen and plasmin by a variety of conformational methods. A summary of a portion of the results obtained[19,20,124] is given in Table VII. Clearly, at levels of ε-ACA which saturate its effect on the property of interest, molecules which possess the amino-terminal peptide, Glu_1–Lys_{76}, undergo, as revealed by $S^0_{20,w}$ values, perhaps among the most dramatic ligand-induced conformational alterations heretofore described. On the other hand, molecules not possessing this peptide, e.g., Lys_{77}-Pg and Lys_{77}-Pm, although fully capable of binding ε-ACA (vide infra), do not appear to undergo a conformational alteration of this magnitude, as revealed by the values of $S^0_{20,w}$. The same trends are noted in examination of a rotational property of the system, ρ_h.[20] Here, a dramatic decrease in the rotational relaxation time of Glu_1-Pg and Glu_1-Pm is noted upon addition of ε-ACA (0.1 M) to the system, indicating a "loosening" of the internal structure of the molecules.[20] Again, Lys_{77}-Pg and Lys_{77}-Pm do not undergo significant changes in this parameter upon binding ε-ACA. In the case of all properties examined, Lys_{77}-Pg and Lys_{77}-Pm in the presence or absence of ε-ACA appear to possess a gross conformation similar to Glu_1-Pg or Glu_1-Pm in the presence of saturating levels of ε-ACA. Thus, the amino-terminal peptide (Glu_1–Lys_{76}) appears to significantly control the gross conformation of plasminogen as well as the conformation alterations induced by ε-ACA.

TABLE VIII. Effect of ε-ACA Analogues on Human GLU_1-Pg Conformation

compd	S	ΔF, %[b]	ρ_h, ns[c]
none	5.79		262
Gly, 3-APr[a]	5.72	0	259
4-ABt	4.90	7.2	157
5-APn	4.86	7.0	156
ε-ACA	4.87	7.0	156
7-AHp	4.85	7.1	155
8-AOt	4.88	7.0	160
t-AMCHA	4.79	11.5	160
L-Lys	4.80	7.0	159

[a] In all cases, the concentration of amino acids was 0.1 M. [b] Refers to the change in intrinsic fluorescence of Glu_1-Pg upon addition of the ligand of choice. [c] Refers to the rotational relaxation time.

The above data have been further extended to a study of the ability of analogues of ε-ACA to effect the conformational change of human and rabbit Glu_1-Pg. The compounds employed and their effect on the gross conformation of Glu_1-Pg are summarized in Table VIII.[124] The amino acids L-glycine (Gly) and 3-aminopropionic acid (3-APr) do not affect the gross conformation of human Glu_1-Pg. However, 4-aminobutyric (4-ABt) acid through 8-aminooctanoic acid (8-AOt), in which progressive addition of a methylene group between the COO^- and NH_3^+ takes place, allows the conformational change of Glu_1-Pg to occur, as monitored by decreases in the $S^0_{20,w}$ and ρ_h and an increase in the intrinsic fluorescence (ΔF) of the protein. Interposition of a cyclohexane ring between the COO^- and NH_3^+ groups, as in *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid (t-AMCHA), and insertion of an NH_3^+ group α- to the COO^- (L-Lys) do not alter the occurrence of the conformational alteration in Glu_1-Pg.

Since the alterations in the physical properties, examined above, induced by these agents is so large, we thought it possible to titrate these parameters in order to obtain a quantitative measure of their efficacy in promoting the conformation alteration in Glu_1-Pg (similar effects are observed for rabbit Glu_1-Pg and human Glu_1-Pm). As seen from an example of the data obtained in Figure 7, this can be effectively accomplished.[2,124] Here, the total concentration of amino acid at which one-half of the conformational change is produced ($C_{0.5}$) is taken as a measure of the relative ef-

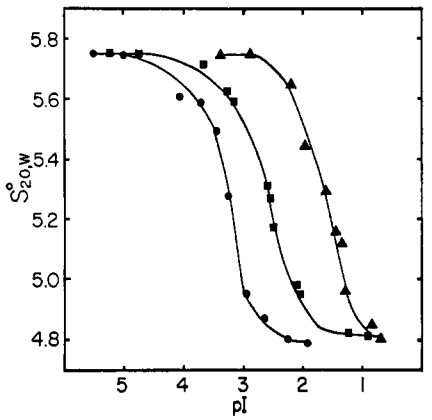


Figure 7. The effect of various concentrations of t-AMCHA (●), ϵ-ACA (■) and L-lysine (▲), plotted as the negative logarithm of the amino acid concentration (pI), on the $S^0_{20,w}$ of human Glu_1-Pg.

