

Chemical Reviews

Volume 81, Number 5

October 1981

Recent Advances in the Chemistry of the Fibrinolytic System

FRANCIS J. CASTELLINO

Department of Chemistry, University of Notre Dame, Notre Dame, Indiana 46556

Received February 16, 1981

Contents

I. Introduction	431
II. Molecular Properties of Plasminogen	431
III. Molecular and Kinetic Properties of Plasmin	434
A. Physical and Chemical Properties	434
B. Kinetic Properties with Synthetic Substrates	434
C. Plasminolysis of Human Fibrinogen and Fibrin	436
IV. Mechanism of Activation of Plasminogen	437
A. Urokinase	437
B. Streptokinase	438
C. Kinetic Mechanism	439
V. Interaction of Antifibrinolytic Amino Acids with Plasminogen	440
A. Chemical and Physical Studies	440
B. Functional Studies	442
VI. Interaction of Human Plasmin with α_2 -Antiplasmin	443
VII. References	444

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



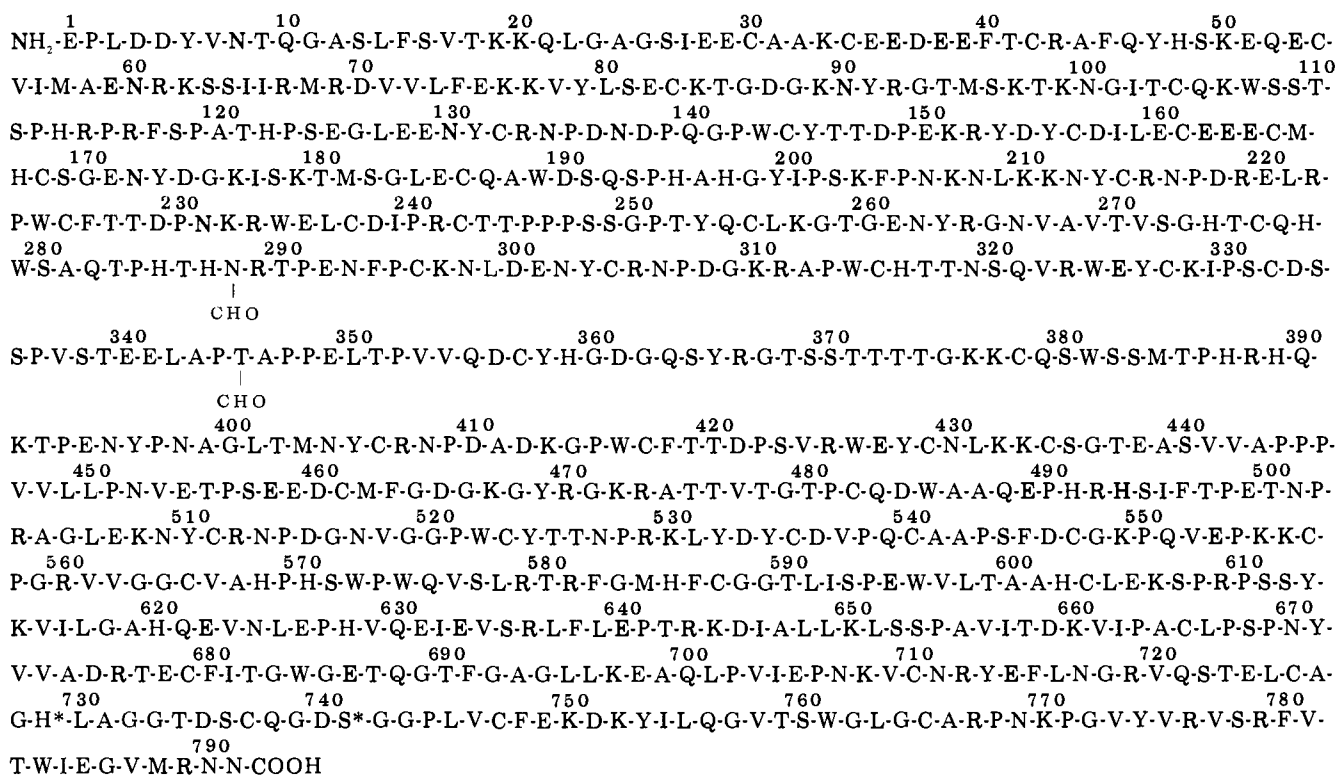
Francis J. Castellino was born in Pittston, PA. He received a B.S. in Chemistry from the University of Scranton in 1964, an M.S. in Biochemistry from the University of Iowa in 1966, and a Ph.D. in Biochemistry from the same university in 1968. At Iowa, his work, with Robert Barker, centered around the 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.

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

CHART I



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 modification² 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 rabbit⁶ and human³ systems. For rabbit plasminogen, F-1 was resolved into at least five subforms by isoelectric focusing techniques,⁶ with *pI* values 6.20–7.78. On the other hand, F-2 was resolved into at least five subforms, with *pI* 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 ~92000 (F-2)–94000 (F-1).⁴ The amino-terminal amino acid residue of each variant is Glu,⁸ and the

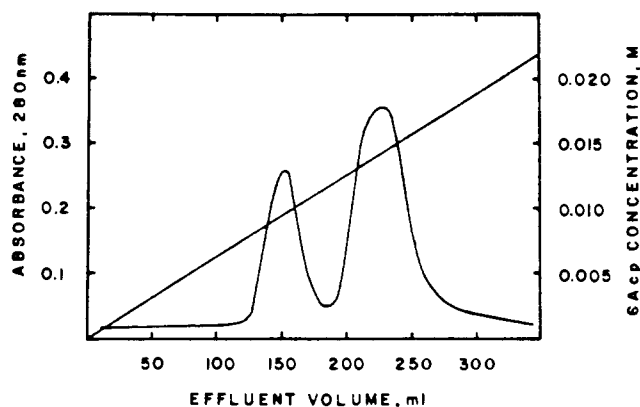
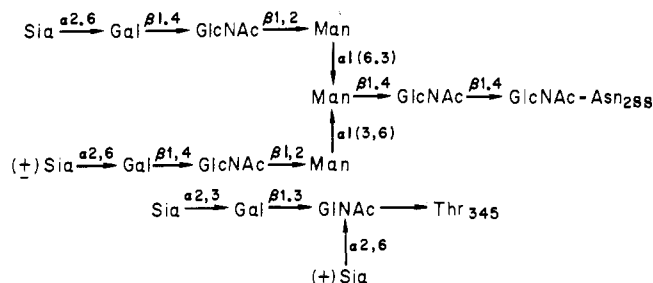


Figure 1. Elution of human Glu₁-Pg variants by affinity chromatography on Sepharose-lysine (taken from ref 2).

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 believed¹⁰ (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 Asn₂₈₈ and the other at Thr₃₄₅. The sequences are



On the other hand, native human F-2 only contains the Thr-based oligosaccharide. The residues labeled (\pm) 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 Glu₁-plasminogen). Exposure of Glu₁-plasminogen (Glu₁-Pg) to the protease, plasmin, for very short times results in cleavage of the amino terminal 76 residues from Glu₁-Pg, yielding a molecule of plasminogen which is of great importance to several properties of the fibrinolytic system, Lys₇₇-plasminogen (Lys₇₇-Pg). Lys₇₇-Pg possesses a molecular weight of approximately 83 000 (F-2)–85 000 (F-1).⁴ Quite dramatic conformational differences exist between Glu₁-Pg and Lys₇₇-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 $S_{20,w}^0$ value of Glu₁-Pg is approximately 5.7 S whereas the same value for Lys₇₇-Pg is approximately 4.7 S.¹⁹ The difference in the $S_{20,w}^0$ values is much too large to be accounted for by the molecular weight difference in the two molecules. The rotational relaxation time of Glu₁-Pg has been found to be approximately 262 ns and that for Lys₇₇-Pg is approximately 160 ns.²⁰ Thus, Lys₇₇-Pg appears to be more asymmetric and more internally flexible than Glu₁-Pg.

