Topography of the High-Affinity Lysine Binding Site of Plasminogen As Defined with a Specific Antibody Probe†

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ABSTRACT: An antibody population that reacted with the high-affinity lysine binding site of human plasminogen was elicited by immunizing rabbits with an elastase degradation product containing kringles 1-3 (EDP I). This antibody was immunopurified by affinity chromatography on plasminogen–Sepharose and elution with 0.2 M 6-aminohexanoic acid. The eluted antibodies bound [^{125}I]EDP I, [^{125}I]Glu-plasminogen, and [^{125}I]Lys-plasminogen in radioimmunoassays, and binding of each ligand was at least 99% inhibited by 0.2 M 6-aminohexanoic acid. The concentrations for 50% inhibition of [^{125}I]EDP I binding by tranexamic acid, 6-aminohexanoic acid, and lysine were 2.6, 46, and 1730 μM, respectively. Similar values were obtained with plasminogen and suggested that an unoccupied high-affinity lysine binding site was required for antibody recognition. The antiserum reacted exclusively with plasminogen derivatives containing the EDP I region (EDP I, Glu-plasminogen, Lys-plasminogen, and the plasmin heavy chain) and did not react with those lacking an EDP I region [miniplasminogen, the plasmin light chain or EDP II (kringle 4)] or with tissue plasminogen activator or prothrombin, which also contain kringles. By immunoblotting analyses, a chymotryptic degradation product of M_r 20 000 was derived from EDP I that retained reactivity with the antibody. The high-affinity lysine binding site was equally available to the antibody probe in Glu- and Lys-plasminogen and also appeared to be unoccupied in the plasmin-α_2-antiplasmin complex. α_2-Antiplasmin inhibited the binding of radiolabeled EDP I, Glu-plasminogen, or Lys-plasminogen by the antiserum, suggesting that the recognized site is involved in the noncovalent interaction of the inhibitor with plasminogen. The binding of [^{125}I]EDP I to fibrin was also inhibited by the antiserum. These observations provide independent evidence for the role of the high-affinity lysine binding site in the functional interactions of plasminogen with its primary substrate and inhibitor.

Plasmin, the serine protease derived from the zymogen plasminogen is the primary fibrinolytic enzyme in the circulation. Regulation of the fibrinolytic system is dependent upon plasminogen activators and upon inhibitors of these activators and of plasmin (Castellino, 1981; Collen, 1980). α_2-Antiplasmin is the primary physiologic inhibitor of plasmin (Collen, 1976; Moroi & Aoki, 1976; Mullertz & Clemmensen, 1976). In addition to the active site, which is located on the light chain of plasmin (Groskopf et al., 1969) the heavy chain is important in establishing the specificity of the enzyme for fibrin (Wiman & Wallen, 1977) and in regulating plasminogen activation (Claeys & Vermylen, 1974) and inactivation of plasmin by α_2-antiplasmin (Christensen & Clemmensen, 1977; Wiman & Collen, 1978). The structural features of the heavy-chain region of plasminogen that participate in these interactions are associated with the disulfide-looped kringle regions of the molecule (Sottrup-Jensen et al., 1978). These structures exhibit the capacity to bind lysine or lysine analogues such as 6-aminohexanoic acid (6-AHA)[1] or tranexamic acid (AMCA) (Markus et al., 1978a,b, 1979, 1981). The ω-aminocarboxylic acids influence the rate of plasminogen activation by various plasminogen activators (Claeys & Vermylen, 1974; Violand et al., 1978; Walter et al., 1975; Thorsen & Mullertz, 1974) and the rate of inactivation by α_2-antiplasmin (Christensen & Clemmensen, 1977; Wiman & Collen, 1978). They additionally interfere with the binding of plasminogen to fibrinogen and fibrin (Wiman & Wallen, 1977; Rokoczi et al., 1978; Thorsen, 1975; Lucas et al., 1983). In addition, the modulatory effects on plasminogen activation by other molecules such as histidine-rich glycoprotein (Lijnen et al., 1980) and thrombospondin (Silverstein et al., 1984) are also abrogated by lysine analogues. Thus, the lysine binding sites play a critical role in establishing the specificity and kinetics of the plasminogen system.

Plasminogen contains five kringles, each comprised of approximately 80-90 amino acids that exhibit considerable sequence homology to one another (Sottrup-Jensen et al., 1978). In addition, homologous structures are also found in a number of other proteins including urokinase, tissue plasminogen activator, and prothrombin (Magnusson et al., 1975; Pennica et al., 1983; Gunzler et al., 1982), and it has been suggested that the kringles are basic building units of proteins that function in their interactions with mediators such as protein cofactors (Neurath, 1985). Glu-plasminogen, the native form of the zymogen, contains one high-affinity (9 μM for 6-AHA) and 5 low-affinity (5 mM for 6-AHA) lysine binding sites (Markus et al., 1978a). Lys-plasminogen, which is generated by limited plasmic proteolysis in the amino terminal region of Glu-plasminogen, appears to express a new lysine binding

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[1] Abbreviations: EDP I, elastase degradation product of plasminogen consisting of the amino acid sequences Tyr-79–Val-337 or Tyr-79–Val-353; EDP II, elastase degradation product of plasminogen consisting of the amino acid sequence Val-354–Ala-439; 6-AHA, 6-aminohexanoic acid; AMCA, tranexamic acid; Glu-plasminogen, the native form of plasminogen with N-terminal Glu; Lys-plasminogen, a proteolytic derivative of Glu-plasminogen with NH_2-terminal Met-68, Lys-77, or Val-78; KIU, kallikrein inactivator unit (1 KIU represents 140 ng of active trasylol); SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2).

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site of intermediate affinity (Markus et al., 1978b). The high-affinity lysine binding site appears to be associated with kringle 1 of both forms of plasminogen (Lerch et al., 1980) and has been implicated in the interactions of plasminogen with fibrin (Rokoczi et al., 1978; Thorsen, 1975; Lucas et al., 1983), α_2-antiplasmin (Christensen & Clemmensen, 1977; Wiman & Collen, 1978), and thrombospondin (Silverstein et al., 1984).

