Generation of forms of fragment E with differing thrombin-binding properties during digestion of fibrinogen by plasmin

Christopher A. GOODWIN, Vijay V. KAKKAR and Michael F. SCULLY*

Thrombosis Research Institute, Emmanuel Kaye Building, Manresa Road, Chelsea, London SW3 6LR, U.K.

Two principal forms of fragment E are generated upon digestion of fibrinogen by plasmin, according to the concentration of enzyme used. At a high concentration of plasmin (above 10 $\mu\text{g/ml}$), a form lacking fibrinopeptide A (FpA) at the *N*-terminus of the $\text{A}\alpha$ -chain was generated. This form of fragment E caused a dose-dependent increase in thrombin clotting times but had no measurable inhibitory activity towards thrombin cleavage of D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide. At a low concentration of plasmin ($< 1 \mu\text{g/ml}$), fragment E containing 35–40% of the original amount of FpA was present in the terminal digest. The FpA-containing form of fragment E inhibited thrombin cleavage of fibrinogen, inhibited amidolytic activity and bound to the enzyme with an affinity 3-fold tighter than fibrinogen itself ($K_d 4.1 \pm 0.3 \mu\text{M}$ as opposed to $12.7 \pm 1.8 \mu\text{M}$). During digestion of fibrinogen at low plasmin concentration, up to 65% of the FpA was cleaved just subsequent to the progressive release of $\text{B}\beta$ -(1–42)-peptide, and the Arg-16–Gly-17 bond of the $\text{A}\alpha$ -chain became relatively stable towards plasmin action when present in fragment E (and possibly fragment Y). It is proposed that both forms of fragment E can inhibit clotting by binding to the fibrin(ogen)-recognition site (anion-binding exosite) of thrombin. The FpA-containing form of fragment E can also inhibit binding that occurs distal to the P_1 site and thereby interfere with amidolysis of the peptide substrate. Our finding of a lability of the Arg-16–Gly-17 bond in the early phase of digestion may provide an alternative explanation of the increased FpA concentrations observed during thrombolytic therapy.

INTRODUCTION

Fibrinogen (340 kDa) is a thrombin-coagulable multifunctional glycoprotein occurring in the blood of vertebrates. The molecule consists of three pairs of non-identical polypeptide chains (termed $\text{A}\alpha$, $\text{B}\beta$ and γ) covalently linked by disulphide bonds [1]. When fibrinogen is treated with thrombin a partial proteolysis occurs with the release of the small peptides fibrinopeptide A (FpA) and B (FpB) from the *N*-termini of the $\text{A}\alpha$ - and $\text{B}\beta$ -chains respectively [2].

Fibrinogen is also liable to digestion by plasmin, generating characteristic fragments. At first there is the removal of a large fragment (45 kDa) from the *C*-terminus of each of the $\text{A}\alpha$ -chains and also of a small peptide [$\text{B}\beta$ -(1–42)-peptide] from the *N*-terminus of each of the $\text{B}\beta$ -chains [3–5]. The fibrinogen-degradation products at this point, fragment X (240 kDa), consists of two distal D domains linked to a central E domain [6,7]. The terminal stages of the digestion proceed by an asymmetric cleavage of one D domain (94 kDa) to generate fragment Y (150 kDa). Fragment Y is further digested to fragment D and fragment E (55 kDa), the latter representing the *N*-terminus of the original fibrinogen molecule. Both terminal products (fragment D and fragment E) have anticoagulant properties that are effected by different mechanisms. Fragment D, which on a molar basis is approx. 6-fold more potent as an anticoagulant than is fragment E [8], acts as an inhibitor of fibrin polymerization by binding to sites complementary to it on the E domain of fibrin monomer [9,10]. The anticoagulant property of fragment E is due to its ability to act as a competitive inhibitor of the action of thrombin upon fibrinogen [11].

In the present study we have investigated the thrombin-binding properties of fragment E. Conditions were established under which it is possible to generate either of two different forms of fragment E, with or without FpA respectively. Both

forms inhibit thrombin activity but with different properties considered to be related to their ability to bind to the two recognition sites for fibrinogen on the thrombin molecule.