TABLE IX. $C_{0.5}$ for Various Amino Acids for Human GLU_1-Pg

amino acid	$C_{0.5}$ (mM) as revealed by S	ΔF	ρ_h
4-ABt	20.7	19.2	
5-APn	4.9	4.0	
ϵ-ACA	2.4	3.0	2.5
7-AHp	17.4	16.9	
8-AOt	32.5	33.0	
t-AMCHA	0.6	0.5	0.4
L-Lys	26.0	25.1	27.2

fectiveness of each agent. Although the example in Figure 7 represents titration of the $S^0_{20,w}$ value of Glu_1-Pg by three of these agents, viz., t-AMCHA, ϵ-ACA, and L-Lys_2, titration of the intrinsic fluorescence could as easily be employed for this purpose.[124] Values obtained for $C_{0.5}$ with a variety of amino acids, as revealed by titration of $S^0_{20,w}$, ΔF, and ρ_h, are listed in Table IX. From these data, for straight-chain α,ω-amino acids, it appears as though the effectiveness is maximal for ϵ-ACA. Insertion of an α-NH_2 group (L-Lys) detracts from the efficacy of these agents, while inclusion of a rigid ring system (as in t-AMCHA) appears to enhance the potency of these agents. Since c-AMCHA did not produce the conformational alternation in Glu_1-Pg, it would appear as though stereospecificity of the amino acid is an important consideration in the effectiveness of these amino acids.

An additional conclusion can be drawn from the data of Table IX. Essentially the same $C_{0.5}$ value for each amino acid is obtained by the three independent techniques for monitoring the conformational change. This suggests that each parameter is a monitor of the same conformational alteration. The nature of this alteration, produced by these amino acids, appears to be a generation of a more asymmetric and internally flexible form of the protein in the presence of these amino acids. Additionally, there would seem to be an alteration in the environment of a tryptophan(s) residue as a result of this alteration in conformation. This latter point is also obvious from the circular dichroism (CD) data published by Sjoholm et al.[125] These investigators observed a change in the CD spectrum of human plasminogen upon interaction with ϵ-ACA. The major spectral changes were in the wavelength range 280–305 nm, consistent with environmental alterations of Trp and Tyr in plasminogen, in the presence of ϵ-ACA.

The interaction of t-AMCHA and ϵ-ACA with human plasminogen and a number of its elastase-generated fragments has been studied. Rickli and Otavsky[126] initially discovered that the human Lys_{77}-Pm heavy chain (Lys_{77}-Arg_{560}), prepared by selective reduction of the two disulfide bonds linking the Lys_{77}-Pm heavy and light chains, was bound to a column of Sepharose–lysine columns, whereas the light chain was not retarded by this column. Studies from our laboratory[127] extended these observations to the isolated heavy chain of human Glu_1-Pm (Glu_1–Arg_{560}), and similar conclusions were drawn. In addition, we found that the alteration of the $S^0_{20,w}$ value of Glu_1-Pg and Glu_1-Pm by compounds of the ϵ-ACA class also occurred with the isolated heavy chain of Glu_1-Pm. The $C_{0.5}$ value of ϵ-ACA, toward Glu_1-Pg, was virtually identical with that for the Glu_1-Pm heavy chain. These results strongly suggest that ϵ-ACA (and its analogues) interact with plasminogen via its latent plasmin heavy chain.