Other functional low molecular weight plasminogen molecules and large peptide fragments have been isolated upon limited proteolysis of Glu₁-Pg with elastase.¹⁰ After treatment of Glu₁-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, Val₄₄₂-plasminogen¹⁰ (Val₄₄₂-Pg) and Leu₄₄₉-plasminogen (Leu₄₄₉-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, Val₃₅₄-plasminogen (Val₃₅₄-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 zy-

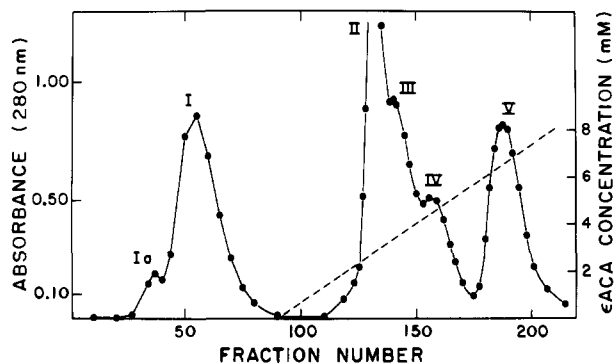


Figure 2. Elution of the limited elastase-digested products of human Glu₁-Pg (affinity variant 2) from Sepharose–lysine. The dashed line refers to the concentration of ϵ -ACA (taken from ref 23).

mogens, 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 Tyr₇₉–Val₃₃₇ and Tyr₇₉–Val₃₅₃¹⁰ (collectively termed K1–3) can be readily isolated,²³ from peak III, a peptide consisting of residues Val₃₅₄–Ala₄₃₉¹⁰ can be easily obtained, and from peak V a peptide comprising residues Tyr₇₉–Ala₄₃₉ can be purified.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 Lys₇₇-Pg, K1–3, Val₄₄₂-Pg (actually a mixture of Val₄₄₂-Pg and Leu₄₄₉-Pg), and K4. The latter three peptides represent fragments of the entire structure of Lys₇₇-Pg. In Lys₇₇-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, Val₄₄₂-Pg, and K4. All thermal transitions found in Lys₇₇-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 Lys₇₇-Pg. This strongly supports the argument that these particular peptides exist as independent domains in the intact Lys₇₇-Pg structure. Similar conclusions are reached when the thermograms of Val₃₅₄-Pg and K1–3 (two components of Lys₇₇-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 Tyr₇₉ to Arg₅₆₀) 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

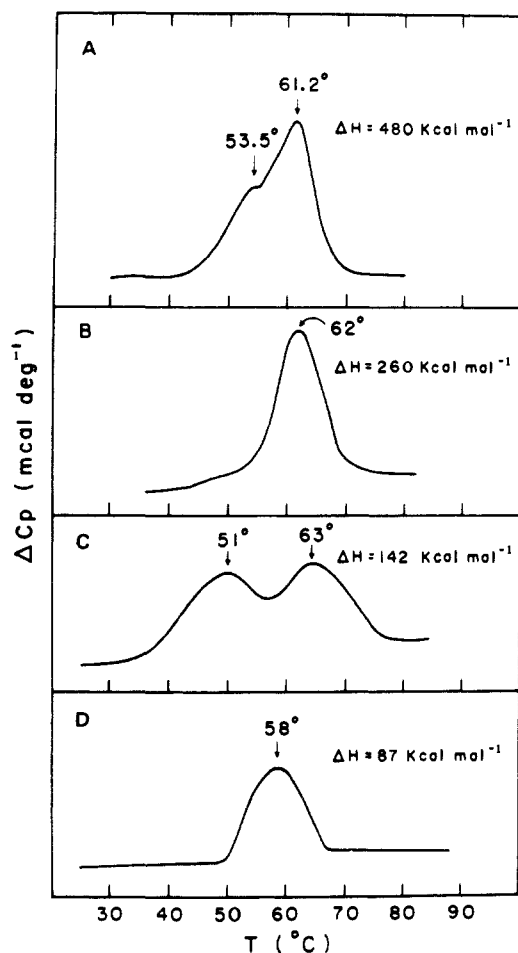


Figure 3. Thermal denaturation profiles of human $\text{Lys}_{77}\text{-Pg}$ (A) and the elastase-produced fragments K1-3 (B), $\text{Val}_{442}\text{-Pg}$ (C), and K4 (D) in the absence of $\epsilon\text{-ACA}$. The T_m positions and the ΔH values are indicated (taken from ref 23).

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 $\text{Arg}_{560}\text{-Val}_{561}$ is essential for the catalytic activity to be expressed.²⁵ Here, no peptides are released, since disulfide bond linkages at $\text{Cys}_{557}\text{-Cys}_{565}$ and $\text{Cys}_{547}\text{-Cys}_{665}$ stabilize the plasmin molecule. As a consequence of plasmin formation, autolysis of $\text{Lys}_{76}\text{-Lys}_{77}$ occurs, with covalent liberation of the amino-terminal 76 residues, yielding the final plasmin.⁴ This plasmin is termed $\text{Lys}_{77}\text{-plasmin}$ ($\text{Lys}_{77}\text{-Pm}$). Native plasmin thus consists of a heavy chain, $M_r \sim 58\,000\text{--}63\,000$ ⁴ disulfide linked to a light chain, $M_r\ 23\,000\text{--}26\,000$.^{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 $\text{Lys}_{77}\text{-plasmin}$ ($\text{Lys}_{77}\text{-Pm}$). Several other plasmins have been isolated, each based upon the particular plasminogen molecule undergoing activation. Activation of either $\text{Glu}_1\text{-Pg}$ or $\text{Lys}_{77}\text{-Pg}$ yields $\text{Lys}_{77}\text{-Pm}$. However,

$\text{Glu}_1\text{-Pm}$ can be prepared when $\text{Glu}_1\text{-Pg}$ is activated under conditions which result in immediate inactivation of the plasmin formed (since plasmin, by autolysis, will cleave the $\text{Lys}_{76}\text{-Lys}_{77}$ peptide bond in $\text{Glu}_1\text{-Pm}$, yielding $\text{Lys}_{77}\text{-Pm}$). In the presence of the Kunitz basic pancreatic trypsin inhibitor (PTI), $\text{Glu}_1\text{-Pm}$ can be prepared from either rabbit $\text{Glu}_1\text{-Pg}$ ^{26,27} or human $\text{Glu}_1\text{-Pg}$.^{4,28} However, the resulting $\text{Glu}_1\text{-Pm}$ will not be active, since the inhibitor will be bound to the active center. $\text{Val}_{442}\text{-Pm}$ is readily obtained upon activation of $\text{Val}_{442}\text{-Pg}$.^{21,29} This plasmin possesses the same light chain as $\text{Lys}_{77}\text{-Pm}$ but contains a fragment of the $\text{Lys}_{77}\text{-Pm}$ heavy chain consisting of residues $\text{Val}_{442}\text{-Arg}_{560}$, of approximate molecular weight 14 000. Additionally, $\text{Val}_{354}\text{-Pm}$ can be obtained upon activation of $\text{Val}_{354}\text{-Pg}$.²² Again, this plasmin contains the identical light chain of $\text{Lys}_{77}\text{-Pm}$, but only a fragment of the $\text{Lys}_{77}\text{-Pm}$ heavy chain ($\text{Val}_{354}\text{-Arg}_{560}$), of approximate molecular weight 28 000.

The physical properties of plasmin have not been extensively studied. The interaction of $\text{Lys}_{77}\text{-Pm}$ with columns of Sepharose-lysine is virtually identical with that of $\text{Lys}_x\text{-Pg}$,³⁰ indicating that the antifibrinolytic amino acid binding sites of $\text{Lys}_{77}\text{-Pg}$ are conserved in $\text{Lys}_{77}\text{-Pm}$. A similar finding applies to $\text{Glu}_1\text{-Pm}$. The $S_{20,w}^0$ value of human $\text{Lys}_{77}\text{-Pm}$ is 4.80 S, a value approximately the same as that found for $\text{Lys}_{77}\text{-Pg}$.¹⁹ The $S_{20,w}^0$ value found for human $\text{Glu}_1\text{-Pm}$ (complexed to the above plasmin inhibitor) is 5.68 S, again very close to that of $\text{Glu}_1\text{-Pg}$.¹⁹ Thus, the conformational differences observed between $\text{Glu}_1\text{-Pg}$ and $\text{Lys}_{77}\text{-Pg}$ are also found between $\text{Glu}_1\text{-Pm}$ and $\text{Lys}_{77}\text{-Pm}$.

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 741³³ 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 fragments³⁶ 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 proacelerin,⁴² antihemophilic factor,⁴² ACTH,⁴³ glucagon,⁴³ and γ -globulin.⁴⁴

Regarding nonphysiologic substrates, plasmin possesses tryptic-like specificity. This enzyme hydrolyzes proteins and peptides at lysyl and arginyl bonds.⁴⁵ Plasmin possesses esterolytic activity toward basic amino acid esters⁴⁶ 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 study⁴⁸ 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

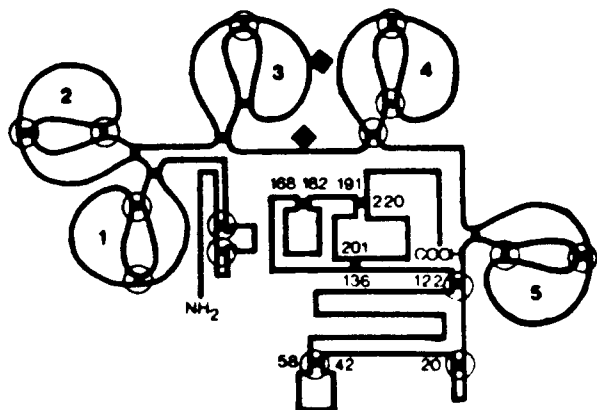


Figure 4. Proposed structure of human plasminogen, drawn to emphasize the kringle structures (taken from ref 10).