The primary structure of plasminogen is known (Wiman & Wallen, 1975a,b; Wiman, 1977; Sottrup-Jensen et al., 1978), and cDNA for the molecule has been characterized (Malinowski et al., 1984). In addition, techniques such as differential scanning calorimetry have provided information on the domain structure of the molecule (Castellino et al., 1981; Novokhatny et al., 1984). An independent and powerful means of analysis of plasminogen structure and function can be provided by antibody probes. Both polyclonal (Plow & Collen, 1981; Hochschwender & Laursen, 1981) and monoclonal antibodies (Ploplis et al., 1982) have been elicited to the low-affinity lysine binding site associated with kringle 4 of plasminogen. These antibodies have been used to characterize the structure and function of this region. The interaction of several of these antibodies with the kringle 4 containing derivative of plasminogen required that the lysine binding site in this region be unoccupied (Plow & Collen, 1981; Hochschwender & Laursen, 1981; Ploplis et al., 1982). Monoclonal antibodies to the kringle 1–3 region have also been described (Ploplis et al., 1982; Cummings et al., 1984), but the reaction of these antibodies with the region was not influenced by lysine analogues despite the assignment of the high- as well as low-affinity lysine binding sites to this region. In the present study, we describe the characterization of an antibody to the kringle 1–3 region which recognizes the functional high-affinity lysine binding site of plasminogen. This antibody has been utilized to examine the accessibility of this region in various plasminogen derivatives and to probe the role of the high-affinity lysine binding site in the interactions of plasminogen with its primary substrate and inhibitor.

Materials and Methods

Plasminogen and Its Derivatives. Glu-plasminogen was isolated from fresh human plasma or Cohn fraction III by affinity chromatography on lysine–Sepharose in the presence of 1 mM benzamidine and 0.02% NaN_3 (Deutsch & Mertz, 1970). Lys-plasminogen was prepared by a modification of a published method (Sodetz & Castellino, 1975). Glu-plasminogen, 4 mL at 0.6 mg/mL in 0.05 M Tris-HCl, pH 8.0, 0.05 M lysine, 0.02% NaN_3, was incubated with 0.4 caseinolytic unit of plasmin (Kabi, Stockholm, Sweden) at 37 °C for 2 h. Following absorption of plasmin onto a column of soybean trypsin inhibitor coupled to Affi-gel beads (Bio-Rad, Richmond, CA), the material that did not bind was dialyzed against 0.1 M sodium phosphate pH 7.3, 0.05 M 6-AHA. Lys-plasminogen was further purified on an I-125 molecular exclusion column (Waters, Milford, MA) by high-pressure liquid chromatography. Lys-plasminogen was distinguished from Glu-plasminogen on 5% reduced polyacrylamide–SDS gels (Weber & Osborn, 1969) in 3 M deionized urea as described (Sodetz & Castellino, 1975). All plasminogen derivatives were extensively dialyzed vs. PBS (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, containing 0.02% sodium azide) to reduce the concentration of 6-AHA to <1 nM.

Digestion of Glu-plasminogen with porcine elastase was performed according to the method of Sottrup-Jensen et al. (1978). Briefly, 145 mg of lyophilized Glu-plasminogen in 10 mL of 0.3 M NH_4HCO_3, pH 8.3, containing 7000 units of trasylol (FBA Pharmaceuticals, New York, NY), was incubated with 0.45 mg of porcine elastase (Calbiochem, La Jolla, CA) for 3.3 h at 22 °C. Diisopropyl phosphofluoridate was added to a final concentration of 1.5 mM, and the mixture was stirred for 30 min. Solid NH_4HCO_3 was added to a concentration of 0.55 M, and the mixture was stirred at 4 °C for 20 h and applied to a column (32.5 × 2.5 cm) of lysine–Sepharose equilibrated with 0.1 M NH_4HCO_3, pH 8.3. The material that did not bind to the column contained miniplasminogen (which contains kringle 5 and the latent light-chain region of plasmin). This derivative was further purified by passage over a Sephadex G-75 column (65 × 2.5 cm) equilibrated with 0.1 M NH_4HCO_3, pH 8.3, 0.02% NaN_3, followed by repassage over a lysine–Sepharose column. The material that bound to the first lysine–Sepharose column was eluted with 0.2 M 6-AHA and contained EDP I (kringles 1–3) and EDP II (kringle 4). These were separated from each other by molecular exclusion chromatography on the Sephadex G-75 column. Amino terminal residues of the EDP I preparations were determined as described by Allen (1981). The amino acid composition of the preparations was determined following hydrolysis in vacuo at 110 °C in 6 N HCl for 24 h, using a Beckman 6300 amino acid analyzer.

The plasmin–α_2-antiplasmin complex was formed in a 10% excess of α_2-antiplasmin and was purified by affinity chromatography on lysine–Sepharose followed by molecular exclusion chromatography on Ultrogel AcA44 (Wiman & Collen, 1977, 1979). The plasmin light and heavy chains were isolated following mild reduction and alkylation of plasmin. The light chain was obtained as the unbound fraction from lysine–Sepharose, and the heavy chain was eluted with 0.2 M 6-AHA (Rickli & Otavsky, 1975). The purity of all derivatives was assessed by SDS–polyacrylamide gel electrophoresis, and a homogeneity of >90% for each derivative was indicated by the staining patterns.

Antibody Preparation. Rabbit antiserum to EDP I was prepared by injecting 100 μg of EDP I initially in complete Freund's adjuvant, followed by biweekly injections in incomplete Freund's adjuvant. An early bleeding following the fourth immunization was used for this study. The antiserum was precipitated in the presence of 50% saturated $(NH_4)_2SO_4$, dialyzed against PBS, and immunopurified by affinity chromatography on plasminogen–Sepharose, equilibrated with PBS containing 100 μM phenylmethanesulfonyl fluoride. The column was washed until the absorbance at 280 nm of fractions was <0.005. Bound antibody was eluted sequentially, first with 0.2 M 6-AHA, pH 7.0, and then with 0.2 M glycine HCl, pH 2.2. The eluates were immediately and exhaustively dialyzed against PBS.

Radioimmunoassay for EDP I. EDP I was radiolabeled by a modified chloramine-T procedure (McConahey & Dixon, 1966), and extensive dialysis in PBS was used to remove free ^{125}I. Typically, trichloroacetic acid (10%) precipitated 95% of the radioactivity of [^{125}I]EDP I, 93% of [^{125}I]Glu-plasminogen, and 85% of [^{125}I]Lys-plasminogen. Specific activities of these ligands ranged from 1 to 5 μCi/μg.

The radioimmunoassay system was of the double antibody type, employing goat anti-rabbit immunoglobulin to achieve precipitation. The assay buffer was 0.04 M borate buffer, pH 8.3, containing 0.024 M NaCl, 19 KIU/mL trasylol, 0.002 M EDTA, 20 U/mL heparin, and 2% heat-inactivated and $BaSO_4$-precipitated normal rabbit serum. [^{125}I]EDP I, [^{125}I]Glu-plasminogen, or [^{125}I]Lys-plasminogen was used at final concentrations of 0.7 nM, and all other aspects of the assays were the same as previously described (Plow et al.,

1979). Percent inhibition was calculated relative to controls lacking competitors, and antigen binding capacities (ABC, 33%) were calculated from the dilution of antiserum required to achieve 33% binding of the ligand (Minden & Farr, 1967).

