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Role of Lysine Binding Regions in the Kinetic Properties of Human Plasmin†

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ABSTRACT: Human plasmin derivatives, possessing heavy chains greatly reduced in size, have been utilized to assess the role of the heavy chain in the steady- and pre-steady-state kinetic parameters of this enzyme, as well as its equimolar complex with streptokinase. Native human plasmin (M_r = 85 000) possesses a K_m of 0.40 ± 0.05 mM and a k_c of 19.8 ± 1.0 s^{-1} toward the peptide substrate, NH_2-D-Val-Leu-Lys-*p*-nitroanilide (S-2251). Plasmin c, which contains kringles 4 and 5 and the light chain, and Val_{442}-plasmin, which contains kringle 5 and the light chain, possess steady-state kinetic parameters similar to those of each other and native plasmin (contains kringles 1–5 and the light chain). When each of the above plasmins is present in an equimolar complex with streptokinase (SK), the K_m values are reduced and the k_c values increased toward those of S-2251, when compared to their values in the absence of SK. Pre-steady-state kinetic parameters have been obtained for each of these enzymes toward the substrate, *p*-nitrophenyl *p'*-guanidinobenzoate (NPGB). Acylation rate constants, k_2, for native plasmin (Lys_{77}-Pm) and plasmin c (Pm-c), as well as their equimolar complexes, were 0.26–0.31 s^{-1}. On the other hand, the k_2 for this substrate with Val_{442}-plasmin and its equimolar SK complex was 0.57–0.60 s^{-1}. The values of K_s for Lys_{77}-Pm and Pm-c with NPGB were very similar to each other and significantly lower than that for Val_{442}-plasmin. K_s values for this substrate were significantly reduced for the equimolar SK complexes with these enzymes. A high-performance liquid chromatography assay was developed in order to monitor the specific degradation of fibrinogen by plasmin. Utilizing this assay, we have shown that Lys_{77}-Pm and Val_{442}-Pm were very similar in regard to their mode of action toward human fibrinogen. This result was surprising since Val_{442}-Pm does not retain the antifibrinolytic amino acid binding sites, which are presumably important in the interaction of plasmin with fibrin(ogen).

Human plasminogen (Pg)[1] is a single-chain plasma zymogen, with a molecular weight of ~92 000 (Rickli & Cuendet, 1971; Rickli & Otavsky, 1973; Violand & Castellino, 1976). Its activated product, human plasmin (Pm), is a two-chain disulfide-linked serine protease (Robbins et al., 1967; Summaria et al., 1967), with a molecular weight of ~85 000 (Violand & Castellino, 1976).

Native plasminogen and plasmin bind a certain group of amino acids, represented by L-lysine and ε-aminocaproic acid (ε-ACA), which possess antifibrinolytic properties. These amino acid binding sites are principally located in the latent or actual plasmin heavy chain (Rickli & Otavsky, 1975; Gonzalez-Gronow et al., 1977), which comprises the first 560 amino acid residues of plasminogen. At least one additional site is likely located in the actual plasmin light chain and is responsible for the weak competitive inhibition of plasmin by some of these agents (Brockway & Castellino, 1971). Markus et al. (1978) demonstrated that human plasminogen contains one high-affinity site for ε-ACA of K_D = 9 μM and at least five additional sites of average K_D = 5 mM. Other laboratories have shown that the high-affinity site is likely implicated in the interaction of plasmin(ogen) with fibrin (Thorsen, 1975; Wiman & Wallen, 1977), α_2-antiplasmin (Moroi & Aoki, 1976), and a histidine-rich plasma glycoprotein (Linjen et al., 1980). The weak plasminogen antifibrinolytic sites are felt to be responsible for the marked conformational alterations produced in native plasminogen by these agents (Violand et al., 1978) and the stimulation of the activation of native plasminogen by urokinase (Claeys & Vermylen, 1974).

Limited digestion of human plasminogen with elastase has been shown to yield a species of human plasminogen, of molecular weight ~38 000 (Val_{442}-Pg), lacking a major portion of the latent plasmin heavy chain and all of its corresponding antifibrinolytic amino acid binding sites (Sottrup-Jensen et al., 1977). In a similar fashion, we have been able to isolate another low molecular weight variant of human plasminogen,

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[1] Abbreviations used: NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; SK, streptokinase; iPr_2PF, diisopropyl fluorophosphate; PTI, pancreatic trypsin inhibitor; HPLC, high-performance liquid chromatography; ε-ACA, ε-aminocaproic acid; Pm, plasmin; Pg, plasminogen; Glu-Pg, human plasminogen containing Glu as the amino-terminal amino acid (native, circulating human plasminogen); Tris, tris(hydroxymethyl)-aminomethane; KIU, kallikrein inactivation unit; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CTA, Committee of Thrombolytic Agents unit.