Quantitative studies of the binding of ϵ-ACA and t-AMCHA with human plasminogen have been recently performed on well-characterized forms of the zymogen. Unfortunately, the two affinity chromatography forms of plasminogen were not resolved for any of the studies described below, possibly cloaking even more interesting conclusions. Markus et al.,[128] in contrast to earlier findings,[129,130] discovered that human Glu_1-Pg contained at least two noninteracting classes of binding sites for ϵ-ACA. One strong ϵ-ACA binding site of K_d = 9 μM and approximately five weaker sites of average K_d = 5 mM were obtained. L-Lysine interacted much more weakly than ϵ-ACA with human Glu_1-Pg, possessing a K_d of 320 μM for its strongest site.[128] On the other hand, t-AMCHA, while possessing one strong binding site for human Glu_1-Pg at approximately the K_d (1.1 μM) for ϵ-ACA, occupied its 4–5 weaker sites with a considerably lower average K_d (0.75 mM) than did ϵ-ACA.[131] Comparison of these results with the $C_{0.5}$ values for ϵ-ACA, L-Lys, and t-AMCHA (Table IX) strongly suggests that the conformational alteration of Glu_1-Pg induced by these amino acids results from their binding to the weak class of sites on Glu_1-Pg. The relative effectiveness of t-AMCHA, ϵ-ACA, and L-Lys in producing this conformational alteration can be completely explained by their relative strengths of interaction with the weak class of sites on Glu_1-Pg. The strong site for ϵ-ACA and t-AMCHA appears to have little influence on the gross conformation of Glu_1-Pg.

The mode of binding of t-AMCHA and ϵ-ACA to human Lys_{77}-Pg has also been investigated.[131,132] In the case of t-AMCHA, one strong site was shown to exist on Lys_{77}-Pg, of K_d = 2.2 μM, slightly weaker than the corresponding strong site for t-AMCHA on Glu_1-Pg. A slightly weaker t-AMCHA site on Lys_{77}-Pg, K_d of 36 μM, and two further sites, average K_d = 103 μM, were found. Similarly, one strong ϵ-ACA site (K_d = 35 μM), a weaker site of K_d of 260 μM, and approximately four additional sites of average K_d = 10 mM were obtained with human Lys_{77}-Pg. Thus, subtle differences do exist in the interaction of ϵ-ACA and analogues with Glu_1-Pg and Lys_{77}-Pg. In general, these involve (1) a decrease in the strength of interaction of the strong binding site in Lys_{77}-Pg compared to Glu_1-Pg, (2) a significant increase in affinity of the second site occupied by Lys_{77}-Pg compared to Glu_1-Pg, and (3) a decrease in affinity for

any additional sites for ε-ACA on Lys_{77}-Pg compared to Glu_1-Pg.

The binding isotherms of ε-ACA to the isolated "kringle" regions of human plasminogen have been determined. K1 has been isolated by gel filtration from a chymotryptic digest of K1–3.[133] This fragment possessed approximately one site for ε-ACA, of K_d 16.7 μM. K4 also possessed one site for ε-ACA, of K_d 36.4 μM. The affinity of K1 and K4 for antifibrinolytic amino acids is lost upon photooxidation of His but is not affected upon modification of Arg.[134] It has been suggested, from studies of this type, that His and possibly Asp are important for interaction of ε-ACA with K1 and K4. The value of the K_d for ε-ACA of 16.7 μM for K1 is slightly higher than the K_d value of 9 μM found for ε-ACA to the tightest site on human Glu_1-Pg[128] and slightly lower than the value of 35 μM found for the tightest site on human Lys_{77}-Pg.[131] Addition of a NH_2-terminal peptide (Glu_1–Met_{57}) to K1 allows the K_I value for the single ε-ACA site to be decreased to 12.5 μM,[133] a value nearly identical with that of the tightest ε-ACA site of Glu_1-Pg.[128] Thus, it appears reasonable to assume that the K1 region of human plasminogen contains the tight ε-ACA binding site, although the results are not entirely unambiguous. Specifically, the K_d value obtained for the K4 region, 36.4 μM, could also reflect the tight ε-ACA binding site, K_d = 35 μM, on Lys-Pg.[131] Assigning the ε-ACA site in this manner would of K_d to uncertainties as to the nature of the K1 site in intact plasminogen. The NH_2-terminal peptide (Glu_1–Met_{57}) does not influence the nature of the binding of ε-ACA to K4. Therefore, only a tentative but nonetheless a stimulating model of the role of the kringle structures in the binding antifibrinolytic amino acids could be assumed. One fact does, however, seem certain. The data of Figure 2 clearly show that all kringle regions can interact to some degree with ε-ACA, since all are retarded on the Sepharose–lysine affinity column. The one possible exception is K5, since Val_{442}-Pg (containing K5) is not significantly retarded by the column (see Figure 2).