To test whether the antibody recognized epitopes with multiple affinities for ω-aminocarboxylic acids, the inhibition curves obtained by titration of ω-aminocarboxylic acids into the binding assays were analyzed in either a one- or a two-site model using the Ligand program (Munson & Rodbard, 1980). The concentration of bound lysine analogue was calculated by multiplying the percent radiolabeled plasminogen derivative displaced from the antibody in the presence of the lysine analogue by the concentration of [^{125}I]plasminogen derivative in the assay. The free concentration of ω-aminocarboxylic acid was determined by subtracting the concentration bound from the concentration added. By this method the number of molecules of lysine analogues bound per molecule of plasminogen derivative does not affect the fit of the data in a one- or two-site model.

Fibrin Binding Assay. A quantity of 100 μg of fibrinogen containing 50 KIU trasylol was dried in each well of microtiter plates at 37 °C overnight. Thrombin, 0.1 units, was incubated with the fibrinogen for 1 h at 22 °C, and the wells were washed 3 times with 150-μL aliquots of 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.01% Tween 80, 50 KIU/mL trasylol, 1 mg/mL bovine serum albumin, and 1 mg/mL gelatin. For the last wash, the buffer contained 0.1 unit/mL hirudin and was incubated with the fibrin for 30 min.

To assay [^{125}I]EDP I binding to fibrin, 0.8 mL of [^{125}I]EDP I at 0.7 nM, in 0.04 M borate buffer, pH 8.3, containing 1000 KIU/mL trasylol, was preincubated with 1.6 mL of 0.3 M 6-AHA, buffer, or antibody at 4 °C for 18 h. Duplicate 100-μL samples were placed in the microtiter wells containing fibrin and incubated for 1 h at 22 °C on a shaker. The test solutions were aspirated, and the wells were washed 3 times and counted for radioactivity. Minimal radioactivity bound to wells without fibrin. Percent specific inhibition of fibrin binding by the various antisera was calculated by assuming that residual binding in the presence of 6-AHA was nonspecific

$$1 - \frac{\text{cpm exptl} - \text{cpm in presence of 6-AHA}}{\text{cpm buffer} - \text{cpm in presence of 6-AHA}} \times 100$$

Immunoblotting. Immunoblotting was performed according to the method of Towbin et al. (1979) as modified by Lammle et al. (1986). Samples were electrophoresed on 15% SDS–polyacrylamide gels (Laemmli, 1970) and electrophoretically transferred, for 1.5 h at 250 mA, to nitrocellulose. The nitrocellulose sheets were treated as described by Johnson et al. (1984), reacted with EACA-eluted antibody for 13 h, and developed with radioiodinated goat anti-rabbit IgG (Biorad, Richmond, CA) at 0.5 μg/mL for 1 h. The sheets were washed an additional 5 times prior to autoradiography.

Other Proteins. Thrombospondin was prepared by a modification of a published method (Lawler et al., 1978) as described from our laboratory (Wolff et al., 1986). The thrombospondin was homogeneous as judged on polyacrylamide gels in the presence of SDS and migrated with an apparent molecular weight of 450000 under nonreducing and 150000 under reducing conditions on 10% gels in the buffer system of Laemmli (1970). The ability of the thrombospondin to bind plasminogen was tested as described by Silverstein et al. (1984). This procedure resulted in binding of 44% of the added [^{125}I]Glu-plasminogen to thrombospondin coupled to plastic microtiter wells and the interaction was inhibited by 6-AHA.

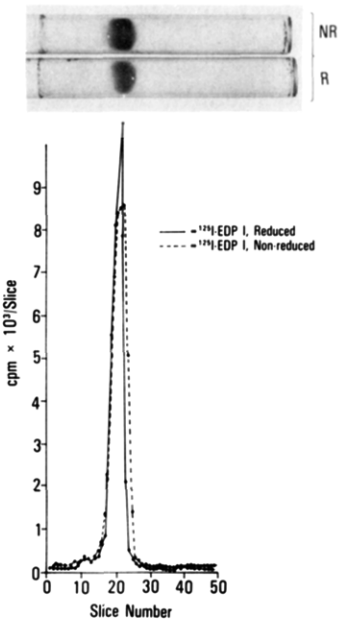

FIGURE 1: Electrophoretic characterization of EDP I and [^{125}I]EDP I. Upper panel: Electrophoresis of 10 μg of EDP I on 10% SDS–polyacrylamide gels under reducing (R) or nonreducing (NR) conditions. Lower panel: radioiodinated EDP I was run on 10% SDS–polyacrylamide gels. Gels were slices in 1-mm sections and counted for radioactivity.

Fibrinogen was prepared as previously described (Keckwick et al., 1955). Prothrombin was a gift from Dr. Daryl Fair, Research Institute of Scripps Clinic. Tissue plasminogen activator from melanoma cells and α_2-antiplasmin were gifts from Dr. Desire Collen, Leuven, Belgium. The possibility that the α_2-antiplasmin preparations were contaminated with plasminogen was tested in a radioimmunoassay employing the glycine-HCl eluate as the antibody and [^{125}I]Glu-plasminogen or [^{125}I]EDP I as ligands. In order to eliminate the influence of the interaction of α_2-antiplasmin with lysine binding sites of these ligands, the assays were performed in the presence of 0.2 M 6-AHA. No plasminogen was detectable in the α_2-antiplasmin preparation at a sensitivity of 0.05 nM plasminogen, and, when diluted into the radioimmunoassays, the maximal possible concentration of plasminogen that could be present would not compete with any of the radiolabeled ligands for binding to the antibody.

Protein concentrations were determined spectrophotometrically at 280 nm by using published extinction coefficients ($E^{1\%}_{1cm}$ 16.8 for plasminogen (Wallen & Wiman, 1972), 22.5 for EDP I, 25 for EDP II, 14 for miniplasminogen (Nilsson et al., 1982), 6.7 for α_2-antiplasmin (Wiman & Collen, 1977), and 15.5 for fibrinogen (Mihalyi, 1968)).