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- <i>p</i> -nitrophenyl ester	1.4×10^{-5}	22.3
Lys ₇₇ -Pm ^a	Cbz-L-lysine- <i>p</i> -nitrophenyl ester	1.5×10^{-5}	22.3
Lys ₇₇ -Pm ^b	α - <i>N</i> -tosyl-L-arginine methyl ester	7.7×10^{-4}	23.0
Val ₄₄₂ -Pm ^c	α - <i>N</i> -tosyl-L-arginine methyl ester	4.2×10^{-3}	45.0
Lys ₇₇ -Pm ^d	α - <i>N</i> -benzoyl-L-arginine ethyl ester	1.9×10^{-4}	12.0
Val ₄₄₂ -Pm ^d	α - <i>N</i> -benzoyl-L-arginine ethyl ester	1.8×10^{-4}	12.6
Lys ₇₇ -Pm ^d	D-Val-Leu-Lys- <i>p</i> -nitroanilide	1.2×10^{-4}	11.7
Val ₄₄₂ -Pm ^d	D-Val-Leu-Lys- <i>p</i> -nitroanilide	1.3×10^{-4}	10.4
Lys ₇₇ -Pm ^d	Bz-Phe-Val-Arg- <i>p</i> -nitroanilide	9×10^{-4}	10.0
Val ₄₄₂ -Pm ^d	Bz-Phe-Val-Arg- <i>p</i> -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 NH₂-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

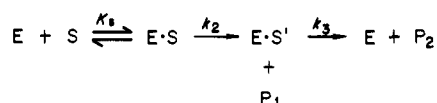


TABLE II. Steady-State Kinetic Values of the Various Plasmins toward the Peptide Substrate, S-2251, at 37 °C, pH 7.4

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 , μ M	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, P₁ is the first product released (alcohol), E·S' is the acyl-enzyme intermediate, and P₂ 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 Lys₇₇-Pm and Val₃₅₄-Pm are nearly identical. However, the K_s for Val₄₄₂-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 Val₄₄₂-Pm than for either Lys₇₇-Pm or Val₃₅₄-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 Lys₇₇-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 α , B β , γ), stabilized by disulfide bonds.⁵⁴⁻⁵⁸ Molecular weights of 64 000, 56 000, and 47 000 have been reported for the A α , B β , and γ chains, respectively.⁵¹ Plasmin begins its digestion of fibrinogen by attack at several locations in the A α chain^{52,59-61} (step 1), leading to release of several A α chain peptides (fragments A, B, and C) and the first detectable residual fibrinogen product, fragment X₁, of M_r ~240 000-265 000.^{62,63} Fragment X₁ consists of a degraded A α chain (A α') of M_r 25 000 and intact B β and γ chains.⁵¹ Other forms of fragment X (e.g., fragment X₂) exist which have partially degraded B β and γ chains.^{51,64} The origin of some of these variants is also shown in Figure 5. Fragment X₂ can arise (step 2) from fragment X₁ by symmetrical cleavage at the COOH terminal of the two B β chains, leading to a pair of β' chains of individual molecular weight 52 000 (these

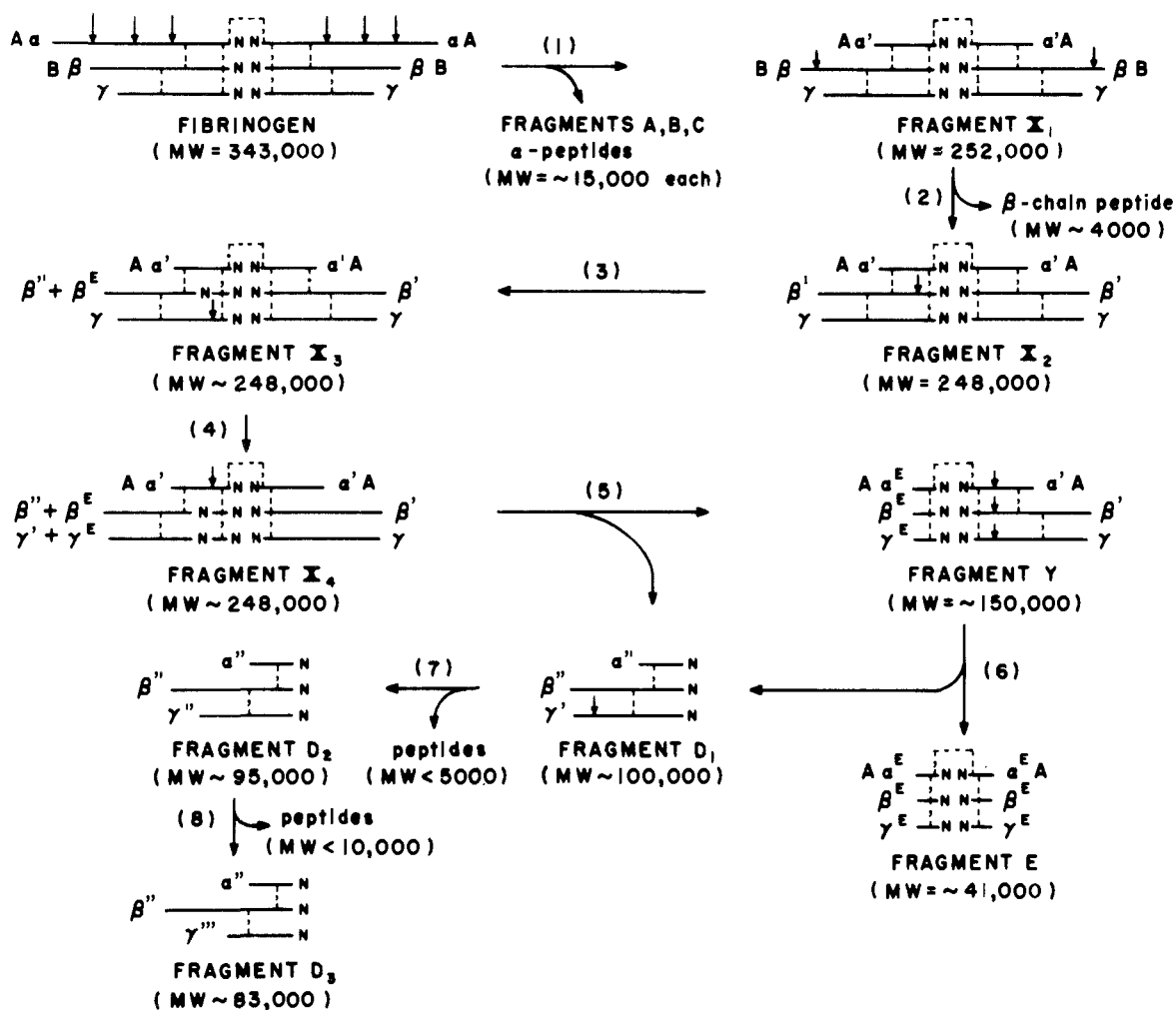


Figure 5. Proposed pathway(s) of the plasminogenolysis of human fibrinogen (taken from ref 51).

chains have also lost fibrinopeptide B). Since fragment D could not arise from fragment X₂ without additional peptide bond cleavages in fragment X₂, other structural variants of fragment X, exemplified by fragment X₃ and fragment X₄, must exist.⁵¹ These latter molecules have not been isolated and characterized. However, these variants would arise by unsymmetrical plasminolysis at the NH₂ 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 X₄, plasminolysis at the NH₂-terminal portion of the Aα' chain leads to release of the well-known fibrinogen fragments D and Y⁶³ (step 5). Purified fragment D, of M_r ~ 83 000–100 000,^{52,63} consists of a degraded Aα chain (α'') of M_r ~ 15 000, a degraded Bβ chain (β'') of M_r ~ 44 000, and a series of γ chains, γ' (fragment D₁), γ'' (fragment D₂), and γ''' (fragment D₃), 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.⁶⁶ Degradation of the γ chains, beyond γ', in fragment D can be inhibited by addition of Ca²⁺ to the plasminolysis mixture.⁶⁷ Fragment Y⁶³ (resulting from step 5 of Figure 5) with a total molecular weight of approximately 155 000,⁶³ is an unsymmetrical molecule con-

sisting of the Aα', β', and γ chains, described above, and extensively degraded Aα (Aα^E), Bβ (B^E), and γ (γ^E) chains.⁵¹ The molecular weights of the Aα^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 Aα', Bβ', 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 Aα^E, β^E, and γ^E (Aα^E₂B^E₂γ^E₂). Fragments Y, D, and E are not clottable by thrombin.⁶³

On the basis of the amino acid sequence of the Aα 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 Lys₅₀₈,⁷⁰ Lys₅₈₃,⁷⁰ and Lys₅₃₉⁶¹ to produce known fragments. Another site (or sites) of plasmin digestion may exist of Lys₄₁₃, Lys₄₁₈, Lys₄₂₁, Lys₄₂₇, or Arg₄₂₄ to produce an Aα 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 Aα and Bβ 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.⁸⁸ The two Lys cross-link donors are believed to be contributed from among the following five; Lys₅₃₉, Lys₅₅₆, Lys₅₆₂, Lys₅₈₀, and Lys₅₈₃.⁶⁸

The mode of action of Lys₇₇-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 B β 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 A α^E and γ^E chains do not contain cross-link sites), differences have been found in immunochemical properties⁸⁰ and plasma survival times⁸¹ 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 \sim 44 500, and one γ' - γ' cross-linked chain of total M_r \sim 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 A α^E and A α' sites have been cleaved from the molecule). This species should contain

two A α^E chains, two A α' 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 A α' chains of fragment X.⁵² Thus, fragment X contains $n + 4$ A α' 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

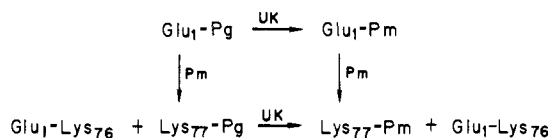
Activators of plasminogen have been found to be present in a variety of sources. The two most widely studied activators have been urokinase and streptokinase.