Additional information regarding the nature of the interaction of ε-ACA with human plasminogen can be obtained from the DSC thermograms of this protein and its fragments in the presence of ε-ACA. The data are shown in Figure 8. The thermogram for Lys_{77}-Pg (Figure 8A) shows that ε-ACA stabilized the molecule to thermal denaturation (compare the data of Figure 8A to that of Figure 3A). In the presence of quantities of ε-ACA which are sufficient to saturate its sites on Lys_{77}-Pg, one region of the Lys_{77}-Pg molecule appears to possess a significantly elevated T_m of 74–76 °C. This thermal region, also present in K1–3 (Figure 8B), likely reflects binding of ε-ACA to K1. In the presence of ε-ACA, the major thermal transition(s) of Lys_{77}-Pg are shifted to a T_m of 66 °C, approximately 5 °C higher than that of native Lys_{77}-Pg. This region, also observed in K1–3, suggests that additional ε-ACA binding sites are present on K1–3. The thermogram of Val_{442}-Pg (Figure 8C) indicates that only a portion of the molecule has undergone a shift in T_m to 65 °C whereas the 50 and 61 °C T_m values are unchanged from those observed for Val_{442}-Pg in the absence of ε-ACA (Figure 3C). This reflects binding of ε-ACA to Val_{442}-Pg, possibly to K5. If so, this binding is very weak since Val_{442}-Pg is only

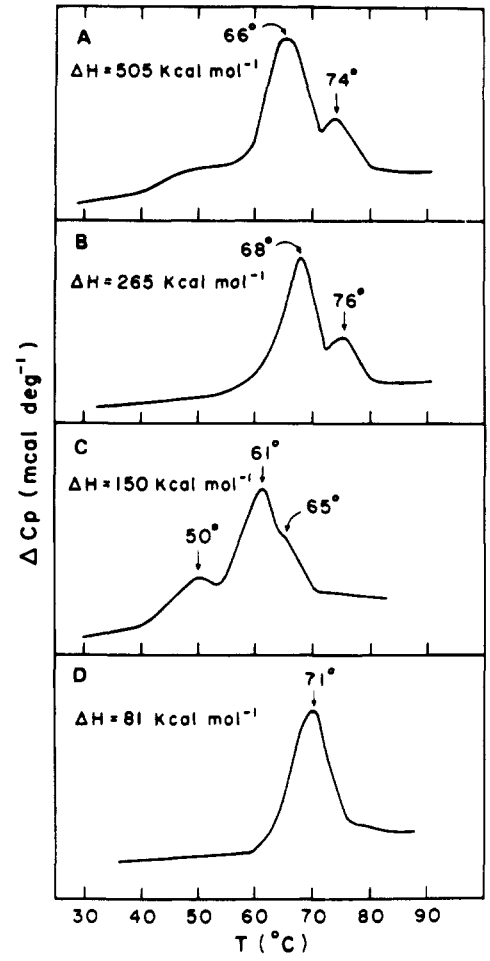


Figure 8. As in Figure 3 except that the ε-ACA concentration was 50 mM (taken from ref 23).

slightly retarded by the Sepharose–lysine column (Figure 2). Finally, the T_m of the thermogram of K4 (Figure 8D) in the presence of ε-ACA is considerably shifted from its value in the absence of ε-ACA (Figure 3D). This observation clearly is consistent with interaction of ε-ACA with K4. Since the ΔH values of K1–3, K4, and Val_{442}-Pg (the totality of these peptides represents the entire structure of Lys_{77}-Pg), when summed is approximately equal to the total ΔH of Lys_{77}-Pg, we conclude that the "kringle" structures likely exist as separate domains in plasminogen in the presence of ε-ACA. From the above results, it does appear as though each of the "kringle" structures are capable of interaction with ε-ACA.