RESULTS

The EDP I fragment, which contains kringles 1–3, was isolated from porcine elastase digests of human plasminogen. This derivative exhibited a broad, single band when analyzed by polyacrylamide gel electrophoresis under reducing and nonreducing conditions (Figure 1) and had an estimated molecular weight of 37500. Under optimal staining conditions, several preparations of EDP I used during the course of these studies could be resolved into 3–4 bands. Such heterogeneity has been observed previously (Vali & Patthy, 1982) and is due to alternative cleavages at either Val-337 or Val-353 of plasminogen by elastase as well as to heterogeneity at the glycosylation sites in the fragments (Lijnen et al., 1981). All preparations had tyrosine as the predominant amino terminal residue, corresponding to Tyr-79 of plasminogen. The amino

Table I: Amino Acid Composition of Isolated EDP I[a]

amino acid	EDP I	predicted[b]	amino acid	EDP I	predicted[b]
lysine	67	69	glycine	65	65
histidine	40	40	alanine	32	32
arginine	60	61	valine	32	24
aspartic acid	131	134	methionine	11	12
threonine	96	101	isoleucine	22	24
serine	97	89	leucine	43	45
glutamic acid	109	113	tyrosine	51	52
			phenylalanine	17	16
proline	124	121			

[a] Expressed as residues per 1000 total amino acid residues. [b] Predicted based on sequence data for plasminogen residues 79–353 (Sottrup-Jensen et al., 1975).

acid composition of a representative EDP I preparation is shown in Table I and is highly consistent with that predicted from the amino acid sequence (Sottrup-Jensen, et al., 1978). When radioiodinated, [^{125}I]EDP I had the same mobility as nonlabeled EDP I on a 10% polyacrylamide gel (Figure 1), and 90% of the radioactivity applied to the gel was recovered within the EDP I band.

Rabbit antibodies to EDP I were bound to plasminogen–Sepharose and sequentially eluted with 0.2 M 6-AHA and 0.2 M glycine-HCl, pH 2.2. After dialysis, the capacity of the antibody fractions to bind [^{125}I]EDP I was assessed in double-antibody radioimmunoassays. No residual antibody was detected in the unbound fraction, indicating complete depletion of antibodies reacting with EDP I. This indicated the absence of antibodies that reacted exclusively with this elastase degradation product but not with plasminogen. As shown in Figure 2A, the 6-AHA eluate bound EDP I. Its antigen binding capacity, ABC-33%, was 22 mol EDP I bound/mL. This reaction was completely abrogated by inclusion of 0.2 M 6-AHA in the assay. In contrast, the glycine-HCl eluate was only modestly affected by 6-AHA (Figure 2B); the difference in ABC-33% in the presence and absence of 0.2 M 6-AHA was 2-fold. On the basis of ABC-33%, the distribution of [^{125}I]EDP I binding activity in the 6-AHA and the glycine-HCl eluates was 5% and 95%, respectively. To further characterize the 6-AHA eluate, three ω-aminocarboxylic acids were serially diluted into the radioimmunoassay. As shown in Figure 3, each lysine analogue produced a concentration-dependent inhibition curve and yielded complete inhibition of antibody binding of [^{125}I]EDP I at high concentrations. The concentrations of the ω-aminocarboxylic acids required for 50% relative inhibition were 2.6, 46, and 1730 μM for AMCA, 6-AHA, and lysine, respectively. When [^{125}I]Glu-plasminogen or [^{125}I]Lys-plasminogen were used as radiolabeled ligands, similar dose-dependent inhibition curves were obtained with the lysine analogues. The values for 50% relative inhibition of the binding of these radiolabeled ligands by the lysine analogues are summarized in Table II. With each ligand, the potency of the lysine analogues as inhibitors was AMCA > 6-AHA > lysine. The 50% inhibition values for each lysine analogue with the three different ligands were not significantly different. For example, with AMCA, 50% inhibition of

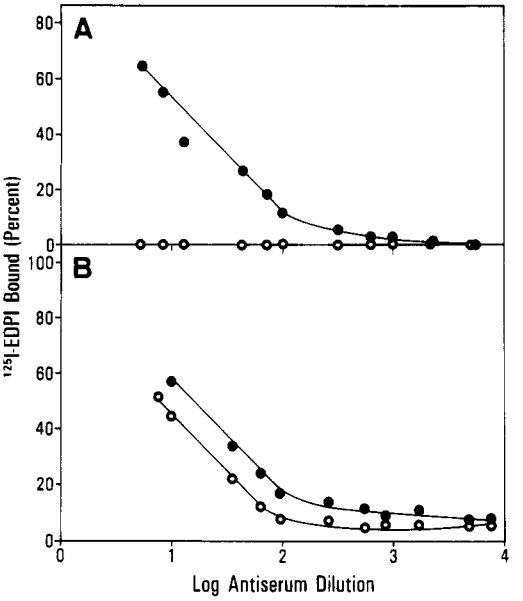

FIGURE 2: Immunopurification of antibodies to EDP I: binding of [^{125}I]EDP I by antibody subpopulations in the presence or absence of 0.2 M 6-aminohexanoic acid. (A) Antibody bound to plasminogen Sepharose and eluted with 0.2 M 6-aminohexanoic acid. (B) Antibody bound to plasminogen–Sepharose and eluted with 0.2 M glycine–HCl, pH 2.2. The concentration of [^{125}I]EDP I was 0.22 nM. Closed circles indicate antibody plus buffer. Open circles indicate antibody plus 0.2 M 6-aminohexanoic acid.

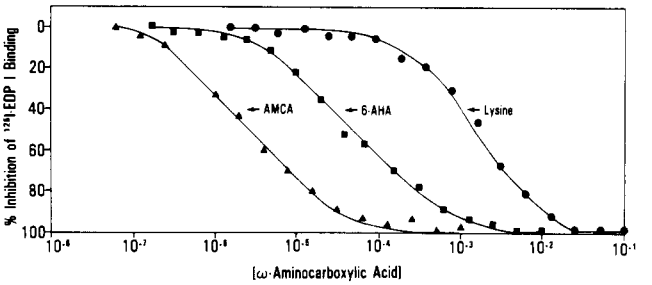

FIGURE 3: Inhibition by ω-aminocarboxylic acids of the binding of [^{125}I]EDP I (0.22 nM) by the 6-AHA-eluted antibodies. The final concentrations of AMCA, 6-aminohexanoic acid (6-AHA), and lysine are indicated on the horizontal axis. The antibodies were utilized at a dilution that bound 54% of [^{125}I]EDP I.

[^{125}I]EDP I, [^{125}I]Glu-plasminogen and [^{125}I]Lys-plasminogen occurred at 2.6, 2.0, and 2.3 μM AMCA. These results suggest that an unoccupied lysine binding site must be available for the antibodies in the 6-AHA eluate to react with these ligands. From a comparison of the 50% inhibition values to the known binding affinities of these ω-aminocarboxylic acids for plasminogen (Markus et al., 1978a,b, 1979, 1981), it is concluded that the recognized epitope(s) is associated with the high-affinity lysine binding site of plasminogen.

