A Unique Proteolytic Fragment of Human Fibrinogen Containing the Aα COOH-terminal Domain of the Native Molecule*

(Received for publication, December 21, 1989)

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The COOH-terminal portion of the Aα chain of human fibrinogen is highly susceptible to proteolytic degradation. This property has prevented isolation of the COOH-terminal domain of fibrinogen for the direct investigation of its functional characteristics. Human fibrinogen was degraded with hematin, a fibrinogenolytic protease from the posterior salivary glands of the leech, Haementeria ghilianii. Two initial fragments, Yhem1 and Dhem1, produced by cleavage through the three polypeptide chains in the connector region, were characterized and shown to retain the entire Aα COOH-terminal domain. Late cleavages by hematin occurred in the Aα chain COOH-terminal region to produce fragments Yhem and Dhem with shorter Aα chain remnants. Fragments Dhem were isolated from an intermediate hematin digest of fibrinogen using anion-exchange chromatography. Fragment Dhem1 was separated further from Dhem fragments with shorter α chain remnants by affinity chromatography on immobilized plasma fibronectin. Fragment Dhem1 represents a unique proteolytic fragment of fibrinogen containing an intact Aα chain COOH-terminal region. NH2-terminal sequence analysis of isolated chains from fragment Dhem1 located hematin cleavage sites in the connector region to Aα Asn<sup>105</sup>-Asn<sup>106</sup>, Bβ Lys<sup>130</sup>-Gln<sup>131</sup>, and γ Pro<sup>150</sup>-Asn<sup>157</sup>. The specific interaction of fragment Dhem1 with immobilized fibronectin indicated that the binding site probably was located within the COOH-terminal 111 amino acids of the Aα chain. The overall pattern of fibrinogen cleavage by hematin is similar to that of plasmin, yet hematin cleaves preferably before attacking the other chains (20-24). The Aα chains are also cleaved first by trypsin (6).

Fibrinogenolytic enzymes have been discovered in leukocytes (13) and platelets (14) and have been implicated in an alternative in vivo pathway for fibrinolysis (15). Although leukocyte and platelet proteases give different cleavage patterns than plasmin, the blood cell enzymes attack the Aα chain first (16-19). Fibrinogenolytic proteases isolated from some snake venoms also cleave fibrinogen in the Aα chains preferably before attacking the other chains (20-24). The Aα chains are also cleaved first by trypsin (6).

The giant South American leech Haementeria ghilianii contains a fibrinogen-degrading protease, hematin, which is present in the posterior and anterior salivary glands (25). The present study aimed at elucidating the pattern of fibrinogen degradation by hematin from the posterior salivary glands of the leech. Structural characterization of fibrinogen degradation fragments was performed and a unique fragment called Dhem1 was retained intact COOH terminus of the native molecule, was purified. The structure of fragment Dhem1 was characterized, and its binding to plasma fibronectin was demonstrated.

**EXPERIMENTAL PROCEDURES**

Reagents—HEPES<sup>1</sup> (U. S. Biochemical Corp., Cleveland, OH), Trit, DTT, hemazamide, 4-chloro-1-naphthol (Sigma), plasmin (Kabi, Stockholm, Sweden), Trasylol (Mobay Chemical Corp., New York, NY), EDTA (MCB Manufacturing Chemists Inc., Cincinnati, OH), ammonium bicarbonate (Mallinkrodt Inc., Paris, KY), CaCl<sub>2</sub> as a 1 M solution (BDH Biochemicals, Poole, England), Geltain-Sepharose, Sepharose CL-6B, Sepharose 4B (Pharmacia LKB Biotechnology), ultrapure, electrophoresis-grade acrylamide (Polysciences Inc., Warrington, PA), SDS, Affi-Gel 15, horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Bio-Rad), trifluoroacetic acid (Fischer), acetonitrile (Fisher), Iodoacetic acid (Eastman Kodak, Rochester, NY), was recrystallized freshly from heptane.

Human band I fibrinogen was purified from pooled plasma as described (24). Purified fibrinogen contained over 95% intact Aα chains as assessed by SDS-PAGE of the reduced sample. Freeze-dried posterior salivary glands from the leech H. ghilianii were a gift from Dr. Gunther S. Steent (Department of Molecular Biology, University of California, Berkeley, CA).