MATERIALS AND METHODS

Materials

Human fibrinogen (grade L) and the synthetic thrombin substrate S-2238 (D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide hydrochloride) were obtained from KabiVitrum (Uxbridge, Middx., U.K.). Purified FpA, snake venom Atroxin and DEAE-Sepharose were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Human plasmin was prepared as described previously [12]. Human α - and γ -thrombin were kindly provided by Dr. J. Fenton (Albany, NY, U.S.A.). Hypersil 3-ODS C_{18} h.p.l.c. columns were obtained from HPLC Technology (Macclesfield, Cheshire, U.K.). DEAE-cellulose was obtained from Whatman Labsales (Maidstone, Kent, U.K.) and Ultrogel AcA44 was from LKB Quality Chemicals (Croydon, Surrey, U.K.). The antibodies used in the immunoelectrophoresis experiments were obtained from Behring Diagnostics (Hounslow, Middx., U.K.). All other reagents were of at least analytical grade and were used without further purification.

Purification of the terminal digestion products of plasmin action upon fibrinogen

Human fibrinogen was dissolved in buffer A [0.1 M-triethanolamine/HCl buffer, pH 7.8, containing 0.1 M-NaCl and 0.1% poly(ethylene glycol) 6000] to a concentration of 7.5 mg/ml and a small amount of an insoluble fraction was removed by centrifugation. The solution was prewarmed to 37 °C and plasmin was added to a final concentration of 1.0 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$. The mixture was incubated for 4 h before addition of aprotinin (1000 kallikrein-inhibitor units) and the temperature of the solution

Abbreviations used: FpA, fibrinopeptide A; FpB, fibrinopeptide B.

* To whom correspondence should be addressed.

was then raised to 100 °C for 5 min (precipitating all D-domain-containing fragments [13]). The digest was centrifuged at 2000 *g* for 20 min at 4 °C to remove the heat-aggregated material. The supernatant was dialysed against 10 mM-sodium carbonate buffer, pH 8.9, containing 0.1 M-NaCl at 4 °C and run over a 10 cm × 3 cm column of DEAE-cellulose equilibrated with the same buffer [14]. The column was washed with this buffer until absorption at 280 nm fell to zero and it was then eluted with the same buffer but 0.2 M with respect to NaCl. The eluted material was dialysed against water at 4 °C and freeze-dried. The freeze-dried material was resuspended in a small volume of buffer A and chromatographed on a 100 cm × 2.5 cm column of Ultrogel AcA44 equilibrated with the same buffer at 4 °C and flow rate of 40 ml/h. Fractions containing fragment E, identified by SDS/PAGE or electroimmunoassay (methods described below), were pooled, concentrated by ultrafiltration (Amicon PM-10 membrane under *N*₂ pressure) and stored frozen at -20 °C. The concentration of fragment E was calculated from the reported $A_{1\text{cm},280}^{1\%}$ value of 10.2 [8].

Fragment D was generated by plasmin digestion of fibrinogen (1.0 µg/ml) as described above but in the presence of 10 mM-CaCl₂. After termination of the reaction by addition of aprotinin, fragment D was purified by Zn²⁺ chelate chromatography as described previously [15].

Time course of plasmin action on fibrinogen

The digestion was carried out as described above for the purification of terminal digestion products except that the reaction mixture was subsampled at timed intervals. Heat-treated and untreated samples were stored frozen at -20 °C.

Analytical techniques

SDS/PAGE. SDS/PAGE was carried out with the Tris/glycine discontinuous buffer system described by Doucet & Trifaró [16]. Samples were prepared as described in the presence and in the absence of 10 mM-2-mercaptoethanol.

Electroimmunoassay. Samples were investigated immunologically for fragments D and E by using the method of Laurell [17]. A buffer system of 24 mM-Tricine/70 mM-Tris/1 mM-EDTA, pH 8.6, was used in a 2% agarose gel. Antibodies to fragment E (Clotimmun FSP-E) and fragment D (Clotimmun FSP-D) were used at 50 µl for each gel of volume 5.4 ml.

Determination of FpA and FpB. FpA and FpB were measured on a Hypersil 3-ODS C₁₈ h.p.l.c. column (0.46 cm × 15 cm) according to the method of Southan *et al.* [18]. Peptide concentrations were quantified by comparing peak area to a calibration curve prepared from the supernatant of solutions of fibrinogen of known concentration digested with α-thrombin (10 NIH units/ml for 2 h at 37 °C) and heat-treated.