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 \sim 200 μ M and the k_c determined to be \sim 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 believed⁹⁰⁻⁹² that the molecular mechanism of activation of Glu₁-Pg to Lys₇₇-Pm, by UK, involved, first, UK-catalyzed cleavage of the Lys₇₆-Lys₇₇ bond in Glu₁-Pg, by UK, yielding Lys₇₇-Pg, and, second, UK-catalyzed cleavage of the Arg₅₆₀-Val₅₆₁ peptide bond in Lys₇₇-Pg, yielding Lys₇₇-Pm. We initially demonstrated that when activation of rabbit Glu₁-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 Glu₁-Pm was formed.²⁶ Subsequently, this was shown to be the case also for activation of human Glu₁-Pg.^{4,93} Thus, UK was capable of cleavage of the Arg₅₆₀-Val₅₆₁ peptide bond without prior cleavage of the Lys₇₆-Lys₇₇ peptide bond. Further, we demonstrated for both the human⁴ and rabbit systems²⁷ that plasmin served as an enzyme for cleavage of the Lys₇₆-Lys₇₇ peptide bond in the Glu₁-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 as⁴

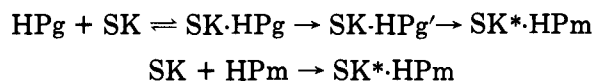


The initial step in the activation is cleavage of the Arg₅₆₀-Val₅₆₁ peptide bond in Glu₁-Pg, forming Glu₁-Pm. This form of plasmin, autocatalytically, cleaves the Lys₇₆-Lys₇₇ peptide bond in the Glu₁-Pm heavy chain, yielding Lys₇₇-Pm. Either Glu₁-Pm or Lys₇₇-Pm can catalyze cleavage of the Lys₇₆-Lys₇₇ peptide bond in any Glu₁-Pg not yet activated, forming Lys₇₇-Pg, which is readily activated^{94,95} to Lys₇₇-Pm. The exact pathway for formation of Lys₇₇-Pm, from Glu₁-Pg, is greatly dependent upon the pH, temperature, and buffer components employed.⁴

B. Streptokinase

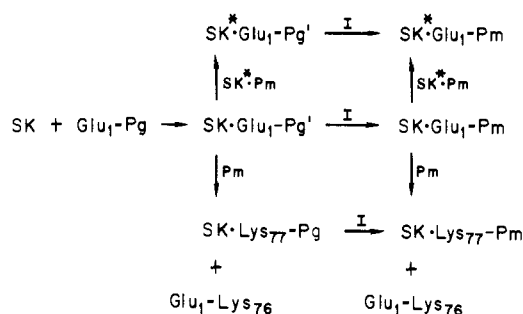
Streptokinase (SK) is a catabolic byproduct of B-hemolytic streptococcus and can catalyze activation of plasminogen in a species specific fashion. No synthetic substrate has yet been found for SK. The only protein substrates known are plasminogens from human,⁹⁶ monkey,^{97,98} baboon,⁹⁷ chimpanzee,⁹⁷ 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 it has no proteolytic activity, of itself, and the question arises as to the manner in which it can catalyze cleavage of the Arg₅₆₀-Val₅₆₁ 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 Arg₅₆₀-Val₅₆₁ 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



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 Arg₅₆₀-Val₅₆₁ 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 shown¹⁰¹ 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 Glu₁-Pg form the SK·Glu₁-Pg' complex, containing an active site in the plasminogen moiety, which through an intramolecular conversion (I) yielding SK·Glu₁-Pm. In the presence of small amounts of free plasmin (Pm), the SK·Glu₁-Pg' complex is converted to SK*·Lys₇₇-Pg', which, through an intramolecular conversion, yields SK*·Lys₇₇-Pm. This latter species can also form from SK·Glu₁-Pm, in the presence of small amounts of free plasmin (Pm). SK·Glu₁-Pg' can also be converted to SK*·Glu₁-Pg', by SK*·Pm. The SK*·Glu₁-Pg' can also, through an intramolecular conversion, be converted to SK*·Glu₁-Pm, which can also be formed from SK·Glu₁-Pm, by SK*·Pm. Most of these species are transiently formed, and the most stable form of plasminogen activator is SK*·Lys₇₇-Pm.

Although neither Glu₁-Pm nor Lys₇₇-Pm is capable of cleavage of the Arg₅₆₀-Val₅₆₁ peptide bond in Glu₁-Pg or Lys₇₇-Pg, catalytic levels of the SK*·Lys₇₇-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 Lys₇₇-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 Lys₇₇-Pm unaffected by the addition of SK, suggesting that access of small molecules to the plasmin active site is not in-

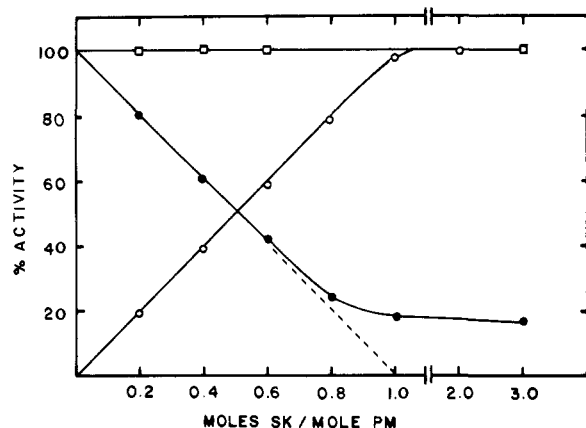


Figure 6. Activity of human $\text{Lys}_{77}\text{-Pm}$ toward TAME (\square), azocasein (\bullet), and bovine plasminogen (\circ), as a function of added streptokinase (taken from ref 106).

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*· $\text{Lys}_{77}\text{-Pm}$	0.26 ± 0.04	28.8 ± 1.3	0.13 ± 0.02
SK*· $\text{Val}_{354}\text{-Pm}$	0.28 ± 0.04	33.5 ± 1.3	0.09 ± 0.02
SK*· $\text{Val}_{442}\text{-Pm}$	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*· $\text{Lys}_{77}\text{-Pm}$ 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 $\text{Lys}_{77}\text{-Pm}$ upon addition of SK. Maximal activator activity is accomplished at a 1:1 molar ratio of SK and $\text{Lys}_{77}\text{-Pm}$. Thus, it appears as though plasminogen is specifically directed into the plasmin active site of the SK*· $\text{Lys}_{77}\text{-Pm}$ 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 $\text{Lys}_{77}\text{-Pm}$, $\text{Val}_{354}\text{-Pm}$, and $\text{Val}_{442}\text{-Pm}$ 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

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*· $\text{Lys}_{77}\text{-Pm}$	1.4 ± 0.3	0.31 ± 0.05
SK*· $\text{Val}_{354}\text{-Pm}$	1.9 ± 0.3	0.28 ± 0.05
SK*· $\text{Val}_{442}\text{-Pm}$	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	$k_{\text{Pg}}/K_{\text{Pg}}$, $\mu\text{M}^{-1} \text{min}^{-1}$, for		
	Glu ₁ -Pg	Lys ₇₇ -Pg	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 -Pg ^b	39.0	nd	nd
SK·Val ₄₄₂ -Pg ^b	14.8	nd	nd
UK (S1)	23.9	13.6	nd
UD (S2)	27.4	26.5	16.2

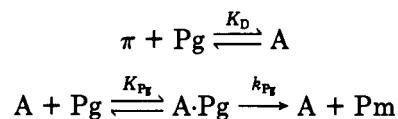
^a Data from ref 114. ^b It has not been determined whether SK actually exists as SK* in these complexes.

^c Not determined.

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.