Extraction of Leech Glands—Posterior salivary glands from the leech H. ghilianii were extracted as described (33). Freeze-dried glands were placed in 20 mM HEPES, 10 mM CaCl<sub>2</sub>, pH 7.8 buffer, and

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1 The abbreviations used are: HEPES, N-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; CM-Dhem1, carboxymethylated fragment Dhem1; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; mAb, monoclonal antibody.
allowed to stand for 15 min at room temperature. The mixture was then allowed to cool to 4 °C before homogenization. Homogenization was with a Tri-R-stir-R Model K41 homogenizer (Tri-R instruments, Rockville Center, NY). The procedure was carried out in four 10-s bursts. In between homogenizations, the preparation was allowed to cool to 4 °C. The homogenized leech glands were centrifuged at 12,000 × g in a fixed angle rotor using a Sorvall RC-2 centrifuge. The supernatant was taken and stored at 4 °C while the leech gland pellet was re-extracted. The combined supernatants were then centrifuged at 100,000 × g, aliquoted, and stored at −20 °C. Extracts prepared in this manner were stable indefinitely at −20 °C.

Digestion of Band I Fibrinogen with Hementin—Band I fibrinogen, 2 mg/ml final concentration, was digested with 8 µg/ml of gland clonal antisera against fibrinogen, fragment D, fragment E, and the Aα chain were obtained in rabbits. The antisera were absorbed with normal human serum. Antisera to fragment D were also absorbed with human plasma, and the antisera against epitopes on the Aα chain COOH terminus were obtained and characterized as previously described (27). Monoclonal antisera against epitopes on the Aα chain COOH terminus were obtained and characterized as previously described (28). The immunoblot procedure repeated. Fragments Dhem, were then eluted from the column using a linear gradient composed of 35 ml of 0.01 M phosphate, 1.0 M NaCl, 4.0 M urea, pH 7.4, as the gradient buffer. Fragment Dhem, was eluted at a urea concentration of 1.0 M.

Reduction and Carboxymethylation of Fragment Dhem—The reduction and carboxymethylation of polyepitope chains derived from purified fragment Dhem, was done by a modification of the procedure of Bewley et al. (31). One mg of lyophilized fragment Dhem, was dissolved in 1.0 ml of 0.2 M Tris-HCl, 0.0 M guanidine HCl buffer. DTT was added to the fragment Dhem, solution at a 1500-fold molar excess. Reduction was allowed to proceed at 30 °C for 30 min. The reaction mixture was cooled to room temperature using ice water. Iodoacetic acid was added in a 3.0-fold molar excess over DTT. Incubation took place in the dark at room temperature for 20 min and on ice for 30 min. Unreacted DTT and iodoacetic acid were removed by gel filtration. The carboxymethylated chains of fragment Dhem, were analyzed by 10.0-10.0% SDS-PAGE under nonreducing conditions.

Separation of Reduced, Carboxymethylated Fragment Dhem Chains by Revers-phase HPLC—Four hundred µg of lyophilized CM-Dhem, chains were dissolved in a 1.0 ml (v/v) of 0.1% trifluoroacetic acid, 10% acetic acid, to 1.0 mg/ml final concentration. Chromatography was carried out on a Vydac C8 reverse-phase HPLC column, 25 cm × 4.6 mm inner diameter (Vydac, Hesperia, CA), using a Waters HPLC system with a dual pump and an automated gradient controller (Waters Assoc., Inc., Milford, MA). The start was 0% acetonitrile (Bio-Rad).

Characterization of the Polypeptide Chain Composition of Fibrinogen Fragments by Two-dimensional SDS-PAGE—In the first dimension, the fragments were separated by SDS-PAGE on a 3.5-5.0% polyacrylamide slab gel gradient under nonreducing conditions. A 1.0 X 13-cm lane with separated fragments was cut out and incubated in 10 mM DTT at 37 °C for 30 min. The reduced gel was placed at a right angle onto a 9.0% polyacrylamide slab gel and sealed with 1.0% bromphenol blue dye front reached the bottom of the gel. Protein spots were visualized by Coomassie Brilliant Blue R-250 staining. A similar gel was also electroblotted onto nitrocellulose and incubated with antibodies to the Aα chain of fibrinogen. Bands were visualized with Protein A Gold (Bio-Rad).

Separation of Fibrinogen Fragments by Anion-exchange Chromatography—Anion-exchange chromatography of a hematin digest of fibrinogen was carried out on a Mono Q HR 5/5 column with Pharmacia FPLC system with an automated gradient controller (Pharmacia). Samples were prepared for chromatography by extensive dialysis against 0.05 M ammonium bicarbonate, 1 mM EDTA, pH 8.0, followed by filtration of the sample through a 0.22 µm Millipore syringe filter (Millipore Corp., Bedford, MA). The sample was loaded onto the column in 0.05 M ammonium bicarbonate, pH 8.0, and washed to remove any unbound protein. Bound protein was eluted with a linear NaCl gradient in ammonium bicarbonate buffer, pH 8.0: (a) from 0 to 0.2 M in 40 ml, (b) from 0.2 to 0.5 M in 3 ml; and (c) 3-ml isocratic elution at 0.5 M NaCl. Protein was eluted at 1.0 ml/min and collected in 0.65-ml fractions. Six mg of protein were separated per run.