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of molecular weight of ~51 000 (Pg-c), consisting of a slightly less extensively degraded latent plasmin heavy chain and containing one of the amino acid binding regions.[2]

The existence of these two low molecular weight forms of human plasminogen allows a unique opportunity to fruitfully investigate the role of the latent plasmin heavy chain, and, consequently, the antifibrinolytic amino acid binding sites, in the kinetic properties of human plasmin. This paper represents the results of such a study.

Materials and Methods

Proteins. Native plasminogen (Glu-Pg) has been prepared by affinity chromatography (Deutsch & Mertz, 1970) as previously described (Brockway & Castellino, 1972). Affinity chromatography variant 2 has been used in all studies reported herein. Val_{442}-Pg was purified as previously described (Powell & Castellino, 1980). The method of purification of Pg-c will appear shortly (Castellino & Powell, 1981). Streptokinase was purified from Kabikinase (A B Kabi), as described earlier (Castellino et al., 1976), and urokinase was a gift from Dr. W. H. Holleman, Abbott Laboratories.

Lys_{77}-plasmin (Lys_{77}-Pm) was prepared by activation of Glu-Pg with catalytic levels of urokinase. Specifically, lyophilized Glu-Pg was dissolved in a buffer consisting of 25 mM Tris-HCl/50 mM L-lysine/25% glycerol (v/v), pH 7.4, at 4 °C, to a final concentration of 1.5–2.0 mg/mL. Urokinase, dissolved in the same buffer, was then added to this solution to a final concentration of 100 CTA units/mL. The mixture was allowed to incubate at room temperature for 6 h, followed by incubation at 4 °C for 48 h, and stored at −20 °C. The concentration of active plasmin was then determined by using the NPGB[1] burst assay of Chase & Shaw (1967). Plasmin c (Pm-c) was obtained by activation of Pg-c, and Val_{442}-plasmin (Val_{442}-Pm) was obtained by activation of Val_{442}-Pg by this same procedure. In all cases, active plasmin concentrations were at least 85% of the original plasminogen concentration. Equimolar streptokinase–plasmin complexes, with each of the above plasmin preparations, were prepared by addition of a 1.1-fold molar excess of streptokinase, dissolved in 25 mM L-lysine, to an aliquot of the desired plasmin stock solution.

Human fibrinogen was purified from fresh human plasma by modification of the earlier procedures of Blomback & Blomback (1959) and Longas et al. (1980). All steps were carried out at room temperature. A quantity of 800 mL of plasma was immediately adjusted to 1 mM in iPr_2PF and 40 KIU/mL of PTI (Trasylol). This solution was then percolated over a Sepharose–lysine (2.0 × 25 cm) column, in order to remove plasminogen (Brockway & Castellino, 1972). Solid $(NH_4)_2SO_4$ was slowly added to the plasma thus obtained, to a final concentration of 25% (w/v). After being stirred for 30 min, the suspension was subjected to centrifugation at 21000*g*. The pellet was resuspended in 20 mM sodium citrate/0.15 M NaCl/10 mM benzamidine, pH 7.4, to 160 mL. A quantity of 16 mL of $Al(OH)_3$ (unflavored Wyeth Amphojel) was then added and stirred for 15 min. The suspension was then centrifuged at 27000*g* for 15 min, and the pellet was discarded. A total of 5 mL of 40% (w/v) PEG-6000 was added to the solution, and the suspension was stirred for 30 min, prior to centrifugation for 15 min at 27000*g*. The pellet was then dissolved in 30 mL of 20 mM sodium citrate/0.15 M NaCl/10 mM benzamidine, pH 7.4 and the temperature adjusted to 15 °C. Solid glycine was then added to a final concentration of 2.1 M. The suspension was stirred for 15 min and subjected to centrifugation at 27000*g*. The pellet was redissolved in a minimal volume of 20 mM citrate/0.15 M NaCl, pH 7.4, dialyzed against H_2O, and lyophilized. This material was at least 95% clottable.

Porcine pancreatic elastase was purchased, in a purified form, from the Sigma Chemical Co. Bovine pancreatic trypsin inhibitor was obtained from FBA Pharmaceuticals.