The possibility that the antiserum might also recognize the ω-aminocarboxylic acid binding site of intermediate affinity expressed on Lys-plasminogen was considered. Binding of AMCA or 6-AHA to [^{125}I]Lys-plasminogen was analyzed by

Table II: Concentrations of ω-Aminocarboxylic Acids Required To Produce 50% Inhibition of the Binding of Plasminogen Derivatives by the 6-AHA-Eluted Antibody

ω-aminocarboxylic acid	concn required for 50% inhibition (μM)		
	[^{125}I]EDP I	[^{125}I]Glu-plasminogen	[^{125}I]Lys-plasminogen
lysine	1730 ± 82[a]	973 ± 315[a]	nd
6-aminohexanoic acid	46 ± 2[a]	45 ± 1[a] (9)[c]	14 ± 5[a] (35)[e]
AMCA	2.6 ± 0.1[a] (1.1)[f]	2.0 ± 0.8[a] (1.1)[d]	2.3 ± 0.1[b] (2.2)[d]

[a] Average of two determinations. [b] Average of three determinations. [c] Reported in Markus et al. (1978a). [d] Reported in Markus et al. (1979). [e] Reported in Markus et al. (1978b). [f] Reported in Markus et al. (1981).

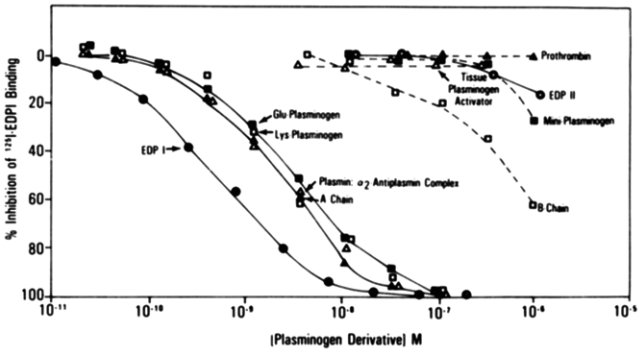

FIGURE 4: Competitive inhibition by plasmin(ogen) derivatives of the binding of [^{125}I]EDP I by the 6-AHA-eluted antibodies. The antibody dilution used bound 51% of the [^{125}I]EDP I (0.22 nM) present in the radioimmunoassay for all competitors except the plasmin B chain, which was assayed with the antibodies at a dilution that bound 40% of the [^{125}I]EDP I (0.22 nM) present in the radioimmunoassay.

using the Ligand computer program (Munson & Rodbard, 1980). The data could be fit to a one-site model. When a two-site model was tested, the mean square error was increased 1.5-fold for AMCA and 14-fold for EACA, compared to a one-site fit. Therefore, a model in which the antibody recognizes only one epitope with a single affinity for ω-aminocarboxylic acids is most consistent with the experimental data.

The kringles of plasminogen exhibit a high degree of primary structural homology with one another as well as with the kringle structures of other proteins (Sottrup-Jensen et al., 1978; Pennica et al., 1983; Gunzler et al., 1982). Therefore, the reactivity of the 6-AHA eluate with various plasminogen derivatives containing kringles was analyzed (Figure 4). Only plasminogen derivatives containing the EDP I region were effective inhibitors of the binding of [^{125}I]EDP I by the antibodies in the 6-AHA eluate. The inhibitory derivatives included EDP I, Glu-plasminogen, the plasmin A chain, and the plasmin–α_2-antiplasmin complex. Of these derivatives, EDP I was the most effective inhibitor, suggesting that other regions of plasminogen may affect the accessibility of the EDP I region to the antibody probe. Minimal inhibition (cross-reactivity or a contamination with an EDP I-containing fragment of $\leq$0.6% relative to Glu-plasminogen) was observed with derivatives lacking an EDP I region [miniplasminogen, the plasmin light chain, or EDP II (kringle 4)]. Tissue plasminogen activator and prothrombin, which also contain kringle regions, did not react with the antiserum. Glu-plasminogen and Lys-plasminogen produced similar inhibition curves, suggesting that the high-affinity lysine binding site was equally available on both molecules. (This issue is addressed in greater detail below.) The plasmin heavy chain and the plasmin–α_2-antiplasmin complex produced superimposable inhibition curves and were more effective competitors than Glu- or Lys-plasminogen. This result suggests that the high-affinity lysine binding site is unoccupied in the plasmin–α_2-antiplasmin complex and that the conformation of the plasmin moiety is altered in the complex to render it more accessible to antibody.

To further examine the availability of the high-affinity lysine binding site in plasminogen forms, competitive inhibition curves were constructed with [^{125}I]Glu-plasminogen, [^{125}I]Lys-plasminogen, or [^{125}I]EDP I as ligand. Table III summarizes the concentrations of competitors required for 50% inhibition of antibody binding of these ligands by EDP I, Glu-plasminogen, and Lys-plasminogen. In all cases, the immunizing antigen, EDP I, was a more effective competitor. The inhibitory capacities of Glu- and Lys-plasminogen were very similar, and the differences were in all cases less than twofold.

Table III: Competitive Inhibition by Plasminogen Derivatives of the Binding of ^{125}I-Radiolabeled Ligand by the 6-AHA-Eluted Antibodies

radiolabeled ligand	competitor concn required for 50% binding inhibn of ^{125}I-ligand to anti-EDP I ($\times 10^{-9}$M)		
	EDP I	Glu-plasminogen	Lys-plasminogen
EDP I	0.73	1.3	2.2
Glu-plasminogen	0.37	1.6	2.8
Lys-plasminogen	0.23	0.79	1.3

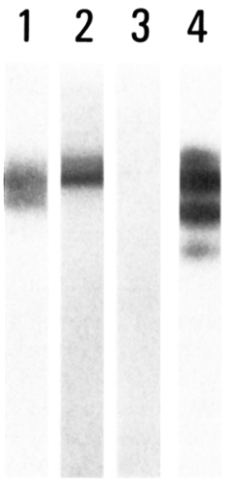

FIGURE 5: Immunoblotting of EDP I and a chymotryptic digest of EDP I with the 6-AHA-eluted antibodies. EDP I (7.7 mg/mL) was digested with chymotrypsin (0.67 mg/mL) in 0.1 M Tris buffer, pH 8.1, for 16 h at 37 °C. Samples were run on 15% polyacrylamide gels in SDS, electrophoretically transferred to nitrocellulose, and reacted with the 6-AHA-eluted antibody. Reactions were detected with [^{125}I]goat anti-rabbit IgG followed by autoradiography. Lane 1, 5 μg of EDP I under nonreducing conditions. Lane 2, 10 μg of EDP I under reducing conditions; Lane 3, 10 μg of EDP I under reducing conditions and the immunoblotting was performed in 0.2 M 6-AHA. Lane 4, 40 μg of chymotryptic digest of EDP I under nonreducing conditions.