Purification and Immobilization of Plasma Fibrinogen—Plasma fibrinogen was purified from fresh human plasma using affinity chromatography on immobilized gelatin by a modification of the procedure by Engvall and Ruoslahti (30). Sixty mg of purified fibrinogen was coupled to 4 ml of Affi-Gel 15 (Bio-Rad) according to the manufacturer's instructions. The coupling efficiency was >95%.

Separation of Fragment Dhem, from Dhem Fragments with Shorter α Chain Remnants by Affinity Chromatography on Immobilized Plasma Fibrinogen—A preparation containing fragments Dhem, was obtained after chromatography of a hematin digest on Mono Q followed by chromatography on immobilized anti-E antibodies. One mg of the preparation was applied to a column containing 200 mg of immobilized fibrinogen. The sample was applied in phosphate-buffered saline, pH 7.4, and washed with the same buffer at a flow rate of 1.0 ml/min. 0.65-ml fractions were collected. The elution buffer was then switched to 0.01 M phosphate, 1.0 M NaCl, pH 7.4, and the elution procedure repeated. Fragments Dhem, were then eluted from the column using a linear gradient composed of 35 ml of 0.01 M phosphate, 1.0 M NaCl, pH 7.4, as the start buffer and 35 ml of 0.01 M phosphate, 1.0 M NaCl, 4.0 M urea, pH 7.4, as the gradient buffer. Fragment Dhem, was eluted at a urea concentration of 1.0 M.

**Table I**

Molecular mass comparison between fibrinogen fragments

The average sizes for plasmic fragments determined by many investigators using many methods are listed in the left-hand column. The sizes calculated for fibrinogen fragments produced by hematin digestion are characterized by assigned name and molecular weight, calculated from protein standards. The nomenclature for fibrinogen fragments produced by hematin has been derived from that for plasmic fragments.

| Fragment | Mass (kDa) | Fragment | Mass (kDa) | Mass differences (kDa) |
|----------|------------|----------|------------|-----------------------|
| X        | 250        | Yhem 1   | 204        | Yhem 1 – Y plasmin = 49 |
| Y        | 155        | Yhem 2   | 192        | Yhem 2 – D plasmin = 40 |
| D        | 100        | Yhem 3   | 183        | Yhem 3 – Y plasmin = 12 |
| E        | 50         | Dhem     | 140        | Dhem – D plasmin = 12 |
|          |            | Dhem 2   | 128        | Dhem 2 – D plasmin = 9 |
|          |            | Dhem 3   | 119        | Dhem 3 – D plasmin = 7 |
|          |            | Dhem 4   | 102        | Dhem 4 – D plasmin = 5 |
|          |            | Ehem     | 62.5       | Ehem – E plasmin = 38 |
Unique Fibrinogen Fragments Produced by Hementin—The pattern of fibrinogen digestion by hementin was determined by characterizing the structures of fragments formed in a timed digest (Fig. 1). The molecular weight of each fragment formed, calculated relative to standards on SDS-PAGE, was used for evaluating fragment structures.

Early cleavage of fibrinogen produced two fragments of 204 and 140 kDa simultaneously. The two fragments persisted as the major products for the first 8 h of digestion (Fig. 1). Late cleavages produced additional lower molecular weight fragments after 24 h (Fig. 1). The sum of the sizes of the earliest two fragments was calculated at 344 kDa, a value comparable to the mass of the intact fibrinogen molecule. Furthermore, release of smaller peptide material was not detected by SDS-PAGE (data not shown). Using this information in conjunction with the documented model of fibrinogen structure and its degradation by plasmin, we postulated that hementin cleaved first through the three polypeptide chains in the connector region to split the fibrinogen molecule asymmetrically. Since plasmin also cleaves through the connector region of fibrinogen, producing the asymmetric fragment Y, followed by fragments D and E (5, 9), nomenclature for describing the fragments produced by hementin was derived from that developed previously for plasmin fragments.

Plasmin cleaves the Aα chains first forming fragment X of 250 kDa (Table I) (5, 9). Hementin cleavage did not result in the formation of a fragment of comparable size. Therefore, the hypothesis that the early fragments Y_{hem} 1 and D_{hem} 1 retain their Aα chain COOH termini was investigated.