Steady-State Assays. The enzymatic activity of each plasmin, as well as that of each equimolar streptokinase–plasmin preparation, was evaluated with the peptide substrate, Val-Leu-Lys-*p*-nitroanilide (S-2251), at 37 °C and pH 7.4, in a buffer consisting of 50 mM Tris-HCl/100 mM NaCl. Substrate hydrolysis was monitored at 405 nm. The substrate concentration was varied from ~0.2 to 2.0 × K_m and plotted by the Eadie–Hofstee method. In all cases described, the final enzyme concentration was in the range of 18×10^{-9}–27×10^{-9} M. K_I values for benzamidine were obtained in the usual fashion. The ϵ^{1M}_{405nm} employed for *p*-nitroanilide was 9620.

Pre-Steady-State Assays. All equations employed, as well as the exact method utilized for these assays, have been previously described (Powell & Castellino, 1980). The substrate used was NPGB.

Assays of Plasmin Digestion of Fibrinogen. All solutions were prepared in buffer consisting of 50 mM Tris-HCl/100 mM NaCl, pH 7.4. The temperature of all assays was 37 °C.

In this procedure, we developed a method employing HPLC in order to monitor the degradation products of plasmin action of human fibrinogen. The products of interest were fibrinogen fragments X, Y, D, and E. The structural relationship of these fragments to native fibrinogen can be found in the paper of Pizzo et al. (1973). The equipment used was purchased from the Beckman Instrument Co. and consisted of a Model 334 gradient liquid chromatograph system, Model 110A pumps, and a Model 421 systems controller. An ISCO Model 1840 variable wavelength monitor, with a built-in recorder, was connected to the outlet of a Beckman Spherogel TSK-3000 SW gel filtration column (7.5 × 600 mm).

A solution of human fibrinogen (1.0 mg/mL), dissolved in 50 mM Tris-HCl/100 mM NaCl, pH 7.4, was prepared. An aliquot of Lys_{77}-Pm or Val_{442}-Pm, dissolved in the same buffer, was added to this solution, at 37 °C, such that the mole ratio of human fibrinogen to plasmin was 200:1. At various times, a quantity of 25 μL of solution was removed, and a small volume aliquot of PTI was added to inhibit further reaction (final PTI to plasmin ratio was 300:1 mol/mol). A total of 20 μL was injected onto the column, which was previously equilibrated at room temperature with a buffer consisting of 50 mM Hepes/200 mM Na_2SO_4, pH 7.4. The protein was eluted from this column with the same buffer, and the eluate was monitored at 280 nm. Similar assays were conducted with various concentrations of ε-ACA added to the incubation buffer of fibrinogen and plasmin.

In all cases, peak areas were calculated with an Apple II minicomputer with the Graphics Tablet hardware. The software used was either provided with the system or written by our laboratory.

$DodSO_4$ Gel Electrophoresis. The methodology employed was as essentially described by Weber & Osborn (1969), except that 6 M urea was substituted for H_2O in the gel solutions. The gels were 5% in acrylamide.

Results

Reduced $DodSO_4$ gel electophoretograms of each of the proteins utilized in this study are presented in Figure 1. Lys_{77}-Pg (gel 1) consists of a single band of previously de-

[2] J. R. Powell, and F. J. Castellino, unpublished experiments.

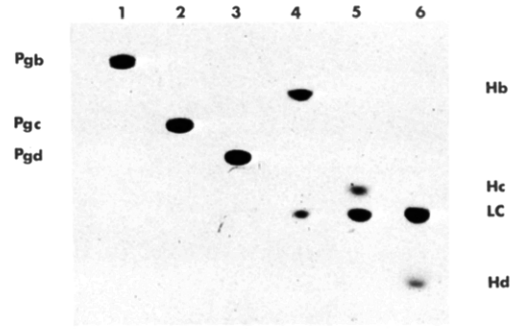

FIGURE 1: Reduced $DodSO_4$ electrophoretograms of Lys_{77}-Pg (Pgb) (gel 1), and its elastase-generated fragments, Pgc (gel 2) and Val_{442}-Pg (Pgd) (gel 3). The corresponding urokinase-catalyzed activation peptides of each plasminogen are shown in gel 4 (for Pgb activation), gel 5 (for Pgc activation), and gel 6 (for Pgd activation). H_b refers to the heavy chain of Lys_{77}-Pm, H_c refers to the heavy chain of Pm-c, and H_d refers to the remaining heavy chain fragment of Val_{442}-Pm (kringle 5). LC represents the light chain of each plasmin.