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:



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 Glu₁-Pg activation, the most effective activator in this list is SK·Val₄₄₂-Pg'. The S-1 and S-2 forms of UK were equally effective. For Lys₇₇-Pg activation, the most effective activator is SK·Glu₁-Pg'. These differences in $k_{\text{Pg}}/K_{\text{Pg}}$ ratios for Lys₇₇-Pg activation are solely due to differences in k_{Pg} .¹¹⁴ Thus, it appears as though the NH₂-terminal portion of Pg, at least to residue 442, is not involved in the value of the Michaelis constant for activation. Regarding Lys₇₇-Pg activation, it appears as though UK form S-1

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

protein	value obtained for				
	$S_{20,w}^0, S$			ρ_h, ns^d	
	N ^a	ϵ^- ACA ^b	$\Delta F, c\%$	N ^a	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 fluorescently 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 Lys₇₇-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 activation¹¹⁵⁻¹²¹ and rabbit plasminogen activation¹²² 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 products¹²² 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 Glu₁-Pg. It was initially recognized by Alkjaersig¹²³ that the $S_{20,w}^0$ 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, Glu₁-Lys₇₆, undergo, as revealed by $S_{20,w}^0$ values, perhaps among the most dramatic ligand-induced conformational alterations heretofore described. On the other hand, mole-

TABLE VIII. Effect of ϵ -ACA Analogues on Human Glu₁-Pg Conformation

compd	S	$\Delta F, \%^b$	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 Glu₁-Pg upon addition of the ligand of choice. ^c Refers to the rotational relaxation time.

cules not possessing this peptide, e.g., Lys₇₇-Pg and Lys₇₇-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 $S_{20,w}^0$. 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 Glu₁-Pg and Glu₁-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, Lys₇₇-Pg and Lys₇₇-Pm do not undergo significant changes in this parameter upon binding ϵ -ACA. In the case of all properties examined, Lys₇₇-Pg and Lys₇₇-Pm in the presence or absence of ϵ -ACA appear to possess a gross conformation similar to Glu₁-Pg or Glu₁-Pm in the presence of saturating levels of ϵ -ACA. Thus, the amino-terminal peptide (Glu₁-Lys₇₆) appears to significantly control the gross conformation of plasminogen as well as the conformation alterations induced by ϵ -ACA.

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 Glu₁-Pg. The compounds employed and their effect on the gross conformation of Glu₁-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 Glu₁-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 NH₃⁺ takes place, allows the conformational change of Glu₁-Pg to occur, as monitored by decreases in the $S_{20,w}^0$ and ρ_h and an increase in the intrinsic fluorescence (ΔF) of the protein. Interposition of a cyclohexane ring between the COO⁻ and NH₃⁺ groups, as in *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid (t-AMCHA), and insertion of an NH₃⁺ group α - to the COO⁻ (L-Lys) do not alter the occurrence of the conformational alteration in Glu₁-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 Glu₁-Pg (similar effects are observed for rabbit Glu₁-Pg and human Glu₁-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-

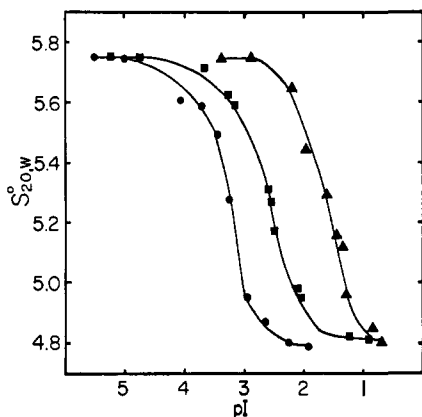


Figure 7. The effect of various concentrations of t-AMCHA (●), ϵ -ACA (■) and L-lysine (▲), plotted as the negative logarithm of the amino acid concentration (pI), on the $S_{20,w}^0$ of human Glu₁-Pg.

TABLE IX. $C_{0.5}$ for Various Amino Acids for Human Glu₁-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 $S_{20,w}^0$ value of Glu₁-Pg by three of these agents, viz., t-AMCHA, ϵ -ACA, and L-Lys₂, 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 $S_{20,w}^0$, 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 -NH₂ 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 alteration in Glu₁-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 Sjöholm 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 Otavsky¹²⁶ initially discovered that the human Lys₇₇-Pm heavy chain (Lys₇₇-Arg₅₆₀), prepared by selective reduction of the two disulfide bonds linking the Lys₇₇-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 laboratory¹²⁷ extended these observations to the isolated heavy chain of human Glu₁-Pm (Glu₁-Arg₅₆₀), and similar conclusions were drawn. In addition, we found that the alteration of the $S_{20,w}^0$ value of Glu₁-Pg and Glu₁-Pm by compounds of the ϵ -ACA class also occurred with the isolated heavy chain of Glu₁-Pm. The $C_{0.5}$ value of ϵ -ACA, toward Glu₁-Pg, was virtually identical with that for the Glu₁-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 Glu₁-Pg contained at least two noninteracting classes of binding sites for ϵ -ACA. One strong ϵ -ACA binding site of $K_d = 9 \mu\text{M}$ and approximately five weaker sites of average $K_d = 5 \text{ mM}$ were obtained. L-Lysine interacted much more weakly than ϵ -ACA with human Glu₁-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 Glu₁-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 Glu₁-Pg induced by these amino acids results from their binding to the weak class of sites on Glu₁-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 Glu₁-Pg. The strong site for ϵ -ACA and t-AMCHA appears to have little influence on the gross conformation of Glu₁-Pg.

The mode of binding of t-AMCHA and ϵ -ACA to human Lys₇₇-Pg has also been investigated.^{131,132} In the case of t-AMCHA, one strong site was shown to exist on Lys₇₇-Pg, of $K_d = 2.2 \mu\text{M}$, slightly weaker than the corresponding strong site for t-AMCHA on Glu₁-Pg. A slightly weaker t-AMCHA site on Lys₇₇-Pg, K_d of 36 μM , and two further sites, average $K_d = 103 \mu\text{M}$, were found. Similarly, one strong ϵ -ACA site ($K_d = 35 \mu\text{M}$), a weaker site of K_d of 260 μM , and approximately four additional sites of average $K_d = 10 \text{ mM}$ were obtained with human Lys₇₇-Pg. Thus, subtle differences do exist in the interaction of ϵ -ACA and analogues with Glu₁-Pg and Lys₇₇-Pg. In general, these involve (1) a decrease in the strength of interaction of the strong binding site in Lys₇₇-Pg compared to Glu₁-Pg, (2) a significant increase in affinity of the second site occupied by Lys₇₇-Pg compared to Glu₁-Pg, and (3) a decrease in affinity for

any additional sites for ϵ -ACA on Lys₇₇-Pg compared to Glu₁-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 μ M. K4 also possessed one site for ϵ -ACA, of K_d 36.4 μ M. 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 μ M for K1 is slightly higher than the K_d value of 9 μ M found for ϵ -ACA to the tightest site on human Glu₁-Pg¹²⁸ and slightly lower than the value of 35 μ M found for the tightest site on human Lys₇₇-Pg.¹³¹ Addition of a NH₂-terminal peptide (Glu₁-Met₅₇) to K1 allows the K_d value for the single ϵ -ACA site to be decreased to 12.5 μ M,¹³³ a value nearly identical with that of the tightest ϵ -ACA site of Glu₁-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 μ M, could also reflect the tight ϵ -ACA binding site, $K_d = 35 \mu$ M, 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 NH₂-terminal peptide (Glu₁-Met₅₇) 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 Val₄₄₂-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 Lys₇₇-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 Lys₇₇-Pg, one region of the Lys₇₇-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 Lys₇₇-Pg are shifted to a T_m of 66 °C, approximately 5 °C higher than that of native Lys₇₇-Pg. This region, also observed in K1-3, suggests that additional ϵ -ACA binding sites are present on K1-3. The thermogram of Val₄₄₂-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 Val₄₄₂-Pg in the absence of ϵ -ACA (Figure 3C). This reflects binding of ϵ -ACA to Val₄₄₂-Pg, possibly to K5. If so, this binding is very weak since Val₄₄₂-Pg is only

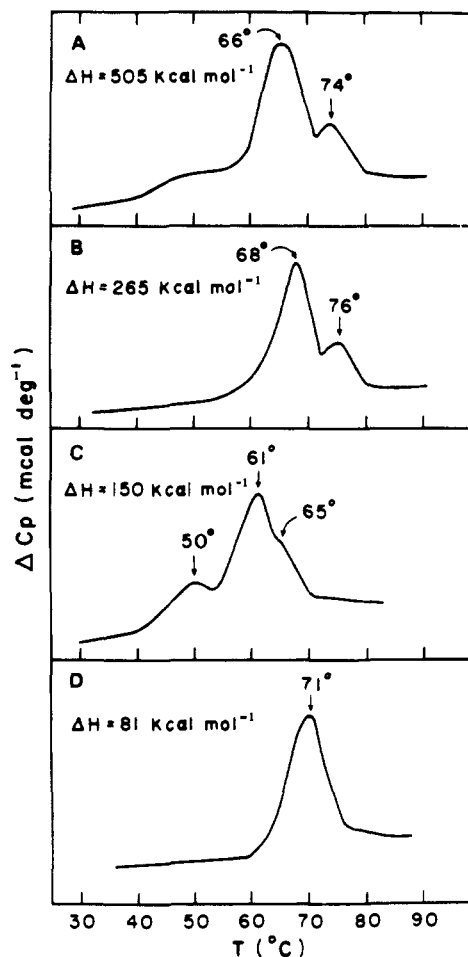


Figure 8. As in Figure 3 except that the ϵ -ACA concentration was 50 mM (taken from ref 23).