The hypothesis is consistent with the difference in mass between fragments produced by plasmin and hementin (Table...
I). The difference in mass between fragments \(Y_{\text{hem}} 1\) and \(Y\) is 49 kDa, corresponding to the mass of the \(A\alpha\) chain COOH-terminal peptide and the \(B\beta\) NH\(_2\)-terminal peptide released from fibrinogen by plasmin. The mass difference of 40 kDa calculated for fragments \(D_{\text{hem}} 1\) and \(D\), is the mass of the \(A\alpha\) chain COOH-terminal peptide.

The structure of fragment \(E_{\text{hem}}\) was evaluated according to its calculated size (62.5 kDa) and its timed appearance in the fibrinogen digest (Fig. 1). Fragment \(E_{\text{hem}}\) first appeared at 4 h, the band intensity increasing with time. The progressive increase in the amount of fragment \(E_{\text{hem}}\) paralleled the decrease in fragments \(Y_{\text{hem}}\). Longer digestion resulted in the retention of a constant amount of fragment \(E_{\text{hem}}\) (data not shown). Therefore, it was concluded that fragment \(E_{\text{hem}}\) is derived from fragments \(Y_{\text{hem}}\) by cleavage through the three polypeptide chains in the connector region in a manner similar to the generation of fragment \(E\) from fragment \(Y\) by plasmin.

Table I lists size differences calculated for fragments \(Y_{\text{hem}} 1\) and \(Y_{\text{hem}} 2\) (12 kDa), \(D_{\text{hem}} 1\) and \(D_{\text{hem}} 2\) (12 kDa), \(Y_{\text{hem}} 2\) and \(Y_{\text{hem}} 3\) (9 kDa), \(D_{\text{hem}} 2\) and \(D_{\text{hem}} 3\) (9 kDa). The data suggested a precursor-product relationship between fragments \(Y_{\text{hem}} 1\) and smaller fragments \(Y_{\text{hem}}\) as well as \(D_{\text{hem}} 1\) and smaller fragments \(D_{\text{hem}}\). The similarity in size of peptides released to form both of the smaller fragments \(Y_{\text{hem}}\) and \(D_{\text{hem}}\) indicated a cleavage in the COOH terminus of each fragment because the two COOH termini are identical. Furthermore, the total mass of material released upon conversion of fragment \(D_{\text{hem}} 1\) to fragment \(D_{\text{hem}} 4\) (38 kDa) is consistent with cleavage in the \(\gamma\) chain as judged from the mass available for cleavage from each chain on the COOH-terminal side of the disulfide ring.

A direct comparison between the cleavage sites of hementin versus plasmin was made by comparing fibrinogen fragments formed by (1) cleavage with hementin, (2) cleavage with hementin followed by plasmin, and (3) cleavage with plasmin. An intermediate hementin digest of fibrinogen (Fig. 2, first lane), after further cleavage with plasmin, produced plasmic fragments X, Y, D, and E (compare middle lane with last lane in Fig. 2) indicating that some plasmic cleavage sites were still available in fibrinogen fragments produced by hementin. All three \(Y_{\text{hem}}\) fragments were cleaved to a single plasmic fragment \(Y\), and the four \(D_{\text{hem}}\) fragments were cleaved to plasmic fragment \(D\), most likely by cleavage of the \(A\alpha\) chain COOH-terminal peptide. The results are consistent with the idea that fragments \(Y_{\text{hem}}\) and \(D_{\text{hem}}\) are similar to their plasmic counterparts with the addition of varying extensions of the \(A\alpha\) chain. The cleavage patterns also indicate that plasmin and hementin have cleavage sites in the connector region which are in close proximity.

On the basis of the parallel structures inferred for hementin and plasmin fragments, an immunologic approach to the characterization of fragments \(Y_{\text{hem}}\), \(D_{\text{hem}}\), and \(E_{\text{hem}}\) was taken using antibodies specific for plasmic fragments D and E. When a control immunoblot of an intermediate hementin digest of fibrinogen was developed with antiserum against fibrinogen, a positive reaction occurred with all components of the digest (Fig. 3, first lane). However, when identical immunoblots were developed with either anti-fragment D or anti-fragment E antibodies, fragments \(Y_{\text{hem}}\) reacted positively under both conditions, fragments \(D_{\text{hem}}\) reacted only with anti-D antibodies, and fragment \(E_{\text{hem}}\) gave a positive reaction only with anti-E antibodies, as predicted (Fig. 3, second and third lanes).