B. Functional Studies

For a period of time, it was believed that the basis for the antifibrinolytic effects of compounds such as ε-ACA rested upon an inhibition of the activation rate of Glu_1-Pg when conformationally altered by ε-ACA. It was initially shown by Thorsen et al.,[135] Thorsen and Astrup,[136] Thorsen and Mullertz,[94] and Claeys and Vermylen[95] that a biphasic pattern of enhancement of the activation of human Glu_1-Pg followed by an inhibition of Glu_1-Pg activation existed upon increase of the ε-ACA concentration in the activation mixture when UK was the activator. On the other hand, only progressive inhibition, with no enhancement, was noted with increasing concentrations of ε-ACA when tissue activator was used in place of UK.[136] The inhibitory

effect of ε-ACA, at high concentrations, involves inhibition of the enzymatic activity of plasmin or UK, or both. The concentration dependence of the ε-ACA enhancement of Glu_1-Pg activation by UK[95,131,136] strongly suggested that only the weak ε-ACA sites were involved in this phenomena and that concentrations of ε-ACA which only saturated its strong binding site on Glu_1-Pg had only a slight inhibitory effect on the activation rate by UK.[131] Since the weak antifibrinolytic amino acid sites on Glu_1-Pg have also been shown to be responsible for the large conformational alteration produced in Glu_1-Pg, as discussed earlier in this review, investigators in this field now agree that conformationally altered Glu_1-Pg is activated much more rapidly than Glu_1-Pg in the absence of ε-ACA. Substantiation of this view has been provided by experiments which have shown that Lys_{77}-Pg, which possesses a gross conformation very similar to that of Glu_1-Pg when Glu_1-Pg is saturated with ε-ACA and like molecules (see preceding section), is activated by UK at a very similar rate as that of Glu_1-Pg in the presence of ε-ACA-like molecules.[95,131,135,136] Further, ε-ACA does not affect the activation rate (or conformation) of Lys_{77}-Pg.[95,131,135,136] Studies from our laboratory have shown that the rates of conversion of human Glu_1-Pg to Glu_1-Pm, as catalyzed by UK, Glu_1-Pm to Lys_{77}-Pm, as catalyzed by Lys_{77}-Pm, and Glu_1-Pg to Lys_{77}-Pg, as catalyzed by Lys_{77}-Pm, are all stimulated by ε-ACA.[124] Therefore, it appears as though all intermediates important to the activation of human Glu_1-Pg to Lys_{77}-Pm which possess the 76 amino acid amino-terminal peptide (and which undergo the conformational transition with ε-ACA) also undergo reactions in this sequence which are stimulated by ε-ACA.

Although the above findings are clearly established, they cannot explain the in vivo antifibrinolytic effect of molecules such as ε-ACA. In fact, the stimulation of activation of Glu_1-Pg, by ε-ACA, should lead to quite the opposite effect, i.e., a fibrinolytic tendency. Some light has been shed on the question by the discovery[137] that Glu_1-Pg possesses a weak affinity for fibrin whereas Lys_{77}-Pg has considerably stronger affinity in that regard. In whole plasma, approximately 4% Glu_1-Pg and 8% Lys_{77}-Pg are specifically bound to the fibrin clot.[138] Plasminogen bound to fibrin can be removed by addition of ε-ACA.[137] The concentration dependence of this effect indicated that the amount of ε-ACA required to reduce binding of Lys_{77}-Pg to one-half its initial value was 8.0×10^{-5} M, and the same value for t-AMCHA was 1.7×10^{-5} M.[138] These values are clearly inconsistent with the involvement of the weak antifibrinolytic sites in plasminogen and suggest that the role of the tight antifibrinolytic site is to mediate interaction of plasminogen and fibrinogen. Resolution of the question as to why Glu_1-Pg interacts more weakly with fibrin than Lys_{77}-Pg has not been clarified, since the tight binding site which exists for ε-ACA on Lys_{77}-Pg also exists on Glu_1-Pg (although not of precisely the same strength). In this regard, it is possible that the strong antifibrinolytic amino acid site on Glu_1-Pg is involved in interaction with the amino-terminal peptide not present in Lys_{77}-Pg which competes with the fibrin interactions of Glu_1-Pg.[139] Thus, at least three effects of ε-ACA on plasminogen and plasmin should exist. First, the antifibrinolytic role of those amino acids at low concentrations could be explained by their displacement of plasmin(ogen) from fibrin, and this effect involves the strong ε-ACA site in plasminogen. Second, at slightly higher ε-ACA concentrations (the region of effective titration of the weak ε-ACA sites), the activation rate of Glu_1-Pg in solution is enhanced. This should not lead to a pronounced fibrinolytic effect since activation would not occur on the fibrin surface. Third, at even higher concentrations, plasmin and UK activity are inhibited by ε-ACA, with K_d values of 58 mM[29] and 10 mM,[140] respectively.