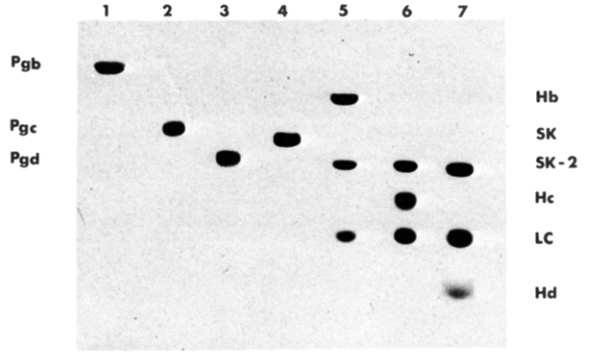

FIGURE 2: Activation of various plasminogens by equimolar levels of streptokinase (SK). The reduced $DodSO_4$ gels represent Lys_{77}-Pg (Pgb) (gel 1), Pgc (gel 2), Val_{442}-Pg (Pgd) (gel 3), and SK (gel 4). These are followed by gels representing the products obtained as a consequence of incubation of equimolar levels of SK with Pgb (gel 5), Pgc (gel 6), and Pgd (gel 7). These incubations were carried out for 10 min in a buffer consisting of 50 mM Tris-HCl/100 mM lysine, pH 7.4, at 37 °C. SK-2 refers to the molecular weight altered SK formed as a consequence of the activation. All other abbreviations are as described in Figure 1.

termined molecular weight of ~84 000 (Violand & Castellino, 1976). Plasminogen c (gel 2) consists of two closely related bands, not resolved here, resulting from lack of complete specificity of elastase digestion of native plasminogen of molecular weight of approximately 50 000–53 000. Val_{442}-Pg (gel 3) also consists of two closely related bands of molecular weight of ~38 000, again not resolved here (Sottrup-Jensen et al., 1977). Lys_{77}-Pm (gel 4) is composed of the well-known disulfide-linked heavy (M_r, ~60 000) and light (M_r, ~24 000) chains. Plasmin c consists of the same light chain, disulfide linked to a degraded heavy chain (M_r, ~28 000), and Val_{442}-Pm consists of the normal plasmin light chain, disulfide linked to a more extensively degraded heavy chain (M_r, ~14 000). Figure 2 illustrates reduced $DodSO_4$ electophoretograms of the components of the equimolar SK complexes of each of these plasmins. Gels 1–4 represent Lys-Pg, Pg-c, Val_{442}-Pg, and SK (M_r, ~44 000) shown for reference. The equimolar SK–native plasminogen complex (gel 5), the equimolar SK–Pm-c complex (gel 6), and the SK–Val_{442}-Pm equimolar complex (gel 7) show the same component plasmin chains noted above, as well as the proteolytically altered form of SK (SK-2) (Brockway & Castellino, 1974).

Representative Eadie–Hofstee plots of the steady-state hydrolysis of S-2251 for Pm-c as well as its equimolar SK complex, in the presence and absence of the competitive inhibitor, benzamidine hydrochloride, are illustrated in Figure 3. From data of this nature, values of K_m and V_{max} for S-2251 and K_I values for benzamidine hydrochloride have been determined for all of the enzymes pertinent to this study. The steady-state kinetic values are listed in Table I. The K_m values for Lys_{77}-Pm, Pm-c, and Val_{442}-Pm are approximately equal to each other and slightly higher than each of the corresponding values for the equimolar streptokinase complexes of these enzymes (also approximately equal to each other). A similar trend is noted for the K_I values for benzamidine hydrochloride. This latter data suggest that binding of this inhibitor to the equimolar streptokinase–plasmin complex is tighter than for uncomplexed plasmin. Again, there seem not to be large differences in this value when comparing the various plasmins to each other and the various equimolar streptokinase–plasmin complexes to each other.

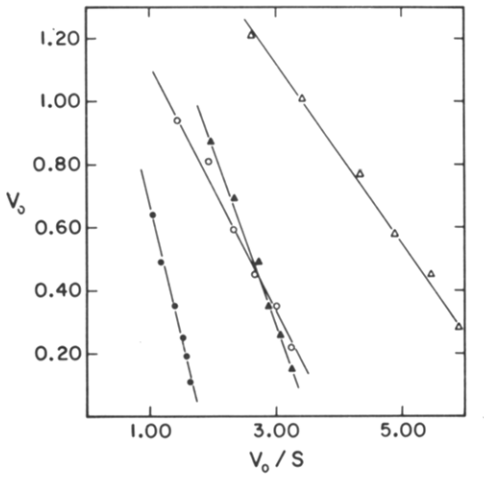

FIGURE 3: Steady-state amidase activity of Pm-c toward Val-Leu-Lys-PNA (S-2251), at pH 7.4 and 37 °C. The symbols represent activity of (○) Pm-c; (●) Pm-c, in the presence of 0.31 M benzamidine hydrochloride, (△) the equimolar SK–Pm-c complex, and (▲) the equimolar SK–Pm-c complex, in the presence of 0.09 M benzamidine hydrochloride. The initial velocities are expressed in units of micromoles of S-2251 hydrolyzed per minute per nanomol of plasmin. The plasmin concentration is expressed in terms of its active-site concentration, as described under Materials and Methods.