The polypeptide compositions of fibrinogen fragments were characterized by two-dimensional nonreduced and reduced SDS-PAGE. The fragments were first separated by SDS-PAGE under nonreducing conditions. After reduction of disulfide bonds, chains were separated in the second dimension (Fig. 4A). The chains of fragment \(Y_{\text{hem}} 1\) comigrated with the chains of the reduced fibrinogen standard, demonstrating that fragment \(Y_{\text{hem}} 1\) contained an intact half of the fibrinogen molecule. The two-dimensional electrophoretic pattern also demonstrated \(A\alpha\) chain cleavage to produce both fragments \(Y_{\text{hem}} 2\) and \(Y_{\text{hem}} 3\). The \(B\beta\) and \(\gamma\) chains remained intact. The \(A\alpha\) chain remnant in fragment \(D_{\text{hem}} 1\) had a calculated mass of 59 kDa, larger than the intact \(B\beta\) chain, signifying the retention of the entire \(A\alpha\) chain COOH-terminal region. The sizes...
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FIG. 5. The reaction of fibrinogen with monoclonal antibodies to the α chain. Identical samples of an electroblotted digest were developed with monoclonal antibodies to the Aα chain COOH terminus. In the first lane a mixture of monoclonal antibodies to epitopes spanning the entire COOH-terminal portion was used. In the second lane, monoclonal antibody (MoAb) 9E9 was used. The epitope of mAb 9E9 has been mapped to Aα<sup>509–583</sup> (28).

of the β and γ chain remnants in fragments D<sub>hem</sub> 1–3 remained the same. The Aα and α chain remnants in fragments Y<sub>hem</sub> and D<sub>hem</sub> shown in Fig. 4A displayed a diagonal pattern indicative of cleavage. The pattern formed because molecular weight differences between fragments were solely due to α chain cleavage. To directly demonstrate that the late cleavages were made only in the α chain, a gel identical to that illustrated in Fig. 4A was electrophoresed onto nitrocellulose and developed with antibodies to the Aα chain. Fig. 4B shows the resulting diagonal pattern of α chain cleavage. Only the Aα chains from fragments Y<sub>hem</sub> 1, Y<sub>hem</sub> 2, D<sub>hem</sub> 1, and D<sub>hem</sub> 2 appeared on the immunoblot, as determined by comparing the distances of the diagonal patterns between the Coomassie Blue-stained gel and the immunoblot.

The next experiment tested the hypothesis that late cleavages in the Aα chain and α chain remnant of fragments Y<sub>hem</sub> 1 and D<sub>hem</sub> 1, respectively, took place in the COOH-terminal portion. A mixture of monoclonal antibodies to epitopes within the α chain COOH-terminal region, or a single monoclonal antibody (mAb 9E9) whose epitope occurs between amino acids 509 and 583 (28), were used to develop similar electroblots of fibrinogen digests. Fig. 5 shows the reaction of fibrinogen, fragments Y<sub>hem</sub> 1–3, and D<sub>hem</sub> 1–3 with the antibody mixture (first lane). However, only fibrinogen, fragment Y<sub>hem</sub> 1, and fragment D<sub>hem</sub> 1 bound mAb 9E9 (second lane). The specific reaction of fragment D<sub>hem</sub> 1 with mAb 9E9 along with the calculated molecular weight for its α chain remnant strongly suggested that the fragment retained the entire COOH terminus of the fibrinogen α chain. Furthermore, the data were consistent with late cleavages occurring only in the COOH terminus of the α chain.

Purification of Fragment D<sub>hem</sub> 1.—To isolate fragment D<sub>hem</sub> 1 from an intermediate hematin digest of fibrinogen, the digest was applied to a Mono Q column, and eluted with a linear NaCl gradient (Fig. 6, elution profile). The first protein peak, eluting between 0.10 and 0.15 M NaCl contained mostly D<sub>hem</sub> fragments with some contaminating fibrinogen (Fig. 6, SDS-PAGE). The second protein peak, eluting between 0.15 and 0.20 M NaCl, contained mostly fibrinogen, Y<sub>hem</sub> fragments, and fragment E<sub>hem</sub> (Fig. 6, SDS-PAGE). Contaminating fibrinogen was removed from the D<sub>hem</sub> pool by affinity chromatography on immobilized anti-E antibodies (data not shown).

Fragment D<sub>hem</sub> 1 on separation from other fibrinogen fragments showed high susceptibility to proteolytic degradation. For example, after storage for 1 month at −20 °C, the majority of fragment D<sub>hem</sub> 1 had degraded α chain COOH-terminal peptides. The degradation of α chain remnants was prevented by (1) storage at −70 °C, (2) use of sterile technique, and (3) the addition of protease inhibitors (data not shown).