Fibrinopeptide content of early and late digestion products of fibrinogen

Early plasmin digestion products of fibrinogen of high molecular mass (fragment X and fragment Y) were concentrated in an Amicon B125 Mini-Concentrator and washed with 4 vol. (equivalent to that of the original sample) of buffer A but with the NaCl concentration increased to 1 M, thereby removing any non-protein-bound fibrinopeptides [FpA, Bβ-(1-42)-peptide]. The concentrate was washed finally with 1 vol. of buffer A alone, then resuspended in 1 vol. of buffer, and the solution was removed from the concentrator. The protein-bound fibrinopeptides were then released by treatment with α-thrombin (10 NIH units/ml final concentration) for 2 h at 37 °C. Then 4 vol. of h.p.l.c. buffer B (stock 50 mM-ammonium acetate adjusted to pH 6.0 with H₃PO₄ and diluted with an equal volume of water) was added and the sample was boiled for 3 min. The

samples were centrifuged at 8000 *g* for 2 min and the supernatants were used directly for h.p.l.c. analysis. Late digestion products were treated as above except that an Amicon B-15 Mini-Concentrator was used to remove the low-molecular-mass peptides. The samples were treated with thrombin as described above for the early digestion products.

Apparent binding affinity of fragment E for thrombin

The apparent binding affinity of fragment E for thrombin was measured as the *K_i* for inhibition of thrombin hydrolysis of synthetic substrate S2238, as described previously, in measuring the affinity of fibrinogen for thrombin [19]. Various concentrations of S2238 from 1.4 to 4.3 times *K_m* (4.7 µM) were incubated with human α-thrombin (final concentration 0.3 nM) alone, with fibrinogen and with purified fragment E. Fibrinogen was not observed to clot under the conditions used. The reactions were carried out in 0.2 ml of buffer A incubated in plastic optical cuvettes in an Abbott Bichromatic Analyzer ABA 100 fitted with a 380 nm/450 nm filter and set at 37 °C. Initial rates of release of *p*-nitroaniline were measured and double-reciprocal plots were constructed. For a competitive inhibitor the change in the *K_m* for amidolysis of S-2238 by thrombin to the apparent *K_m* (*K_m*(app.)) according to the concentration of the inhibitor [Inh] is related to the *K_i* by the following expression:

$$K_i = \frac{[\text{Inh}]}{\frac{K_{m(\text{app.})}}{K_m} - 1}$$

Measurement of the influence of fragment E on the clotting of plasma or fibrinogen solutions by thrombin

The time to clot of citrated normal pooled human plasma (ten donors) or fibrinogen solution was determined with a semi-automatic coagulometer. Human α-thrombin was prepared in a solution of poly(ethylene glycol) 6000 (0.2%) in water at a concentration of 2.5 units/ml. A 200 µl portion of the thrombin solution was delivered to 100 µl of a prewarmed (37 °C) citrated human plasma sample or fibrinogen solution (0.5% in 50 mM-sodium phosphate buffer, pH 6.5) together with various concentrations of fragment E, fragment D or mixtures of both. A timer was started on the addition of the thrombin solution and halted automatically at the point of clotting by a metallic oscillator.

RESULTS

Thrombin-inhibitory forms of fragment E

Fibrinogen was digested by plasmin, and samples of digest were removed at timed intervals and heat-treated and the ability of the products in the supernatant to inhibit thrombin cleavage of the tripeptide *p*-nitroanilide substrate S2238 was tested. During digestion by plasmin at 1.0 µg/ml a progressive appearance of a thrombin-inhibitory activity was observed, reaching a peak after 2 h (Fig. 1). The profile recording the increase in inhibitory activity was observed to run ahead of that recording the concentration of fragment E, as measured immunologically. However, during purification by chromatography on DEAE-cellulose and Ultrogel ACA44 (see the Materials and methods section) the inhibitory activity co-localized with the immunologically measured fragment E (results not shown). At the concentration of plasmin used for the experiment represented in Fig. 1 the inhibitory activity was stable, and no significant decrease in activity was seen when it was tested after incubation at 37 °C for up to 24 h.

At concentrations of plasmin much higher than 1.0 µg/ml a somewhat different pattern was observed, such that at 50 µg/ml

Fig. 1. Generation of thrombin-inhibitory fragment E during digestion of fibrinogen by plasmin

Fibrinogen (7.5 mg/ml) dissolved in buffer A was digested at 37 °C with plasmin (1 μg/ml), and the reaction was terminated at timed intervals by the addition of aprotinin (1000 kallikrein-inhibitor units/ml). Samples were boiled and the supernatants were tested for inhibitory activity towards thrombin cleavage of a single concentration (6.67 μM) of the chromogenic peptide substrate S2238 (○). The concentration of fragment E was measured immunologically (□) as described in Materials and methods section. Samples were measured in duplicate and the data shown here are for a representative experiment.