Table I: Steady-State Kinetic Values of the Various Plasmins toward the Peptide Substrate S-2251 at 37 °C and pH 7.4

enzyme	K_m (mM)	k_c (s^{-1})	K_I[a] (mM)
Lys_{77}-Pm	0.40 ± 0.05	19.8 ± 1.0	0.41 ± 0.05
SK–Lys_{77}-Pm[b]	0.26 ± 0.04	28.8 ± 1.3	0.13 ± 0.02
Pm-c	0.40 ± 0.05	25.3 ± 1.2	0.29 ± 0.05
SK–Pm-c[b]	0.28 ± 0.04	33.5 ± 1.4	0.09 ± 0.02
Val_{442}-Pm	0.36 ± 0.05	25.8 ± 1.2	0.25 ± 0.04
SK–Val_{442}-Pm[b]	0.27 ± 0.04	34.5 ± 1.5	0.09 ± 0.02

[a] Data for benzamidine hydrochloride. [b] Represents the equimolar complex of streptokinase (SK) with the appropriate plasmin.

Examples of plots of the apparent first-order rate constant for hydrolysis of NPGB vs. the concentration of NPGB for Pm-c and the SK–Pm-c complex are shown in Figure 4. From this data, the pre-steady-state rate constants K_s and k_2 have been calculated. Similar plots were employed to evaluate these constants for the other enzyme species used in this study. The pre-steady-state constants obtained are summarized in Table II. Since benzamidine hydrochloride was employed in these experiments to inhibit the acylation rate of each enzyme. The K_I value of benzamidine hydrochloride was determined at pH 8.3 and 30 °C—the pH and temperature at which pre-steady-state assays were performed—in order that these rates

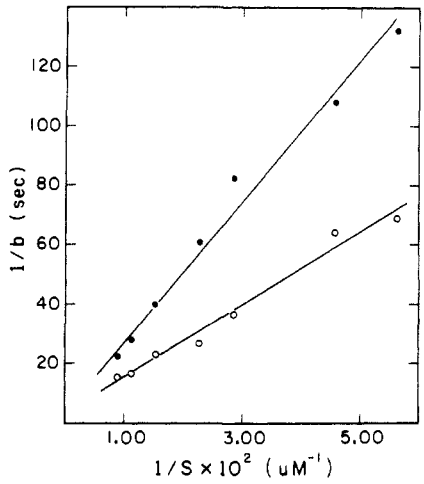

FIGURE 4: Pre-steady-state esterase activity, at pH 8.3 and 30 °C, toward NPGB, in the presence of 20 mM benzamidine hydrochloride. Pm-c activity is shown by (O), and the equimolar SK–Pm-c complex activity is shown by (●).

Table II: Pre-Steady-State Kinetic Parameters of Various Plasmins with the Substrate *p*-Nitrophenyl *p'*-Guanidinobenzoate at 30 °C and pH 8.3

enzyme	K_s (μM)	k_2 (s^{-1})	K_I[a] (mM)	k_2/K_s
Lys_{77}-Pm	5.6 ± 0.7	0.26 ± 0.05	0.31 ± 0.05	0.046
SK–Lys_{77}-Pm[b]	1.4 ± 0.3	0.31 ± 0.05	0.04 ± 0.01	0.221
Pm-c	6.7 ± 0.7	0.28 ± 0.05	0.43 ± 0.05	0.042
SK–Pm-c[b]	1.9 ± 0.3	0.28 ± 0.05	0.06 ± 0.02	0.147
Val_{442}-Pm	11.1 ± 1.0	0.60 ± 0.08	0.17 ± 0.03	0.054
SK–Val_{442}-Pm[b]	2.3 ± 0.4	0.57 ± 0.08	0.03 ± 0.01	0.248

[a] Data for benzamidine hydrochloride from steady-state measurements with S-2251 at 30 °C and pH 8.3. [b] Represents the equimolar complex of streptokinase (SK) and the appropriate plasmin.

should be measurable with routine spectrophotometric equipment. These K_I values are also listed in Table II. The K_s values for Lys_{77}-Pm and Pm-c are not greatly different from each other, but both are considerably lower than the same value for Val_{442}-Pm, indicating that this substrate exhibits tighter binding to the latter enzyme. Once again, it can be noted that when complexed with equimolar SK, the respective plasmins bind this substrate more tightly than free plasmin. Regarding the acylation rate constant, k_2, Lys_{77}-Pm, SK–Lys_{77}-Pm, Pm-c, and SK–Pm-c appear to be acylated at the same rate. This rate is enhanced for Val_{442}-Pm and SK–Val_{442}-Pm.