It has long been hypothesized, but not directly proven, that the binding site for plasma fibronectin on fibrinogen is located at the COOH-terminal end of the Aα chain (32). We tested the hypothesis by using affinity chromatography on immobi-
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**FIG. 7.** The specific binding of fragment Dhem 1 to immobilized fibronectin. A preparation containing a majority of Dhem fragments with degraded α chains was used to demonstrate the specific binding of Fragment Dhem 1 to fibronectin. The initial sample, shown in the first lane was applied in phosphate-buffered saline, pH 7.4. The second lane shows fragments Dhem that did not bind to the column. After washing the column with 1.0 M NaCl, a urea gradient was applied. Fragment Dhem 1 was eluted with 1.0 M urea, 1.0 M NaCl containing buffer (Fig. 7, third lane). The difference in mass between fragment Dhem 1 and fragment Dhem 2, which did not bind, is 12 kDa which corresponds approximately to the COOH-terminal 111 amino acids of the α chain. The results of affinity purification of fragment Dhem 1 indicate that the fibronectin binding site is most likely located within amino acids 499 and 610 at the COOH terminus of the Aα chain.

Purified fragment Dhem 1 was used to determine directly the peptide bonds cleaved in the connector region by hementin. The approach taken was NH$_2$-terminal sequencing of each isolated polypeptide chain remnant derived from the fragment. The covalent bonds between α, β, and γ chain remnants were broken by disulfide reduction and carboxymethylation. α, β, and γ chain remnants were isolated by reverse-phase liquid chromatography on a C$_8$ column. Each chain was subjected to seven or eight cycles on an ABI 430 sequenator (Applied Biosystems, Foster City, CA) with an on-line phenylthiohydantoin analyzer. The resulting sequences were compared with the known amino acid sequence of fibrinogen chains. The NH$_2$-terminal amino acid sequence determined for each chain remnant indicated the hementin cleavage sites in the connector region occurred between Aα Asn$^{496}$-Asn$^{497}$, Bβ Lys$^{102}$-Gln$^{103}$, and γ Pro$^{76}$-Asn$^{77}$ (Fig. 8).

**DISCUSSION**

Posterior gland hementin cleaves purified human fibrinogen in a unique manner. The unique cleavage mechanism was reflected in the isolation of fibrinogen fragment Dhem 1 (Figs. 6 and 7), which retains an intact Aα chain COOH-terminal region passed through the column (Fig. 7, second lane). The column bound only fragment Dhem 1, which was eluted with a 1.0 M urea, 1.0 M NaCl containing buffer (Fig. 7, third lane). The difference in mass between fragment Dhem 1 and fragment Dhem 2, which did not bind, is 12 kDa which corresponds approximately to the COOH-terminal 111 amino acids of the α chain. The results of affinity purification of fragment Dhem 1 indicate that the fibronectin binding site is most likely located within amino acids 499 and 610 at the COOH terminus of the Aα chain.

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Hementin is also produced by the anterior salivary glands of the leech _H. ghilianii_ (25). The pattern of fibrinogen degradation by anterior gland hementin, as assessed by SDS-PAGE (33), seemed similar but not identical to that shown for the posterior gland enzyme. The most significant difference was in the relative rates of cleavage in the Aα chain COOH-terminal region. Anterior gland hementin cleaved in the Aα chain COOH-terminal region much quicker, relative to connector region cleavage, than the posterior gland enzyme. It is for this reason that hementin from posterior salivary glands is more suitable for producing fibrinogen fragments which retain the Aα chain COOH-terminal domain.

The majority of fibrinogenolytic proteases preferentially cleave in the COOH-terminal region of the Aα chain. Plasmin
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(1, 2, 4, 6), trypsin (6, 11), leukocyte (16, 18), and platelet (19) proteases, and proteases from many snake venoms (20-24) all cleave first in the Aa chain COOH-terminal region. The high susceptibility of the Aa chain COOH-terminal region to proteolytic degradation has led to the hypothesis that it is unstructured and surface-oriented (34). However, recent evidence using electron microscopy (35-37), scanning calorimetry (38), and immunochemical techniques (39) support the concept that parts of the Aa chain COOH-terminal region have some ordered secondary structure and are associated noncovalently with the E domain. Our observations revealed increased proteolytic susceptibility of the α chain COOH-terminal region in fragment D_{hem 1}; relative to fibrinogen, supporting the idea that the Aa chain in fibrinogen is protected from proteolysis through its interaction with the remainder of the molecule.