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 Val₄₄₂-Pg (the totality of these peptides represents the entire structure of Lys₇₇-Pg), when summed is approximately equal to the total ΔH of Lys₇₇-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 Glu₁-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 Vermeylen⁹⁵ that a biphasic pattern of enhancement of the activation of human Glu₁-Pg followed by an inhibition of Glu₁-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 Glu₁-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 Glu₁-Pg had only a slight inhibitory effect on the activation rate by UK.¹³¹ Since the weak antifibrinolytic amino acid sites on Glu₁-Pg have also been shown to be responsible for the large conformational alteration produced in Glu₁-Pg, as discussed earlier in this review, investigators in this field now agree that conformationally altered Glu₁-Pg is activated much more rapidly than Glu₁-Pg in the absence of ϵ -ACA. Substantiation of this view has been provided by experiments which have shown that Lys₇₇-Pg, which possesses a gross conformation very similar to that of Glu₁-Pg when Glu₁-Pg is saturated with ϵ -ACA and like molecules (see preceding section), is activated by UK at a very similar rate as that of Glu₁-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 Lys₇₇-Pg.^{95,131,135,136} Studies from our laboratory have shown that the rates of conversion of human Glu₁-Pg to Glu₁-Pm, as catalyzed by UK, Glu₁-Pm to Lys₇₇-Pm, as catalyzed by Lys₇₇-Pm, and Glu₁-Pg to Lys₇₇-Pg, as catalyzed by Lys₇₇-Pm, are all stimulated by ϵ -ACA.¹²⁴ Therefore, it appears as though all intermediates important to the activation of human Glu₁-Pg to Lys₇₇-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 Glu₁-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 discovery¹³⁷ that Glu₁-Pg possesses a weak affinity for fibrin whereas Lys₇₇-Pg has considerably stronger affinity in that regard. In whole plasma, approximately 4% Glu₁-Pg and 8% Lys₇₇-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 Lys₇₇-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 Glu₁-Pg interacts more weakly with fibrin than Lys₇₇-Pg has not been clarified, since the tight binding site which exists for ϵ -ACA on Lys₇₇-Pg also exists on Glu₁-Pg (although not of precisely the same strength). In this regard, it is possible that the strong antifibrinolytic amino acid site on Glu₁-Pg is involved in interaction with the amino-terminal peptide not present in Lys₇₇-Pg which competes with the fibrin interactions of Glu₁-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 concen-

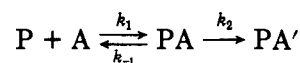
trations could be explained by their displacement of plasmin(ogen) from fibrin, and this effect involves the strong ϵ -ACA site in plasminogen. Second, at slightly higher ϵ -ACA concentrations (the region of effective titration of the weak ϵ -ACA sites), the activation rate of Glu₁-Pg in solution is 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 mM²⁹ 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 adsorbed 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 Lys₇₇-Pm and Val₄₄₂-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 Val₄₄₂-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 Lys₇₇ 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_1 value of fibrinogen toward Lys₇₇-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 Lys₇₇-Pm. These include α_2 -macroglobulin, C1 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 $M_r \sim 70000$.^{145,147} α_2 -Antiplasmin rapidly forms a reversible equimolar complex with Lys₇₇-Pm, which then converts to an irreversible complex.¹⁴⁶ The reaction scheme has been reported to be¹⁴⁷



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 Lys₇₇-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 Val₄₄₂-Pm, these values are $6.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $1.9 \times 10^{-9} \text{ M}$, and $4.2 \times 10^{-3} \text{ s}^{-1}$, 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 Val₄₄₂-Pm (lacking K1-4) than for Lys₇₇-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 $M_r \sim 14\,000$ may be cleaved from the amino terminus of the plasmin light chain as a consequence of complex formation. The rate at which Lys₇₇-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 Lys₇₇-Pm than for Val₄₄₂-Pm. The latter plasmin does not possess the major antifibrinolytic sites. The k_1 for Val₄₄₂-Pm and α_2 -antiplasmin was found to be $6.5 \times 10^5 \text{ M}^{-1}$,¹⁴⁸ a value much smaller than the k_1 of $3.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for Lys₇₇-Pm. Further, ϵ -ACA inhibits k_1 of the plasmin Lys₇₇-Pm- α_2 -antiplasmin reaction with a K_d of approximately $20 \mu\text{M}$ 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 Lys₇₇-Pm (presumed to be present, as it is in Lys₇₇-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 Lys₇₇-Pm and α_2 -antiplasmin, also implicating the plasmin active site as important as in this interaction.¹⁴⁷ When Val₄₄₂-Pm is substituted for Lys₇₇-Pm, ϵ -ACA did not serve as an inhibitor of the interaction, except for weak competition with α_2 -antiplasmin for the active site ($K_d = 58 \text{ mM}$) of Val₄₄₂-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 Val₄₄₂-Pm and α_2 -antiplasmin were essentially the same as for Lys₇₇-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 Lys₇₇-Pm- α_2 -antiplasmin to 50%) for Glu₁-Pg and α_2 -antiplasmin was found to be $4.0 \mu\text{M}$ whereas that for Lys₇₇-Pg was $0.63 \mu\text{M}$. This again indicates that the weaker binding of Glu₁-Pg to α_2 -antiplasmin may result from interaction of the strong ϵ -ACA site in Glu₁-Pg with its amino-terminal peptide (Glu₁-Lys₇₆) competing with interaction of the strong ϵ -ACA site with α_2 -antiplasmin. This peptide, not present in Lys₇₇-Pg, may allow Lys₇₇-Pg to bind more favorably to α_2 -antiplasmin. On the other hand, the K_d for TLCK-inhibited Lys₇₇-Pm for α_2 -antiplasmin was $0.43 \mu\text{M}$. This latter result points to the importance of the intact plasmin active site in the interaction (cf. to the K_d of $1.9 \times 10^{-10} \text{ M}$ in uninhibited Lys₇₇-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 Lys₇₇-Pg heavy chain and K1-3 possess K_d values of $0.91 \mu\text{M}$ and $0.18 \mu\text{M}$ 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 $9.0 \mu\text{M}$, 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 Lys₇₇-Pg to fibrin is decreased to 50% at an α_2 -antiplasmin concentration of approximately $1.1 \mu\text{M}$ (approximately the concentration found in normal plasma).¹⁴⁹ The presence of fibrinogen decreases the k_1 of the Lys₇₇-plasmin- α_2 -antiplasmin reaction to 50% of its value at a fibrinogen concentration of $0.7 \mu\text{M}$. No inhibition of the Val₄₄₂-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 Lys₇₇-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 Lys₇₇-Pm- α_2 -antiplasmin interaction.

VII. References

- (1) Deutsch, D.; Mertz, E. T. *Science (Washington, D.C.)* **1970**, *170*, 1095.
- (2) Brockway, W. J.; Castellino, F. J. *Arch. Biochem. Biophys.* **1972**, *151*, 194.
- (3) Summaria, L.; Spitz, F.; Arzadon, L.; Boreisha, I. G.; Robbins, K. C. *J. Biol. Chem.* **1976**, *251*, 3693.
- (4) Violand, B. N.; Castellino, F. J. *J. Biol. Chem.* **1976**, *251*, 3906.
- (5) Hayes, M. L.; Bretthauer, R. K.; Castellino, F. J. *Arch. Biochem. Biophys.* **1975**, *171*, 651.
- (6) Sodetz, J. M.; Brockway, W. J.; Castellino, F. J. *Biochemistry* **1972**, *11*, 4451.
- (7) Siefiring, G. E.; Castellino, F. J. *J. Biol. Chem.* **1974**, *249*, 1434.
- (8) Wallen, P.; Wiman, B. *Biochim. Biophys. Acta* **1972**, *257*, 122.
- (9) Robbins, K. C.; Summaria, L.; Hsieh, B.; Shah, R. J. *J. Biol. Chem.* **1967**, *242*, 2333.
- (10) Sottrup-Jensen, L.; Claeys, H.; Zajdel, M.; Petersen, T. E.; Magnusson, S. In: "Progress in Chemical Fibrinolysis and Thrombolysis," Raven Press: New York, 1978; Vol. 3, pp 191-209.
- (11) Wiman, B. *Eur. J. Biochem.* **1973**, *39*, 1.
- (12) Wiman, B. *Eur. J. Biochem.* **1977**, *76*, 129.
- (13) Wiman, B.; Wallen, P. *Eur. J. Biochem.* **1973**, *36*, 25.
- (14) Robbins, K. C.; Boreisha, I. G.; Arzadon, L.; Summaria, L. *J. Biol. Chem.* **1975**, *250*, 4044.
- (15) Hayes, M. L.; Castellino, F. J. *J. Biol. Chem.* **1979**, *254*, 8768.
- (16) Hayes, M. L.; Castellino, F. J. *J. Biol. Chem.* **1979**, *254*, 8772.
- (17) Hayes, M. L.; Castellino, F. J. *J. Biol. Chem.* **1979**, *254*, 8777.
- (18) Siefiring, G. E.; Castellino, F. J. *J. Biol. Chem.* **1974**, *249*, 7742.
- (19) Violand, B. N.; Sodetz, J. M.; Castellino, F. J. *Arch. Biochem. Biophys.* **1975**, *170*, 300.
- (20) Castellino, F. J.; Brockway, W. J.; Thomas, J. K.; Liao, H.; Rawitch, A. B. *Biochemistry* **1973**, *12*, 2787.
- (21) Powell, J. R.; Castellino, F. J. *J. Biol. Chem.* **1980**, *255*, 5329.