Fig. 2. Generation of thrombin-inhibitory fragment E during digestion of fibrinogen at various concentrations of plasmin

Fibrinogen (7.5 mg/ml) dissolved in buffer A was digested at various concentrations of plasmin, and the reaction was terminated at timed intervals by the addition of aprotinin (1000 kallikrein-inhibitor units/ml). Samples were boiled and the supernatants were tested for inhibitory activity towards thrombin cleavage of a single concentration (6.67 μM) of the chromogenic peptide substrate S2238, as described in the Materials and methods section. The concentrations of plasmin were 0.1 μg/ml (▼), 1.0 μg/ml (○), 10.0 μg/ml (□) and 50 μg/ml (■). Samples were measured in duplicate and the data shown here are for a representative experiment.

and 10 μg/ml a rapid appearance and disappearance of a thrombin-inhibitory fragment E was observed. Thus after 4 h incubation at 37 °C there was no remaining inhibitory activity, although fragment E could be purified from this terminal digest (Fig. 2). At lower concentrations of plasmin there was a lag in the appearance of thrombin-inhibitory activity in the heat-treated digest that reached a level after 4 h equivalent to that observed after 2 h incubation at a plasmin concentration of 1.0 μg/ml.

Properties of the thrombin-inhibitory forms of fragment E

Fragment E was purified from 4 h digests of fibrinogen after digestion at 1.0 μg of plasmin/ml, termed fragment E_{lp} (for low plasmin) or 10 μg of plasmin/ml, termed fragment E_{hp} (for high

Fig. 3. Effects of fibrinogen and fragment E_{hp} on the hydrolysis of S2238 by thrombin

The amidolytic activity of thrombin was observed at 37 °C in buffer A in the absence (■) and in the presence of fibrinogen (▼) or fragment E_{lp} (●), both at a concentration of 6 μM. Thrombin activity was assayed with various concentrations of S2238, measurements being made as described in the Materials and methods section. The concentration of α-thrombin was 0.3 nM. Results are shown as the means of three determinations.

Fig. 4. Influence of fragment E_{hp} upon the inhibition of thrombin amidolysis of S2238 by fragment E_{lp}

Fragment E_{lp} was mixed at a final concentration of 3.6 μM with thrombin (0.23 nM) and S2238 (6.67 μM) in buffer A, causing a finite inhibition of thrombin activity with respect to a control containing no fragment E_{lp}. The results record the decrease in observed inhibition according to the added concentration of fragment E_{hp} and are the means of three determinations.

plasmin), respectively. The apparent K_i for inhibition of thrombin-catalysed amidolysis of S2238 by fragment E_{lp} was calculated from Lineweaver-Burk plots in the presence and in the absence of a single concentration of the fragment and found to be 4.1 ± 0.3 μM (mean $\pm$ S.E.M.) (Fig. 3). The apparent K_i observed for inhibition by fibrinogen under the same conditions was 12.7 ± 1.8 μM. Fragment E_{hp} had no effect on amidolysis when tested at concentrations up to 25 μM. The apparent K_i towards γ-thrombin (the non-clotting form of human thrombin) was also measured and found to be 23.6 ± 3.2 μM for fragment E_{lp} and 79.1 ± 10.3 μM for fibrinogen. Although fragment E_{hp} had no effect on thrombin amidolytic activity, it did reverse the inhibitory activity of fragment E_{lp}. Upon the addition of increasing concentrations of fragment E_{hp} to a mixture of thrombin, S2238 and fragment E_{lp} there was a progressive decrease in the degree of inhibition of the enzyme (Fig. 4) with an IC_{50} of 15.6 μM.

Both fragment E_{lp} and fragment E_{hp} were inhibitors of the thrombin-induced clotting of fibrinogen, causing doubling in the thrombin time of plasma at 7.2 ± 1.7 M and 15.5 ± 2.5 μM and the

Fig. 5. Influence of fragment E upon the thrombin clotting time of plasma or fibrinogen solution

Purified preparations of fragment E_{ip} (● and ○) and fragment E_{hp} (■ and □) were added at various concentrations to human citrated plasma (● and ■) or fibrinogen solution (○ and □) in 50 mM-sodium phosphate buffer, pH 6.5) (○ and □) in a coagulometer at 37 °C. The reaction was initiated by the addition of thrombin to a concentration of 1.65 NIH units/ml, and the time to clot was measured automatically, by an oscillating probe. Results are shown as the means of three determinations.

thrombin time of fibrinogen solution at $6.9 \pm 2.3 \mu\text{M}$ and $22.3 \pm 4.3 \mu\text{M}$ respectively (Fig. 5).