To further localize and characterize the region of EDP I involved in the interaction with the antibody, EDP I was digested with chymotrypsin at enzyme/substrate ratios (w/w) ranging from 1/5 to 1/100. Under conditions where enzymatic degradation of EDP I was detected, as assessed by SDS–polyacrylamide gel analyses, progressive loss and ultimate abolition of the recognized epitope(s) was observed. When a condition was utilized, 1/12 (w/w) chymotrypsin/EDP I ratio for 16 h, at which some antigenic expression was preserved, the nature of the fragments was examined by immunoblotting of SDS–polyacrylamide gels (Figure 5). The antibody reacted with the EDP I under reducing and nonreducing conditions, and the immunoblotting was blocked by 0.2 M 6-AHA. Under nonreducing conditions, the antibody reacted with several degradation products of EDP I, the smallest of which had an estimated molecular weight of 20 000. The immunoblotting pattern closely reflected the protein staining pattern as species of $M_r \leq 20\,000$ were not detected by Coomassie Blue staining. Under reducing conditions, immunoblotting was variable, perhaps due to variable renaturation of appropriate disulfide bond arrangements, but fragments of less than M_r 20 000 were also not detected. These results demonstrate that the antibody recognizes a region within EDP I and that the lysine-binding function is retained within this smaller region.

Plasminogen can be eluted from fibrin by concentrations of 6-AHA consistent with involvement of a high-affinity lysine

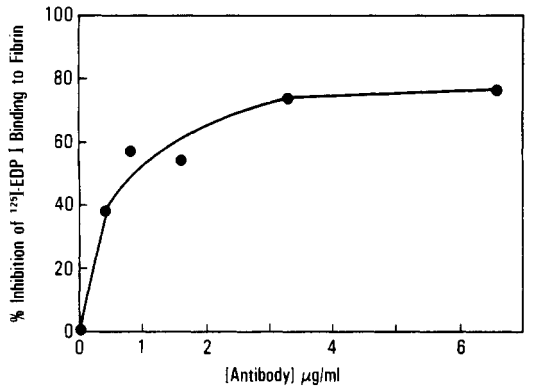

FIGURE 6: Inhibition of binding of [^{125}I]EDP I to fibrin by anti-EDP I. [^{125}I]EDP I (0.23 nM) was preincubated with anti-EDP I or buffer and its ability to bind to fibrin was tested as described in Materials and Methods. The percent inhibition of lysine binding site-mediated binding is shown on the vertical axis.

binding site in the interaction (Wiman & Collen, 1978; Lucas et al., 1983). The effect of the 6-AHA eluate on the binding of [^{125}I]EDP I to fibrin was tested (Figure 6). [^{125}I]EDP I was preincubated with the antibody for 18 h at 4 °C and then incubated with fibrin in microtiter wells for 1 h. When [^{125}I]EDP I was preincubated without antibody, 8% of the total counts bound to fibrin. This percent is consistent with reported values for the incorporation of plasminogen into fibrin clots (Wiman & Wallen, 1977). When [^{125}I]EDP I was preincubated with 0.2 M 6-AHA, the interaction was inhibited by ≥90%, affirming the involvement of the lysine binding sites for the interaction in this system. The 6-AHA eluate produced a dose-dependent inhibition of [^{125}I]EDP I binding to fibrin and the maximum inhibition was approximately 80%. The serum that did not absorb to plasminogen–Sepharose was used as a nonimmune control and produced only 8% inhibition of binding at a protein concentration at which the 6-AHA-eluted antibody produced 55% inhibition of binding. As an additional control, the glycine-HCl eluate was tested and found to be much less inhibitory. At concentrations of the 6-AHA and glycine-HCl eluates that bound an equivalent quantity of [^{125}I]EDP I, the inhibition of [^{125}I]EDP I fibrin binding by these antibody subpopulations was 57% and 17%, respectively. This low inhibitory activity of the glycine-HCl eluate may reflect steric effects or the contribution of a small population of residual antibodies to the high-affinity lysine binding site in the glycine-HCl eluate.

The binding of plasmin(ogen) to α_2-antiplasmin appears to require a free high-affinity lysine binding site (Christensen & Clemmensen, 1977; Wiman & Collen, 1978; Wiman et al., 1979). Since the binding of antibodies in the 6-AHA eluate to plasminogen derivatives required an unoccupied high-affinity lysine binding site, the ability of α_2-antiplasmin to inhibit binding of either [^{125}I]EDP I, [^{125}I]Glu-plasminogen, or [^{125}I]Lys-plasminogen by the antibody was tested. Concentrations of α_2-antiplasmin between 0.7 and 2.5 μM inhibited antibody binding to each ligand by 50%, consistent with the reported dissociation constants for the interaction of α_2-antiplasmin with these ligands (Wiman et al., 1979). In contrast, when concentrations of thrombospondin between 22 and 267 nM were incorporated into the radioimmunoassay, inhibition of antibody binding did not exceed 10%. The reported apparent K_d for the thrombospondin–Glu-plasminogen interaction is 35 nM, and it has been suggested that the interaction involves the high-affinity lysine binding site of plasminogen (Silverstein et al., 1984). This result suggests either that the affinity of soluble thrombospondin for plasminogen is less than for thrombospondin absorbed to plastic or that the interaction is not mediated through the high-affinity lysine binding site as identified with the antibody probe described in this study.

Discussion

In this study, an antiserum to EDP I has been fractionated based on the capacity of 6-AHA and glycine-HCl, pH 2.2, to elute antibody populations from plasminogen–Sepharose. The 6-AHA eluate was characterized and found to react exclusively with plasminogen derivatives containin the EDP I (kringles 1–3) region and not with plasminogen derivatives containing kringles 4 or 5 or with tissue plasminogen activator or prothrombin, which also contain kringle structures. Therefore, the epitopes recognized by these antibodies are not shared by these other kringle-containing proteins although the five plasminogen kringles exhibit 35% structural homology with each other and 25% homology with the two kringles of prothrombin (Sottrup-Jensen et al., 1978). Polyclonal (Plow & Collen, 1981; Hochschwender & Laursen, 1981) and monoclonal (Ploplis et al., 1982) antibodies that react exclusively with EDP II (kringle 4) or with EDP I (Ploplis et al., 1982; Cummings et al., 1984) have previously been described. Thus, the ability of our antiserum to discriminate between kringle structures within plasminogen and between plasminogen and other molecules further establishes the nonidentity of epitopes within these highly homologous regions. In addition, immunoblotting experiments demonstrated that the antibody reacts specifically with several chymotryptic degradation products of EDP I including a fragment of M_r 20000. Further digestion of EDP I with chymotrypsin resulted in loss of the recognized epitope. Several studies (Wiman & Wallen, 1977, Lerch et al., 1980) have indicated that chymotrypsin can produce a fragment of M_r 7000 that corresponds to kringle 1 and retains lysine binding function. Yields of this derivative have, however, not been indicated. The failure of our antibody to immunoblot such a derivative suggests that this fragment may represent a product generated at low yield, which stains poorly with Coomassie Blue and does not transfer well to nitrocellulose, or that the epitope(s) recognized by the antibodies do not reside exclusively in the kringle 1 region. A derivative of M_r 20000 is consistent with a fragment containing kringles 1 and 2.