In order to assess the activity of each plasmin toward fibrinogen, we developed a simple, rapid, and convenient method, utilizing HPLC, to assay the fibrinogen degradation products, fragment Y, fragment D, and fragment E. These fragments can be resolved by this technique, at high ionic strength. The quality of a representative separation is shown in Figure 5. The $DodSO_4$ gel inserts of each of the peaks demonstrate that peak 1 is fragment X, peak 2, fragment Y, peak 3, fragment D, and peak 4, fragment E. Therefore, this assay allows a rapid and quantitative evaluation of the concentrations of these fragments at given times of incubation of fibrinogen with the desired plasmin. Practically, however, when undigested fibrinogen exists in the mixture, it is not well resolved from fragment X. Therefore, we report only the time-dependent evolution of fragments Y, D, and E in this study.

The rate of appearance of the aforementioned human fibrinogen fragments upon digestion with Lys_{77}-Pm is shown in Figure 6, while the appearance of fibrinogen fragments upon digestion with Val_{442}-Pm is shown in Figure 7. The data of

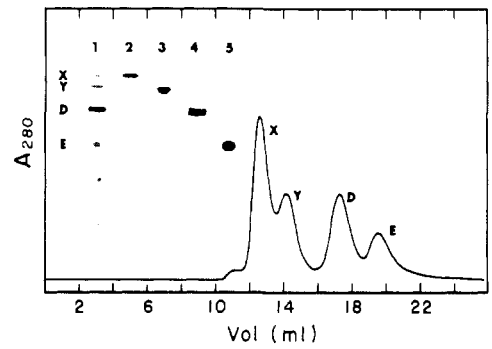

FIGURE 5: Representative elution pattern of the Lys_{77}-Pm-mediated degradation products of human fibrinogen from a column (7.5 × 600 mm) of Spherogel TSK-3000 SW, equilibrated and eluted with a buffer consisting of 50 mM Hepes/200 mM Na_2SO_4, pH 7.4. The fibrinogen (5.0 mg/mL) was incubated with Lys_{77}-Pm for 10 min and the reaction halted with pancreatic trypsin inhibitor, as described under Materials and Methods. A total of 0.02 mL of the digest was injected onto the column at a flow rate of 1 mL/min. The column temperature was 22 °C. The inset depicts $DodSO_4$ gel electrophoretograms of the following: gel 1, plasmic products of fibrinogen; gel 2, pooled fragment X; gel 3, pooled fragment Y; gel 4, pooled fragment D; gel 5, pooled fragment E. Fragment X and remaining fibrinogen (when present) are not well resolved in this system.

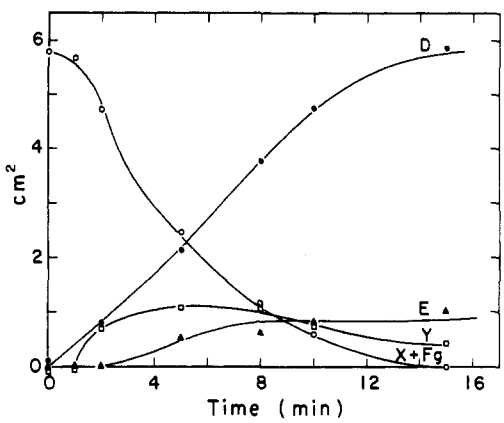

FIGURE 6: Time course digestion of human fibrinogen (Fg) by Lys_{77}-Pm and appearance of plasmic digestion fragments, X, Y, D, and E, of human fibrinogen. The symbols represent (O) Fg fragment X plus remaining Fg, (□) Fg fragment Y, (●) Fg fragment D, and (▲) Fg fragment E. The HPLC chromatographs (Figure 5), obtained at various digestion times, were integrated to give the areas (cm^2) shown on the ordinate. Data in this figure and Figure 7 were obtained at 0.885 mg/mL Fg and 0.07 μM plasmin initial concentrations.

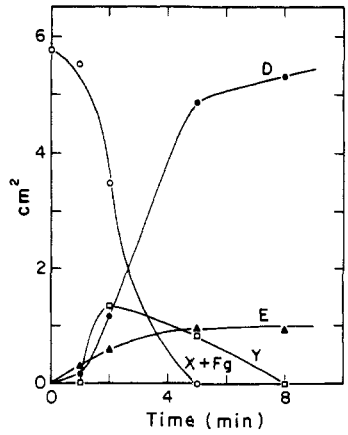

FIGURE 7: Time course digestion of Fg by Val_{442}-Pm. All conditions were as described in Figure 6.