When fragment E_{ip} was pretreated with low concentrations of α -thrombin or Atroxin a loss of amidolysis-inhibitory activity was observed coincident to the removal of FpA. The ability of fragment E_{ip} to inhibit thrombin cleavage of the tripeptide *p*-nitroanilide substrate S2238 was therefore related to the possession of FpA. The FpA content of the fragment was measured by thrombin cleavage and found to be in the range 0.7–0.8 mol/mol of fragment E, about twice that described previously [20,21]. Fragment E_{hp} had no measurable FpA content.

Since the FpA content of fibrinogen is 2 mol/mol the occurrence of 0.7–0.8 mol of FpA/mol of fragment E_{ip} could arise from a population in which 35–40% of molecules contained 2 mol of FpA/mol of fragment E or 70–80% contained 1 mol of FpA/mol (the remainder in both cases comprising fragment E without FpA) or which was some combination of all three forms. Investigation as to which of these populations existed was made by chromatography of fragment E_{ip} on DEAE-Sephacel in 10 mM-sodium carbonate buffer, pH 10.0, containing 0.1 M-NaCl. Fragment E_{ip} was eluted as a broad peak when a salt gradient of 0.1–0.3 M-NaCl was applied. Measurement of the FpA content and thrombin-inhibitory activity of each fraction showed a partial depletion of FpA and inhibitory activity in those fractions at the leading edge of the chromatogram (Fig. 6). The decrease in the area of these curves when compared with the curve expressing concentration of fragment E was equivalent to approx. 27% (Fig. 6). If the 27% non-FpA-containing fragment E in the leading edge is allowed for, the ratio of FpA to fragment E is 1:1 across the entire chromatogram. Such a chromatographic distribution suggests that fragment E_{ip} consists, predominantly, of 20–30% of molecules containing no FpA and 70–80% of molecules containing 1.0 mol of FpA/mol, rather than other combinations (e.g. 60% non-FpA containing and 40% containing 2.0 mol of FpA/mol).

Mechanism of generation of the thrombin-inhibitory forms of fragment E

Fibrinogen was digested with plasmin at 50 $\mu\text{g}/\text{ml}$, 1.0 $\mu\text{g}/\text{ml}$ and 0.4 $\mu\text{g}/\text{ml}$, and the concentrations of free and protein-bound FpA were measured in samples taken at timed intervals. The

Fig. 6. Chromatography of fragment E_{ip} upon DEAE-Sephacel

Fragment E_{ip} (approx. 40 mg) was applied to a 10 cm $\times$ 2.5 cm column of DEAE-Sephacel equilibrated with 10 mM-sodium carbonate buffer, pH 10.0, containing 0.1 M-NaCl. The protein was eluted by a linear gradient, created by progressive mixing of starting buffer (500 ml) with 10 mM-sodium carbonate buffer, pH 10.0, containing 0.3 M-NaCl (500 ml), applied across the column at a flow rate of 60 ml/h and at 22 °C. Fractions (6.5 ml) were collected and analysed for concentration of fragment E (○) and FpA (□) and thrombin-inhibitory activity (●) measured with a single concentration of chromogenic substrate S-2238 (6.67 μM). Protein was eluted at a volume of 390–680 ml of buffer (fractions 31–51).

Fig. 7. High-molecular-mass fragments of fibrinogen during digestion by plasma (1 $\mu\text{g}/\text{ml}$)

Fibrinogen (7.5 mg/ml) dissolved in buffer A was digested at 37 °C at a plasmin concentration of 1 $\mu\text{g}/\text{ml}$. Samples were taken at timed intervals and quenched in SDS/area electrophoresis buffer, boiled for 2 min and run on 10% gels in Tris/glycine buffer [16]. Lane a, molecular-mass standards; lanes b–i, 0 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min and 240 min respectively. Mobilities for fibrinogen (Fg), early fragment X (FX'), late fragment X (FX), fragment Y (FY), fragment D (FD), fragment E (FE) and A α -chain fragment 45 kDa (AF) are indicated.

results show the rapid and total release of FpA during digestion at 50 μg of plasmin/ml and a very slow release at 0.4 $\mu\text{g}/\text{ml}$. At 1.0 μg of plasmin/ml there was no release of FpA in the early stage of digestion, during which a peptide from the C-terminus of the A α -chain (Fig. 7) and also B β -(1–42)-peptide were released (Fig. 8). However, during the release of B β -(1–42)-peptide a