The binding of the 6-AHA eluted antibodies to [^{125}I]EDP I, [^{125}I]Glu-plasminogen or [^{125}I]Lys-plasminogen was inhibited in a dose response manner up to 99% by ω-aminocarboxylic acids. The effectiveness of these lysine analogues as inhibitors of the antigen–antibody interaction was, in decreasing order, AMCA, 6-AHA, and lysine. This order is consistent with the potency of these compounds as inhibitors of fibrinolysis (Markwardt, 1978) as well as with their reported abilities to bind to plasminogen (Markus et al., 1978a,b, 1979, 1981) and suggests that these antibodies require an unoccupied lysine binding site to interact with the plasminogen. Polyclonal and monoclonal antibodies that require an unoccupied lysine binding site in kringle 4 have been reported (Plow & Collen, 1981; Hochschwender & Laursen, 1981; Ploplis et al., 1982), but the described antibodies to EDP I were unaffected in the presence of ω-aminocarboxylic acids (Ploplis et al., 1982; Cummings et al., 1984). From the studies with antibodies to kringle 4, it appeared that the concentrations of lysine analogues required for 50% relative inhibition of ligand–antibody binding were equivalent to their dissociation constants for kringle 4. The issue then arises as to whether the 6-AHA-eluted antibodies recognize the high- and/or low-affinity lysine binding sites of EDP I. The dissociation constants of AMCA for the high-affinity lysine binding site of EDP I, Glu-plasminogen, and Lys-plasminogen were estimated to be 1.1, 1.1,

and 2.2 μM by Markus et al. using ultrafiltration (Markus et al., 1979, 1981). The immunochemical values derived in this study, 2.6, 2.0, and 2.3 μM, are in excellent agreement and suggest that the antibody recognizes only the high-affinity lysine binding site in these derivatives. For 6-AHA the dissociation constants derived immunochemically were 46, 45, and 14 μM for EDP I, Glu-plasminogen, and Lys-plasminogen, respectively, while ultrafiltration experiments yielded 9 and 35 μM for Glu-plasminogen and Lys-plasminogen, respectively (Markus et al., 1978a,b). The immunochemical data also suggest that the dissociation constants of lysine are 38-fold and 22-fold higher than that of 6-AHA for EDP I and Glu-plasminogen, respectively. Previous studies suggested that these differences were 24-fold for EDP II and 14-fold for Glu-plasminogen for the low-affinity lysine binding site of kringle 4 (Plow & Collen, 1981). Thus, the relative differences in affinity for lysine and 6-AHA are similar for both the low- and high-affinity interactions. In sum, the data presented here suggest that the antibodies in the 6-AHA eluate recognize an unoccupied high-affinity lysine binding site.

The high-affinity lysine binding site of plasminogen is required for its interaction with α_2-antiplasmin (Christensen & Clemmensen, 1977; Wiman & Collen, 1978; Wiman et al., 1979), fibrin, and fibrinogen (Rokoczi et al., 1978; Lucas et al., 1983), and it has been suggested that this region is also involved in the interaction of plasminogen with thrombospondin (Silverstein et al., 1984). In addition, plasminogen conformation (Abiko et al., 1969; Brockway & Castellino, 1972; Castellino et al., 1973; Sjoholm et al., 1982) and kinetics of activation (Claeys & Vermylen, 1974; Violand et al., 1978; Walter et al., 1975; Thorsen & Mullertz, 1974) are strongly affected by ω-aminocarboxylic acids. In our study α_2-antiplasmin inhibited the binding of [^{125}I]EDP I, [^{125}I]Glu-plasminogen, or [^{125}I]Lys-plasminogen to the 6-AHA-eluted antibody. In contrast, the plasmin–α_2-antiplasmin complex reacted well with the 6-AHA-eluted antibody. This suggests that the high-affinity lysine binding site is unoccupied in the stabilized form of the complex, even though the high-affinity lysine binding site participates in the initial interaction of plasmin(ogen) with α_2-antiplasmin. A similar conclusion has been suggested from circular dichroism studies of the plasmin–α_2-antiplasmin complex (Nilsson et al., 1982).

The 6-AHA eluted antibodies inhibited the lysine binding site-dependent interaction of [^{125}I]EDP I with fibrin by 76%, supporting a role for the high-affinity lysine binding site in the interaction with fibrin. This result does not necessarily exclude involvement of other lysine or nonlysine binding sites in the interaction. Nonetheless, it provides further evidence that the high-affinity lysine binding site is required for this interaction.

Thrombospondin did not interfere with the interaction of the 6-AHA-eluted antibody and [^{125}I]Glu-plasminogen when present at a concentration 7.6-fold higher than its reported K_d for plasminogen. It has been suggested that thrombospondin interacts with plasminogen through the high-affinity lysine binding site based on an I_{50} for EACA of 200 μM (Silverstein et al., 1984). This value is over 20-fold higher than the reported affinity of Glu-plasminogen for EACA, 9 μM (Markus et al., 1978a). Therefore, it appears that the high-affinity lysine binding site, as identified with the antibody probe described in this study, is not required for the interaction of Glu-plasminogen and thrombospondin. Alternatively, the K_d for the thrombospondin–plasminogen interaction in the soluble phase may be higher than that measured when thrombospondin is absorbed to plastic.

The studies described here provide an alternative method for measuring the affinity of plasminogen and its derivatives for ω-aminocarboxylic acids. In addition it provides a further means of identifying the regions of plasminogen involved in its interactions with substrates, inhibitors, and other molecules that regulate the fibrinolytic system.

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Registry No. 6-AHA, 60-32-2; AMCA, 1197-18-8; L-lysine, 56-87-1; plasminogen, 9001-91-6.

REFERENCES

Abiko, Y., Iwanoto, M., & Tomikawa, M. (1969) *Arch. Biochem. Biophys. 151*, 194.

Allen G. (1981) *Sequencing of Proteins and Peptides*, pp 168–173, North-Holland, Amsterdam.

Brockway, W. J., & Castellino, F. J. (1972) *Arch. Biochem. Biophys. 151*, 194.

Castellino, F. J. (1981) *Chem. Rev. 81*, 431.