Besides hementin, the only fibrinogenolytic proteases which do not cleave the Aa chain COOH terminus preferentially are the β-fibrinogenases from Trimeresurus macropsquama (20) and Trimeresurus gramineus (22) snake venoms, and proteases II and III from Crotalus atrox venom (24). These proteases preferentially cleave an NH2-terminal peptide from the Bβ chain, which contains a polymerization site (7, 40). Recent investigation into the structure of the polymerization site on the Bβ chain NH2 terminus indicated that it resides on an exposed region (40), consistent with its relative sensitivity to proteolytic degradation. It is interesting that the leech H. ghilianii has evolved to produce an anticoagulant protease which does not cleave first in either the Aa chain COOH-terminal or Bβ chain NH2-terminal regions preferred by other enzymes. The originally intended function of the leech protease involved prevention of blood clotting during feeding. A fibrinogenolytic protease would then be required to act more quickly on its substrate than the blood coagulation cascade. Fibrinogenolytic fragments which lack Aa chain COOH-terminal peptides, such as fragment X, still retain >95% coagulability (3, 5). Similarly, the 325-kDa derivative of fibrinogen produced by cleaving the Bβ chain NH2 terminus with protease III from C. atrox venom is >95% coagulable (41). The most efficient way to degrade fibrinogen into non-covalagable species is to cleave the connector region, splitting the molecule, destroying the bivalent function, and in doing so producing fragments which are themselves anticoagulants (5, 9).

Both hementin and plasmin cleave fibrinogen through the three polypeptide chains in the connector region. This was demonstrated by the fact that hementin produced proteolytic fragments of fibrinogen that reacted with anti D and anti-E antibodies (Fig. 3) in a manner similar to that shown for plasminogen fragments Y, D, and E, and the localization of hementin cleavage sites in close proximity to plasmin cleavage sites in the connector region (Fig. 8). Other proteases which presumably cleave fibrinogen in the connector region are trypsin, which produces fragments X, Y, D, and E (6, 11), and leukocyte elastase which produces X-like and D-like fragments (42, 43). Unlike hementin, however, both these enzymes cleave first in the Aa chain COOH-terminal region.

The cleavage sites for hementin in the connector region were first demonstrated (Fig. 1), then proven by sequence analysis (Fig. 8) to exist in close proximity to plasmin cleavage sites. Using the procedure of Chou and Fasman (44) to predict the secondary structure of the area surrounding both hementin and plasmin cleavage sites in the connector region (results not shown), we derived a region of open structure. The prediction is in agreement with Doolittle and colleagues (45) who used similar methods to predict the structure of the connector region as a coiled-coil, interrupted in the middle by an open-structured, protease-sensitive area.

There is little precedent for proteases with specificity for cleaving peptide bonds on the NH2-terminal side of asparagine and glutamine, as demonstrated for hementin. One example is procollagen-N-proteinase that cleaves either Pro-Glu or Ala-Glu peptide bonds to release the procollagen-N-peptides from the collagen triple helix, allowing it to polymerize. Cleavage by procollagen-N-proteinase is also predicted to occur between two regions of triple helix, in an area characterized by lack of interaction between the three polypeptide chains (46).

The significance of the interaction between fibrinogen and fibronecint has long been recognized (47). The affinity purification on immobilized fibronecint of fragment D_{hem 1} from other D_{hem} fragments with shorter α chain remnants, provided convincing evidence that the binding site was within the COOH-terminal 12 kDa of the Aa chain. The direct interaction between fibronecint and the Aa chain COOH-terminal domain of fibrinogen reinforces the significance of this domain in the processes of cell migration and wound healing (47).

Acknowledgments—We would like to thank Dr. Michael W. Mosesson for his helpful suggestions and critical comments during the preparation of the manuscript.