Figures 6 and 7 demonstrate that Val_{442}-Pm is similar to Lys_{77}-Pm in that the rates of fragment production are similar and the pattern of digestion is identical.

Discussion

On the basis of amino acid sequence homology of certain areas of the latent human plasmin heavy chain of human plasminogen with the "kringle" structures in prothrombin (Magnusson et al., 1975), it has been shown (Sottrup-Jensen et al., 1977) that this portion of the plasminogen molecule is represented by five kringle structures, with interconnecting peptide strands. Portions of the molecule containing these kringles have been isolated. Specifically, kringle (K) 1 (Tyr_{79}-Lys_{165} of human Glu_1-Pg) Lerch et al., 1980), K 1–3 (corresponding to residues Tyr_{79}-Val_{337} or Try_{79}-Val_{353} of human Glu_1-Pg), K 4 (corresponding to residues Val_{354}-Ala_{439} of human Glu_1-Pg), Val_{442}-Pg (corresponding to Val_{442}-Asn_{791} of Glu_1-Pg, and containing K 5) (Sottrup-Jensen et al., 1977), K 1–4 (corresponding to residues Tyr_{79}-Ala_{439}) (F. J. Castellino, V. A. Ploplis, J. R. Powell, D. K. Struckland, unpublished experiments), the intact human plasmin heavy chain (corresponding to Tyr_{79}-Arg_{560}, containing K 1–5) (Rickli & Otavsky, 1975; Gonzalez-Gronow et al., 1977), and Pg-c[2] (Val_{354}-Asn_{791} containing K 4 and K 5 have been isolated. Utilization of differential scanning calorimetry has led to the proposal that these kringle regions likely exist as independent domains in the intact Lys_{77}-Pg structure (Castellino et al., 1981).

The isolation of these regions of native plasminogen have allowed some interesting structure–function studies of this molecule to be carried out. For example, it has been found that plasminogen has at least one high-affinity and four to five lower affinity binding sites for antifibrinolytic amino acids (Markus et al., 1978, 1979). It has been shown that K 1–3, K 4 (Sottrup-Jensen et al., 1977), and K 1 (Lerch et al., 1980) bind ϵ-ACA, whereas K 5 does not bind ϵ-ACA strongly (Sottrup-Jensen et al., 1977). Studies such as these have additionally allowed assessment of the role of the various regions of plasminogen in binding to fibrin, binding to α_2-antiplasmin, and binding to SK.

The isolation of the above low molecular weight plasminogens has stimulated studies on the role of the heavy-chain regions in activation of plasminogen and in plasmin kinetics. We have previously found that the activation rate of Val_{442}-Pg, by both urokinase and SK, is very similar to that of Lys_{77}-Pg and that Val_{442}-Pg and Val_{442}-Pm both form equimolar plasminogen activator complexes with SK, as do Lys_{77}-Pg and Lys_{77}-Pm (Powell & Castellino, 1980). Thus, it appears as though the latent or actual plasmin heavy chain is not important to its activation rate, under the conditions previously employed in in vitro systems. Christensen et al. (1979) concluded, on the basis of a pH dependence of hydrolysis of several substrates by Val_{442}-Pm and Lys_{77}-Pm, that the active sites of the enzymes were similar. Data published by Wohl et al. (1980) resulted in a similar conclusion being drawn. In addition, Christensen et al. (1979) demonstrated that the differences seen in inhibition of these two plasmins by α_2-antiplasmin were related only to the loss of heavy-chain ϵ-ACA binding regions in V_{442}-Pm and not due to inherent differences in their catalytic sites. Out steady-state kinetic data, in Table I, essentially agree with this viewpoint and extend the similarities in steady-state kinetic values to Pm-c, which contains the ϵ-ACA binding fragment, K 4. When complexed to SK, these plasmins yield K_m values nearly equal to each other and, in all cases, slightly, but significantly, lower than for each uncomplexed plasmin. The k_c parameters show an opposite trend, indicating that, when complexed to SK, the catalytic effeciency of the plasmin active site toward small substrates is enhanced, compared to uncomplexed plasmin. The K_I values obtained for the competitive inhibitor, benzamidine hydrochloride, suggest, again, that small molecules bind to the active site of each plasmin with the same strenght, but that when plasmin is complexed to SK, the K_I values decrease. Therefore, it appears as though the ϵ-ACA-binding kringles in the native plasmin heavy chain do not greatly influence the steady-state properties of plasmin, or its equimolar SK complex, toward small molecules.