Castellino, F. J., Brockway, W. J., Thomas, J. K., Liao, H. J., & Rawitch, A. B. (1973) *Biochemistry 12*, 2787.

Castellino, F. J., Ploplis, V. A., Powell, J. R., & Strickland, D. R. (1981) *J. Biol. Chem. 256*, 4778.

Christensen, U., & Clemmensen, I. (1977) *Biochem. J. 163*, 389.

Claeys, H., & Vermylen, J. (1974) *Biochim. Biophys. Acta 342*, 351.

Collen, D. (1976) *Eur. J. Biochem. 69*, 209.

Collen, D. (1980) *Thromb. Haemostasis 43*, 77.

Cummings, H. S., Ploplis, V. A., Beals, J. M., & Castellino, F. T. (1984) *Arch. Biochem. Biophys. 230*, 306.

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

Groskopf, W. R., Summaria, L., & Robbins, K. C. (1969) *J. Biol. Chem. 244*, 3590.

Gunzler, W. A., Steffens, G., Otting, F., Kim, S.-M. A., Frankus, E., & Flohe, L. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 1155.

Hochschwender, S. M., & Laursen, R. A. (1981) *J. Biol. Chem. 256*, 11 166.

Johnson, D. A., Gautsch, J. W., Sportsman, J. R., & Elder, J. H. (1984) *Gene Anal. Techn. 1*, 3.

Keckwick, R. A., Mackay, M. E., Nance, M. H., & Record, B. R. (1955) *Biochem. J. 60*, 671.

Laemmli, U. K. (1970) *Nature (London) 227*, 280.

Lammle, B., Berrettini, M., Schwartz, H. P., Heeb, M. J., & Griffin, T. H. (1986) *Thromb. Res. 41*, 747.

Lawler, J. W., Slayter, H. S., & Coligan, J. E. (1978) *J. Biol. Chem. 253*, 8609.

Lerch, P. G., Rickli, E. E., Lergher, W., & Gillessen, D. (1980) *Eur. J. Biochem. 107*, 7.

Lijnen, H. R., Hoylaerts, M., & Collen, D. (1980) *J. Biol. Chem. 255*, 10 214.

Lijnen, R., Van Hoef, B., & Collen, D. (1981) *Eur. J. Biochem. 120*, 149.

Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983) *J. Biol. Chem. 258*, 4249.

Magnusson, S., Peterson, T. G., Sottrup-Jensen, L., & Claeys, H. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B., & Shaw, E., Eds.) p 123, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Malinowski, D. P., Sadler, E. J., & Davie, E. W. (1984) *Biochemistry 23*, 4243.
Markus, G., De Pasquale, J. L., & Wissler, F. C. (1978a) *J. Biol. Chem. 253*, 727.
Markus, G., Evers, J. L., & Hobika, G. H. (1978b) *J. Biol. Chem. 253*, 733.
Markus, G., Priore, R. L., & Wissler, F. C. (1979) *J. Biol. Chem. 254*, 1211.
Markus, G., Camiolo, S. M., Sottrup-Jensen, L., & Magnusson, S. (1981) *Prog. Fibrinolysis 5*, 125.
Markwardt, F. (1978) in *Fibrinolytics and Anti-fibrinolytics* (Markwardt, F., Ed.) p 487, Springer-Verlag, Berlin.
McConahey, P., & Dixon, F. (1966) *Int. Arch. Allergy Appl. Immunol. 29*, 181.
Mihalyi, E. (1968) *Biochemistry 7*, 208.
Minden, P., & Farr, R. S. (1967) in *Handbook of Experimental Immunology* (Weir, D. M., Ed.) p 463, F. A. Davis, Philadelphia, PA.
Moroi, M., & Aoki, N. (1976) *J. Biol. Chem. 251*, 5956.
Mullertz, S., & Clemmensen, I. (1976) *Biochem. J. 159*, 545.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220.
Neurath, H. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 2907.
Nilsson, T., Sjoholm, I., & Wiman, B. (1982) *Biochim. Biophys. Acta 705*, 264–270.
Novokhatny, V. V., & Kudinov, S. A. (1984) *J. Mol. Biol. 179*, 215.
Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ard, C. A., Bennet, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L., Goeddel, D. V., & Collen, D. (1983) *Nature (London) 301*, 214.
Ploplis, V. A., Cummings, H. S., & Castellino, F. J. (1982) *Biochemistry 21*, 5891.
Plow, E. F., & Collen, D. (1981) *J. Biol. Chem. 256*, 10864.
Plow, E. F., DeCock, F., & Collen, D. (1979) *J. Lab. Clin. Med. 93*, 199.
Rickli, E. E., & Otavsky, W. I. (1975) *Eur. J. Biochem. 9*, 441.
Rokoczi, I., Wiman, B., & Collen, D. (1978) *Biochim. Biophys. Acta 540*, 295.
Silverstein, R. L., Leung, L. L. K., Harpel, P. C., & Nachman, R. L. (1984) *J. Clin. Invest. 74*, 1625.
Sjoholm, I., Wiman, B., & Wallen, P. (1982) *Biochim. Biophys. Acta 705*, 264.
Sodetz, J. M., & Castellino, F. J. (1975) *J. Biol. Chem. 250*, 3041.
Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis 3*, 191.
Thorsen, S. (1975) *Biochim. Biophys. Acta 393*, 55.
Thorsen, S., & Mullertz, S. (1974) *Scand. J. Clin. Lab. Invest. 34*, 167.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350.
Vali, Z., & Patthy, L. (1982) *J. Biol. Chem. 257*, 2104.
Violand, B. N., Byrne, R., & Castellino, F. J. (1978) *J. Biol. Chem. 253*, 5395.
Wallen, P., & Wiman, B. (1972) *Biochim. Biophys. Acta 257*, 122.
Walter, P. J., Hill, R. L., & McKee, P. A. (1975) *J. Biol. Chem. 250*, 5926.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Wiman, B. (1977) *Eur. J. Biochem. 76*, 129.
Wiman, B., & Wallen, P. (1975a) *Eur. J. Biochem. 50*, 489.
Wiman, B., & Wallen, P. (1975b) *Eur. J. Biochem. 58*, 539.
Wiman, B., & Collen, D. (1977) *Eur. J. Biochem. 78*, 19.
Wiman, B., & Wallen, P. (1977) *Thromb. Res. 10*, 213.
Wiman, B., & Collen, D. (1978) *Eur. J. Biochem. 84*, 573.
Wiman, B., & Collen, D. (1979) *J. Biol. Chem. 254*, 9291.
Wiman, B., Lijnen, H. R., & Collen, D. (1979) *Biochim. Biophys. Acta 579*, 142.
Wolff, R., Plow, E. F., & Ginsberg, M. H. (1986) *J. Biol. Chem. 261*, 6840.