- (22) Powell, J. R.; Castellino, F. J., unpublished findings.
- (23) Castellino, F. J.; Ploplis, V. A.; Powell, J. R.; Strickland, D. K. *J. Biol. Chem.* 1981, 256, 4778.
- (24) Magnusson, S.; Petersen, T. E.; Sottrup-Jensen, L.; Claeys, H. In "Proteases and Biological Control"; Cold Spring Harbor, New York, 1975; pp 123-149.
- (25) Robbins, K. C.; Summaria, L.; Hsieh, B.; Robbins, K. C. *J. Biol. Chem.* 1967, 242, 2333.
- (26) Sodetz, J. M.; Brockway, W. J.; Mann, K. G.; Castellino, F. J. *Biochem. Biophys. Res. Commun.* 1974, 60, 729.
- (27) Sodetz, J. M.; Castellino, F. J. *J. Biol. Chem.* 1975, 250, 3041.
- (28) Summaria, L.; Arzadon, L.; Bernabe, P.; Robbins, K. C. *J. Biol. Chem.* 1975, 250, 3988.
- (29) Christensen, U.; Sottrup-Jensen, L.; Magnusson, S.; Petersen, T. E.; Clemmensen, I. *Biochim. Biophys. Acta* 1979, 567, 472.
- (30) Siefring, G. E.; Castellino, F. J. *J. Appl. Physiol.* 1975, 38, 114.
- (31) Summaria, L.; Hsieh, B.; Groskopf, R.; Robbins, K. C. *J. Biol. Chem.* 1967, 242, 5046.
- (32) Groskopf, W. R.; Hsieh, B.; Summaria, L.; Robbins, K. C. *J. Biol. Chem.* 1969, 244, 439.
- (33) Groskopf, W. R.; Summaria, L.; Robbins, K. C. *J. Biol. Chem.* 1969, 244, 3590.
- (34) Robbins, K. C.; Bernabe, P.; Arzadon, L.; Summaria, L. *J. Biol. Chem.* 1973, 248, 1631.
- (35) Pizzo, S. V.; Schwartz, M.; Hill, R. L.; McKee, P. A. *J. Biol. Chem.* 1972, 247, 646.
- (36) Kaplan, A. P.; Austen, K. F. *J. Exp. Med.* 1971, 133, 696.
- (37) Ratnoff, O. D.; Naff, G. B. *J. Exp. Med.* 1967, 125, 337.
- (38) Ward, P. A. *J. Exp. Med.* 1967, 125, 337.
- (39) Arroyave, C. M.; Muller-Eberhard, H. J. *J. Immunol.* 1973, 111, 536.
- (40) Harpel, P. C. *J. Clin. Invest.* 1970, 49, 58.
- (41) Brade, V. A.; Nicholson, A.; Bitter-Suermann, A.; Hadding, U. *J. Immunol.* 1974, 113, 1735.
- (42) Allagile, D.; Soulier, J. P. *Semaine Hop. Paris* 1956, 32, 355.
- (43) Mirsky, J. A.; Perisutti, G.; Davis, N. C. *J. Clin. Invest.* 1959, 38, 14.
- (44) Janeway, C. A.; Merler, E.; Rogen, F. S.; Salmon, S.; Crain, J. D. *New Engl. J. Med.* 1967, 278, 919.
- (45) Wallen, P.; Iwanaga, S. *Biochim. Biophys. Acta* 1972, 258, 577.
- (46) Weinstein, M. J.; Doolittle, R. F. *Biochim. Biophys. Acta* 1974, 334, 187.
- (47) Christensen, U.; Mullertz, S. *Biochim. Biophys. Acta* 1974, 334, 187.
- (48) Morris, J. P.; Blatt, S.; Powell, J. R.; Strickland, D. K.; Castellino, F. J., *Biochemistry*, in press.
- (49) Bender, M. L.; Begue-Canton, M.; Blakeley, R. L.; Brubacher, L. J.; Felder, J.; Gunter, C. R.; Kezdy, F. J.; Killheffer, F. J.; Marshall, T. H.; Miller, C. G.; Roeske, R. W.; Stoops, J. K. *J. Am. Chem. Soc.* 1966, 88, 5890.
- (50) Bender, M. L.; Brubacher, C. J. *J. Am. Chem. Soc.* 1965, 88, 5880.
- (51) Pizzo, S. V.; Schwartz, M. L.; Hill, R. L.; McKee, P. A. *J. Biol. Chem.* 1972, 247, 636.
- (52) Ferguson, E. W.; Fretto, L. J.; McKee, P. A. *J. Biol. Chem.* 1975, 250, 7210.
- (53) Mosesson, M. W.; Finlayson, J. S.; Galanakis, D. K. *J. Biol. Chem.* 1973, 248, 7319.
- (54) Blomback, B.; Yamashina, I. *Ark. Kemi.* 1958, 12, 299.
- (55) Henschen, A. *Ark. Kemi.* 1964, 22, 375.
- (56) Laki, K.; Gladner, J. A. *Physiol. Rev.* 1964, 44, 127.
- (57) Johnson, P.; Mihalyi, E. *Biochim. Biophys. Acta* 1965, 102, 467.
- (58) McKee, P. A.; Rogers, C. A.; Marler, E.; Hill, R. L. *Arch. Biochem. Biophys.* 1966, 116, 271.
- (59) Mills, D.; Karparkin, S. *Biochem. Biophys. Res. Commun.* 1970, 40, 206.
- (60) Takagi, T.; Doolittle, R. F. *Biochemistry* 1975, 14, 5149.
- (61) Ferguson, E. W. *J. Lab. Clin. Med.* 1980, 96, 710.
- (62) Fletcher, A. P.; Alkjaersig, N.; Fisher, S.; Sherry, S. *J. Lab. Clin. Med.* 1966, 68, 780.
- (63) Marder, V. J.; Shulman, N. R.; Carroll, W. R. *J. Biol. Chem.* 1969, 244, 2111.
- (64) Mosesson, M. W.; Alkjaersig, N.; Sweet, B.; Sherry, S. *Biochemistry* 1967, 6, 3279.
- (65) Catanzaro, A.; Strathern, J.; Edgington, T. S. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 1971, 30, 339.
- (66) Pizzo, S. V.; Taylor, L. M.; Schwartz, M. L.; Hill, R. L.; McKee, P. A. *J. Biol. Chem.* 1973, 248, 4584.
- (67) Lindsey, G. G.; Brown, G.; Purves, L. R. *Thromb. Res.* 1978, 13, 345.
- (68) Cottrell, B. A.; Strong, D. D.; Watt, K. W. K.; Doolittle, R. F. *Biochemistry* 1979, 18, 5405.
- (69) Watt, K. W. K.; Cottrell, B. A.; Strong, D. D.; Doolittle, R. D. *Biochemistry* 1979, 18, 5410.
- (70) Cottrell, B. A.; Doolittle, R. F. *Thromb. Res.* 1978, 12, 1135.
- (71) Blomback, B. *Ark. Kemi.* 1958, 12, 321.
- (72) Blomback, B.; Blomback, M.; Edman, P.; Hessel, B. *Biochim. Biophys. Acta* 1966, 115, 371.
- (73) Blomback, B.; Hessel, B.; Iwanaga, S.; Reuterby, J.; Blomback, M. *J. Biol. Chem.* 1972, 247, 1496.
- (74) Lorand, L.; Downey, J.; Gotoh, J.; Jacobsen, A.; Tokura, S. *Biochem. Biophys. Res. Commun.* 1968, 31, 322.
- (75) Pisano, J. J.; Finlayson, J. S.; Peyton, M. P. *Science (Washington, D.C.)* 1968, 160, 892.
- (76) Maticic, S.; Loewy, A. G. *Biochem. Biophys. Res. Commun.* 1968, 30, 356.
- (77) Chen, R.; Doolittle, R. F. *Biochemistry* 1971, 10, 4486.
- (78) Pisano, J. J.; Finlayson, J. S.; Peyton, M. P.; Nagai, Y. *Proc. Natl. Acad. Sci. U.S.A.* 1971, 68, 770.
- (79) Pizzo, S. V.; Schwartz, M. L.; Hill, R. L.; McKee, P. A. *J. Biol. Chem.* 1973, 248, 4574.
- (80) Edgington, T. S.; Plow, E. F. *J. Biol. Chem.* 1975, 250, 3393.
- (81) Catanzano, A.; Edgington, T. S. *J. Lab. Clin. Med.* 1974, 83, 458.
- (82) Wallin, R.; Belew, M.; Gerdin, B.; Saldeen, T. *Thromb. Res.* 1980, 19, 441.
- (83) Francis, C. W.; Marder, V. J.; Barlow, G. H. *J. Clin. Invest.* 1980, 66, 1033.
- (84) Landmann, H.; Markwardt, F. *Experientia* 1970, 26, 145.
- (85) Barlow, G. H. *Methods Enzymol.* 1976, 45, 239.
- (86) White, W. R.; Barlow, G. H.; Mozer, M. M. *Biochemistry* 1966, 5, 2160.
- (87) Lesuk, A.; Terminiello, J. H.; Traver, J. H.; Groff, J. H. *Thromb. Diath. Haemorrh.* 1967, 18, 293.
- (88) Soberano, M. E.; Ong, E. B.; Johnson, A. J. *Thromb. Res.* 1976, 9, 675.
- (89) Urokinase Pulmonary Embolism Trials. *Circulation* 1973, 17, Suppl. 11.
- (90) Wiman, B.; Wallen, P. *Eur. J. Biochem.* 1973, 39, 25.
- (91) Rickli, E. E.; Otavsky, W. I. *Biochim. Biophys. Acta* 1973, 295, 381.
- (92) Walther, P. J.; Steinman, H. M.; Hill, R. L.; McKee, P. A. *J. Biol. Chem.* 1974, 249, 1173.
- (93) Summaria, L.; Arzadon, L.; Bernabe, P.; Robbins, K. C. *J. Biol. Chem.* 1975, 250, 3988.
- (94) Thorsen, S.; Mullertz, S. *Scand. J. Clin. Lab. Invest.* 1974, 34, 167.
- (95) Claeys, H.; Vermeylen, J. *Biochim. Biophys. Acta* 1974, 342, 351.
- (96) Milstone, H. *J. Immunol.* 1941, 42, 109.
- (97) McKee, P. A.; Lemmon, W. B.; Hampton, J. W. *Thromb. Diath. Haemorrh.* 1971, 26, 512.
- (98) Wulf, R. J.; Mertz, E. T. *Can. J. Biochem.* 1969, 47, 927.
- (99) Sodetz, J. M.; Brockway, W. J.; Castellino, F. J. *Biochemistry* 1972, 11, 4451.
- (100) Taylor, F. B.; Botts, J. *Biochemistry* 1968, 7, 232.
- (101) Brockway, W. J.; Castellino, F. J. *Biochemistry* 1974, 13, 2063.
- (102) Hummel, B. C. W.; Buck, F. F.; DeRenzo, E. C. *J. Biol. Chem.* 1966, 241, 3474.
- (103) Ling, C.-M.; Summaria, L.; Robbins, K. C. *J. Biol. Chem.* 1967, 242, 1419.
- (104) McClintock, D. K.; Bell, P. H. *Biochem. Biophys. Res. Commun.* 1971, 43, 694.
- (105) Reddy, K. N. N.; Markus, G. *J. Biol. Chem.* 1972, 247, 1683.
- (106) Schick, L. A.; Castellino, F. J. *Biochemistry* 1973, 12, 4315.
- (107) Kosow, D. P. *Biochemistry* 1975, 14, 4459.
- (108) Bajaj, S. P.; Castellino, F. J. *J. Biol. Chem.* 1977, 252, 492.
- (109) Markus, G.; Werkheiser, W. C. *J. Biol. Chem.* 1964, 239, 2637.
- (110) DeRenzo, E. C.; Boggiano, B.; Barg, W. F. *J. Biol. Chem.* 1967, 242, 2428.
- (111) Summaria, L.; Ling, C.-M.; Groskopf, W. R. *J. Biol. Chem.* 1968, 243, 144.
- (112) Buck, F. F.; Boggiano, E. *J. Biol. Chem.* 1971, 246, 2901.
- (113) Wohl, R. C.; Arzadon, L.; Summaria, L.; Robbins, K. C. *J. Biol. Chem.* 1977, 252, 1141.
- (114) Wohl, R. C.; Summaria, L.; Robbins, K. C. *J. Biol. Chem.* 1980, 255, 2005.
- (115) Takada, A.; Takada, Y.; Ambrus, J. L. *J. Biol. Chem.* 1970, 245, 6389.
- (116) Takada, A.; Takada, Y.; Ambrus, J. L. *Biochim. Biophys. Acta* 1972, 263, 610.
- (117) Chesterman, C. N.; Cederholm-Williams, S. A.; Allington, M. J.; Sharp, A. A. *Thromb. Res.* 1977, 10, 421.
- (118) Takada, A.; Takada, Y. *Thromb. Res.* 1978, 13, 325.
- (119) Camiolo, S. M.; Markus, G.; Evers, J. L.; Hobika, G. H. *Int. Congr. Biochem. Abstr., 18th* 1979, 154.
- (120) Camiolo, S. M.; Markus, G.; Evers, J. L.; Hobika, G. H. *Thromb. Res.* 1980, 10, 421.
- (121) Takada, A.; Mochizuki, K.; Takada, Y. *Thromb. Res.* 1980, 19, 767.
- (122) Violand, B.; Castellino, A.; Castellino, F. J. *Thromb. Res.* 1980, 19, 705.
- (123) Alkjaersig, N. *Biochem. J.* 1964, 93, 171.