Many of the same interpretations can be forwarded on the basis of the pre-steady-state data of Table II. Lys_{77}-Pm and Pm-c possess virtually identical values for K_s for NPGB. The equimolar SK complexes of these plasmins appear to more tightly bind this substrate. Val_{442}-Pm does not bind NPGB as tightly as Lys_{77}-Pm or Pm-c; however, the equimolar SK–Val_{442}-Pm complex possesses a K_s value similar to the equimolar SK complex of the other two plasmins. Acylation rate constants, k_2, for Lys_{77}-Pm, SK–Lys_{77}-Pm, Pm-c, and SK–Pmc are approximately equal to each other and lower than that for Val_{442}-Pm and SK–Val_{442}-Pm. This indicates that subtle differences do exist in the active site of this extensively degraded species of human plasmin.

Finally, we turned our attention to the digestion of fibrinogen by Lys_{77}-Pm and Val_{442}-Pm. The rationale for this aspect of our studies was provided by the proposed importance of the ϵ-ACA sites in the binding of plasmin to fibrin. These experiments were carried out at fibrinogen concentrations of 5 (data not shown) and 1 mg/mL. Fibrinogen is known to inhibit the amidase activity of plasmin toward D-Val-Leu-Lys-*p*-nitroanilide with K_I values of 1.9 μM for Lys_{77}-Pm and 7.3 μM for Val_{442}-Pm (Wiman et al., 1979). The concentrations of fibrinogen used in the present experiments are within the range of these reported K_I values. At both fibrinogen concentrations, the rate of fragment production was not greatly different for the two plasmin species. Thus, it appears that while the lysine binding sites of plasminogen may be important in the binding of plasminogen to fibrinogen, the active-site interactions of Lys_{77}-Pm and Val_{442}-Pm with fibrinogen are at least as important in the experssion of activity of these enzymes toward fibrinogen. Further studies are required to clearly define the role of the antifibrinolytic amino acid binding sites of plasmin in fibrinolysis.

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Aggregation Patterns in *Cherax destructor* Hemocyanin: Control of Oligomer Distribution by Incorporation of Specific Subunits†

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ABSTRACT: Recent polyacrylamide gel electrophoresis studies on *Cherax destructor* hemocyanin have demonstrated the presence of three further constituent fractions in the alkaline dissociation product in addition to the three subunits reported in earlier work. Two of these recently discovered subunits are monomeric with molecular weights around 75 000, while the third subunit is of similar size to the previously identified dimeric subunit M3′ with a molecular weight near 150 000. The aggregation process is influenced by the presence of calcium ions, particularly in the distribution of hybrid hexameric species. However, the relative proportions, as well as the types of subunits present initially, are of primary importance in determining the oligomer distribution pattern obtained upon reconstitution from alkaline pH to pH 7.8 of selected mixtures of subunits. An additional significant factor in the assembly process has been proposed: the operation of different relative rates of aggregation between different types of subunits. Reconstitution experiments based on these findings substantially explain the complex distribution of oligomeric forms in *C. destructor* hemolymph.

Several recent publications have discussed the subunit heterogeneity of arthropod hemocyanins. Commonly, these hemocyanins are composed of several different subunits of similar molecular weight. For example, hemocyanin from the scorpion *Androctonus australis* has been shown to contain eight different polypeptide chains of molecular weight ~75 000 (Jollès et al., 1979; Lamy et al., 1979a; Markl et al., 1979), and hemocyanin from the spider *Eurypelma californicum* (Lamy et al., 1979a; Markl et al., 1979) seven constituent polypeptide chains. Some other arthropods, studied in less detail, are known to have five or six subunits in their hemocyanins (Markl et al., 1979).

In earlier studies of *Cherax destructor* hemocyanin, three subunits were identified, two monomers of molecular weight about 75 000 and a dimer of molecular weight ~150 000 (Murray & Jeffrey, 1974). Subsequently the role of these subunits in formation of the polymeric aggregates was studied (Jeffrey et al., 1976, 1978). More recently the existence of a fourth subunit has been demonstrated (Jeffrey et al., 1980), while in the present paper we report the identification of two more subunits. The involvement of certain specific subunits in the control of oligomer formation in arthropod hemocyanins is already clear in some instances. Thus, it is known in *Limulus polyphemus* hemocyanin that fraction V is required to form structures larger than 16 S (equivalent to 6 monomers) and fraction IV to form 60 S (equivalent to 48 monomers) from 34 S (equivalent to 24 monomers) (Bijlholt et al., 1979), in *Androctonus* hemocyanin that subunit 1—a heterodimer—is required to form 34 S (Lamy et al., 1977), in *Eurypelma* hemocyanin that subunit 4D—also a heterodimer—is required to form 34 S (Markl et al., 1979), and in *Cherax* hemocyanin that M3′—a homodimer—is required to form 25 S (equivalent

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