At least two activators of plasminogen, e.g., pig heart activator and human vascular activator, have strong affinity for fibrin.[141,142] Therefore, it is not surprising that the presence of fibrin stimulates the activation rate of plasminogen by these activators.[142] On the other hand, activators such as SK and UK are not well adsorbed to fibrin, suggesting that these latter activators likely function, in vivo, by activation of plasminogen not absorbed to the clot.

The above considerations suggest that the K1 region in ε-ACA is likely involved in the binding of plasminogen to fibrin. In order to determine whether this region was important in plasmin–fibrinogen interactions, we have compared the fibrinogen digestion patterns obtained with Lys_{77}-Pm and Val_{442}-Pm. The results showed no large differences in the fibrinogen products obtained or in the rates of formation of these fibrinogen fragments with either plasmin. Since Val_{442}-Pm does not contain K1, 2, 3, or 4, it would appear that, while the K1–4 regions in plasmin may or may not be important for binding of Lys_{77} plasmin and fibrinogen, these regions do not seem to be crucial for plasminolysis of fibrinogen. However, the active site region is clearly important in this regard and must be a determining factor in interactions which are meaningful for plasminolysis of fibrinogen to occur. A similar conclusion has been reached by Wiman et al.,[143] who showed that ε-ACA did not greatly affect the K_I value of fibrinogen toward Lys_{77}-Pm when S-2251 was used as the substrate.

VI. Interaction of Human Plasmin with α_2-Antiplasmin

Several plasma protein proteolytic inhibitors are effective in inhibition of human Lys_{77}-Pm. These include α_2-macroglobulin, $C\bar{1}$ inhibitor, antithrombin III (stimulated by heparin), and α_1-antitrypsin. However, the most important plasma protein inhibitor of plasmin appears to be the recently discovered α_2-antiplasmin.[144-146] This is a single-chain glycoprotein of M_r ~70000.[145,147] α_2-Antiplasmin rapidly forms a reversible equimolar complex with Lys_{77}-Pm, which then converts to an irreversible complex.[146] The reaction scheme has been reported to be[147]

$$P + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PA \xrightarrow{k_2} PA'$$

where P is plasmin and A is α_2-antiplasmin.

The values of k_1, K_d (k_{-1}/k_1), and k_2 have been reported for various forms of plasmin.[148] Affinity chromatography form 1 of human Lys_{77}-Pm possesses a k_1 of 3.8×10^7 M^{-1} s^{-1}, a K_d of 1.9×10^{-10} M, and a k_2 of 4.2×10^{-3} s^{-1}. The values for affinity chromatography form 2 are 1.8×10^7 M^{-1} s^{-1}, 1.8×10^{-10} M, and $4.2 \times$

10^{-3} s^{-1}, respectively. For Val_{442}-Pm, these values are 6.5×10^5 M^{-1} s^{-1}, 1.9×10^{-9} M, and 4.2×10^{-3} s^{-1}, respectively.[148] These findings have been used to implicate the antifibrinolytic amino acid binding sites in the fast step of the interaction since k_1 is much lower for Val_{442}-Pm (lacking K1–4) than for Lys_{77}-Pm. The slow irreversible step (k_2) is the same for each form of plasmin, presumably showing that these sites are not important in this phase of the reaction.[148] The final complex (PA′) is a 1:1 complex of α_2-antiplasmin and the plasmin light chain.[145] No peptide bonds appear to be cleaved in the α_2-antiplasmin, but a peptide of M_r ~14 000 may be cleaved from the amino terminus of the plasmin light chain as a consequence of complex formation. The rate at which Lys_{77}-Pm reacts with this inhibitor, in the initial phase, which is nearly diffusion controlled, suggests that α_2-antiplasmin may be the major inhibitor of plasmin in plasma. Several other lines of evidence point to the fact that the antifibrinolytic amino acid binding sites play an important role in the initial (fast) binding of α_2-antiplasmin to plasmin(ogen). As was seen above, the K_d for α_2-antiplasmin was much lower for Lys_{77}-Pm than for Val_{442}-Pm. The latter plasmin does not possess the major antifibrinolytic sites. The k_1 for Val_{442}-Pm and α_2-antiplasmin was found to be 6.5×10^5 M^{-1},[148] a value much smaller than the k_1 of 3.8×10^7 M^{-1} s^{-1} for Lys_{77}-Pm. Further, ϵ-ACA inhibits k_1 of the plasmin Lys_{77}-Pm–α_2-antiplasmin reaction with a K_d of approximately 20 μM[137] (concentration of ϵ-ACA required to reduce k_1 to 50% of its value in the absence of ϵ-ACA). This finding suggests that the strong ϵ-ACA binding site on Lys_{77}-Pm (presumed to be present, as it is in Lys_{77}-Pg) is important to the initial interaction of these molecules. It has been further demonstrated that substrate molecules such as S-2251 greatly inhibit the interaction between Lys_{77}-Pm and α_2-antiplasmin, also implicating the plasmin active site as important as in this interaction.[147] When Val_{442}-Pm is substituted for Lys_{77}-Pm, ϵ-ACA did not serve as an inhibitor of the interaction, except for weak competition with α_2-antiplasmin for the active site (K_d = 58 mM) of Val_{442}-Pm.[29] This is as expected if the antifibrinolytic amino acid binding sites play a significant role in the interaction of plasmin with the inhibitor. The effects of substrate on the reaction between Val_{442}-Pm and α_2-antiplasmin were essentially the same as for Lys_{77}-Pm.