- (124) Violand, B. N.; Byrne, R.; Castellino, F. J. *J. Biol. Chem.* 1978, 253, 5795.
- (125) Sjöholm, Wiman, B.; Wallen, P. *Eur. J. Biochem.* 1973, 39, 471.
- (126) Rickli, E. E.; Otavsky, W. I. *Eur. J. Biochem.* 1975, 59, 441.
- (127) Gonzalez-Gronow, M.; Violand, B. N.; Castellino, F. J. *J. Biol. Chem.* 1977, 252, 2175.
- (128) Markus, G.; DePasquale, J. L.; Wissler, F. C. *J. Biol. Chem.* 1978, 253, 727.
- (129) Abiko, Y.; Iwamoto, M.; Tomikawa, M. *Biochim. Biophys. Acta* 1969, 185, 424.
- (130) Iwamoto, M. *Thromb. Diath. Haemorrh.* 1975, 33, 573.
- (131) Markus, G.; Evers, J. L.; Hobika, G. H. *J. Biol. Chem.* 1978, 253, 733.
- (132) Markus, G.; Priore, R. L.; Wissler, F. C. *J. Biol. Chem.* 1979, 254, 1211.
- (133) Lerch, P. G.; Rickli, E. E.; Lergier, W.; Gillessen, D. *Eur. J. Biochem.* 1980, 107, 7.
- (134) Lerch, P. G.; Rickli, E. E. *Biochim. Biophys. Acta* 1980, 625, 374.
- (135) Thorsen, S.; Kok, P.; Astrup, T. *Thromb. Diath. Haemorrh.* 1974, 32, 325.
- (136) Thorsen, S.; Astrup, T. *Thromb. Diath. Haemorrh.* 1974, 32, 306.
- (137) Thorsen, S. *Biochim. Biophys. Acta* 1975, 393, 55.
- (138) Rakozci, I.; Wiman, B.; Collen, D. *Biochim. Biophys. Acta* 1978, 540, 295.
- (139) Wiman, B.; Wallen, P. *Eur. J. Biochem.* 1975, 50, 489.
- (140) Lorand, L.; Condit, E. V. *Biochemistry* 1965, 4, 265.
- (141) Thorsen, S.; Glas-Greenwalt, S.; Astrup, T. *Thromb. Diath. Haemorrh.* 1972, 28, 65.
- (142) Pepper, D. S.; Allen, R., paper presented at 3rd Wondschoten Conference on Screening Methods for Detection of a Tendency to Thrombosis, 1977.
- (143) Wiman, B.; Linjen, H. R.; Collen, D. *Biochim. Biophys. Acta* 1979, 579, 142.
- (144) Moroi, M.; Aoki, A. *J. Biol. Chem.* 1976, 251, 5956.
- (145) Wiman, B.; Collen, D. *Eur. J. Biochem.* 1977, 78, 19.
- (146) Christensen, U.; Clemmensen, I. *Biochem. J.* 1977, 163, 389.
- (147) Wiman, B.; Collen, D. *Eur. J. Biochem.* 1978, 84, 573.
- (148) Wiman, B.; Boman, L.; Collen, D. *Eur. J. Biochem.* 1978, 87, 143.
- (149) Moroi, M.; Aoki, N. *Thromb. Res.* 1977, 10, 851.
- (150) Wiman, B.; Collen, D. *Nature (London)* 1978, 272, 549.