REFERENCES

1. Mills, D., and Karpaktin, S. (1970) Biochem. Biophys. Res. Commun. 40, 206-211
2. Pizzo, S. V., Schwartz, M. L., Hill, R. L., and McKee, P. A. (1972) J. Biol. Chem. 247, 636-645
3. Sherman, L. A., Mosesson, M. W., and Sherry, S. (1969) Biochemistry 8, 1515-1523
4. Mosesson, M. W., Finlayson, J. S., Umfleet, R. A., and Galanakis, D. (1972) J. Biol. Chem. 247, 5210-5219
5. Marder, V. J., Shulman, N. R., and Carroll, W. R. (1967) Trans. Assoc. Am. Phys. 8, 156-167
6. Mihalvi, E., Weinberg, R. M., Towne, D. W., and Friedman, M. P. (1976) Biochemistry 15, 5377-5381
7. Lahiri, B., and Shainoff, J. R. (1973) Biochim. Biophys. Acta 303, 161-170
8. Mosesson, M. W., Galanakis, D. K., and Finlayson, J. S. (1976) J. Biol. Chem. 249, 4666-4664
9. Budyanski, A. Z., Stahl, M., Kopc, M., Latatto, Z. S., Wegrzynowicz, Z., and Kowalski, E. (1967) Biochim. Biophys. Acta 147, 313-323
10. Fowler, W. E., Fretto, L. J., Erickson, H. P., and McKee, P. A. (1990) J. Clin. Invest. 66, 50-56
11. Norton, P. A., and Slatyer, H. S. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 1661-1665
12. Price, T. M., Strong, U. D., Hudee, M. L., and Doolittle, R. F. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 200-204
13. Rulot, P. H. (1904) Arch. Int. Physiol. Biochim. 1, 152-158
14. Nachman, R. L., and Ferris, B. (1968) J. Clin. Invest. 47, 2530-2540
15. Bow, E. F., and Edinaton, T. S. (1975) J. Clin. Invest. 56, 30-38
16. Gramse, M., Bingenheimer, C., Schmidt, W., Egbring, R., and Havemann, K. (1978) J. Clin. Invest. 61, 1097-1093
17. Bilezikian, S. B., and Nossel, H. L. (1977) Blood 50, 21-28
18. Wintrob, B. V., Coblyn, J. S., Kaempfer, C. E., and Austen, K. F. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5449-5452
19. Kunicki, T. J., Mosesson, M. W., and Pidard, D. (1984) Thromb. Res. 35, 169-182
20. Ouyang, C., and Teng, C. M. (1976) Biochim. Biophys. Acta 420, 298-308
21. Ouyang, C., and Huang, T-F. (1976) Biochim. Biophys. Acta 439, 150-153
22. Ouyang, C., and Huang, T-F. (1979) Biochim. Biophys. Acta 571, 270-283
23. Morau, J. B., and Gereur, C. R. (1981) Biochim. Biophys. Acta 659, 161-165
13676

Unique Fibrinogen Fragment

24. Pandya, B. V., and Budzynski, A. Z. (1984) Biochemistry 23, 460–470
25. Budzynski, A. Z., Olexa, S. A., and Sawyer, R. T. (1981) Proc. Soc. Exp. Biol. Med. 168, 259–265
26. Laemmli, U. K. (1970) Nature 227, 680–685
27. Scheidegger, J. J. (1948) Int. Arch. Allergy Appl. Immunol. 7, 660–672
28. Ewaskiewicz, J. J., O’Brien, J., and Budzynski, A. Z. (1987) Fed. Proc. 46, 2243
29. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
30. Engvall, E., and Ruoslahti, E. (1977) Int. J. Cancer 20, 1–5
31. Bewley, T. A., Dixon, J. S., and Li, C. H. (1968) Biochem. Biophys. Acta 154, 420–422
32. Stathakis, N. E., and Mosesson, M. W. (1977) J. Clin. Invest. 60, 855–860
33. Malinconico, S. M., Katz, J. B., and Budzynski, A. Z. (1984) J. Lab. Clin. Med. 104, 842–854
34. Doolittle, R. F. (1973) Adv. Protein Chem. 27, 1–109
35. Mosesson, M. W., Hainfeld, J., Wall, J., and Haschemeyer, A. E. V. (1981) J. Mol. Biol. 153, 695–718
36. Erickson, H. P., and Fowler, W. E. (1983) Ann. N. Y. Acad. Sci. 408, 146–163
37. Weisel, J. W., Stauffacher, C. V., Bulitt, E., and Cohen, C. (1985) Science 230, 1388–1391
38. Medved, L. V., Gorkun, O. V., and Privalov, P. L. (1983) FEBS Lett. 160, 291–295
39. Cierniewski, C. S., Plow, E. F., and Edgington, T. S. (1984) Eur. J. Biochem. 141, 489–496
40. Pandya, B. V., O’Brien, J., and Budzynski, A. Z. (1989) Thromb. Haemostasis 62, 176
41. Pandya, B. V., Cierniewski, C. S., and Budzynski, A. Z. (1985) J. Biol. Chem. 260, 2994–3000
42. Sterrenberg, L., Nieuwenhuizen, W., and Hermans, J. (1983) Biochim. Biophys. Acta 755, 300–306
43. Sterrenberg, L., van Liempt, G. J., Nieuwenhuizen, W., and Hermans, J. (1984) Thromb. Haemostasis 51, 398–402
44. Chou, P. Y., and Fasman, G. D. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. 47, 45–148
45. Doolittle, R. F., Goldbaum, D. M., and Doolittle, L. R. (1978) J. Biol. Chem. 253, 311–325
46. Dombrowski, K. E., and Prockop, D. J. (1988) J. Biol. Chem. 263, 16545–16552
47. Ruoslahti, E. (1988) Annu. Rev. Biochem. 57, 375–413