These conclusions were verified and extended as the result of a study of the binding of various derivatives of plasminogen to α_2-antiplasmin.[143] The K_d (measured as the amount of the added component required to reduce the k_1 of Lys_{77}-Pm–α_2-antiplasmin to 50%) for Glu_1-Pg and α_2-antiplasmin was found to be 4.0 μM whereas that for Lys_{77}-Pg was 0.63 μM. This again indicates that the weaker binding of Glu_1-Pg to α_2-antiplasmin may result from interaction of the strong ϵ-ACA site in Glu_1-Pg with its amino-terminal peptide (Glu_1–Lys_{76}) competing with interaction of the strong ϵ-ACA site with α_2-antiplasmin. This peptide, not present in Lys_{77}-Pg, may allow Lys_{77}-Pg to bind more favorably to α_2-antiplasmin. On the other hand, the K_d for TLCK-inhibited Lys_{77}-Pm for α_2-antiplasmin was 0.43 μM. This latter result points to the importance of the intact plasmin active site in the interaction (cf. to the K_d of 1.9×10^{-10} M in uninhibited Lys_{77}-Pm) and also points to the fact that plasminogen and plasmin (with a blocked active site) possess similar noncovalent interactions with α_2-antiplasmin. The Lys_{77}-Pg heavy chain and K1–3 possess K_d values of 0.91 μM and 0.18 μM for α_2-antiplasmin, showing the importance of the strong ϵ-ACA site (present in K1 on each peptide) in the interaction. The K_d for K4 toward α_2-antiplasmin is 9.0 μM, a value considerably higher than that for K1–3. K4 lacks the strong ϵ-ACA site.

It has been shown that fibrin and α_2-antiplasmin bind to similar sites in plasmin(ogen).[148] The binding of Lys_{77}-Pg to fibrin is decreased to 50% at an α_2-antiplasmin concentration of approximately 1.1 μM (approximately the concentration found in normal plasma).[149] The presence of fibrinogen decreases the k_1 of the Lys_{77}-plasmin–α_2-antiplasmin reaction to 50% of its value at a fibrinogen concentration of 0.7 μM. No inhibition of the Val_{442}-Pm–α_2-antiplasmin reaction is seen with fibrinogen. Further, fibrinogen fragment E is as effective as fibrinogen in this regard whereas fibrinogen fragment D is slightly less so.[143] On the other hand, it has been observed that the Lys_{77}-plasmin–α_2-antiplasmin complex does not dissociate in the presence of fibrin.[150]

The above findings have led to an assessment of the role of the antifibrinolytic amino acid binding sites in physiological fibrinolysis.[152] Plasminogen, adsorbed to the fibrin surface, through these sites, is activated by activators which are also adsorbed to the fibrin surface. Although free plasmin would be rapidly neutralized by circulating α_2-antiplasmin, plasmin bound to the fibrin would be much less susceptible to such inactivation since its antifibrinolytic amino acid sites are occupied by fibrin and its active site likewise occupied. Both of these regions were shown to be involved in the k_1 of the Lys_{77}-Pm–α_2-antiplasmin interaction.

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