Fig. 8. Concentrations of protein-bound FpA and B β -(1-42)-peptide during digestion of fibrinogen at various concentrations of plasmin

Fibrinogen (7.5 mg/ml) was digested at 37 °C with various concentrations of plasmin: 50 μ g/ml ($\square$ and $\blacksquare$), 1.0 μ g/ml ($\circ$ and $\bullet$) and 0.4 μ g/ml (∇ and $\blacktriangledown$). The concentrations of protein-bound FpA ($\square$, $\circ$ and ∇) and B β -(1-42)-peptide ($\blacksquare$, $\bullet$ and $\blacktriangledown$) were measured in samples taken at timed intervals after separation of peptide material by membrane filtration, as described in the Materials and methods section.

rapid cleavage of FpA occurred (Fig. 8), and this cleavage terminated in coincidence with the appearance of fragment Y in the digestion mixtures (Fig. 7). From that point onwards there was no significant further release of FpA in samples incubated for up to 24 h at 37 °C. It appears, therefore, that FpA present on fragment E_{ip} (Figs. 1 and 8) and probably also when on fragment Y is more stable towards plasmin digestion than FpA on fibrinogen or on fragment X.

DISCUSSION

In this present study we have examined the cleavage of FpA during plasmin degradation of fibrinogen and demonstrate for the first time that the Arg-16-Gly-17 bonds present in the A α -chain are susceptible to cleavage in the initial phase of the digestion. As digestion proceeds this bond appears to become less susceptible to digestion and as a consequence a heterogeneity of fragment E is observed according to the concentration of plasmin used. One form, E_{ip}, retains 35–40 % of the original content of FpA and inhibits both the amidolytic and fibrinogen-cleavage properties of thrombin, whereas the other form, E_{np}, without FpA, inhibits only the fibrinogen-cleavage properties of thrombin. In previous investigations of the primary structure of fragment E there is some variation in reports of the N-terminus of the A α -chain as Ala-1 in early and late digests and Gly-17 (late) or Val-20 (very late) [22–25]. This has led to the proposal that the Arg-16-Gly-17 bond is resistant to primary plasmin digestion and that cleavage occurs only after very prolonged incubation periods. In order to investigate a possible mechanism for the generation of the FpA-containing fragment E we have determined the concentrations of free and protein-bound FpA during digestion of fibrinogen at various concentrations of plasmin. The digests were membrane-filtered to remove free FpA, and the FpA bound to fragments X and Y was measured by thrombin treatment. During digestion at 1.0 μ g of plasmin/ml we observed that 60–70 % of the total FpA was cleaved from the fibrinogen molecule early on, a digestive event that was slightly delayed with respect to the cleavage of the C-terminus of the A α -chain (Fig. 7) and the cleavage of the B β -(1-42)-peptide (Fig. 8). It would appear, therefore, that, contrary to previous reports, the Arg-16-Gly-17 bond is susceptible to digestion when present in fibrinogen and early fragment X with at least 1.2 mol of FpA/mol

being released from the molecule. During subsequent digestion, to fragment Y and then to fragment E, the Arg-16-Gly-17 bond appears to be relatively less susceptible and the product E_{ip} contains 0.8 mol of FpA/mol of fragment E. Our findings would correspond to those of Mihalyi and co-workers [26,27], who observed cleavage of 5.2 bonds/molecule in the rapid phase of plasmin digestion of fibrinogen (i.e. before generation of fragments Y, D and E), four bonds being attributable, presumably, to the cleavage of the C-terminus of the A α -chain and B β -(1-42)-peptide [27].

The thrombin-binding properties of the two different forms of fragment E were then characterized. Interestingly, we find that fragment E_{ip} binds more tightly to thrombin than fibrinogen itself (K_i 4.1 μ M and 12.7 μ M respectively). This difference was also observed when binding was measured directly as the K_m for release of FpA (K_m for fibrinogen 12 μ M that of fragment E_{ip} 5 μ M; C. A. Goodwin, V. V. Kakkar & M. F. Scully, unpublished work). The reason for the tighter binding is not, as yet, explained, but it may arise from the exposure of a secondary binding site or the removal of points of steric hindrance. Furthermore (and contrary to findings made previously with bovine proteins [19]) we observed that human fibrinogen and fragment E_{ip} were bound much more weakly by the non-clotting form of thrombin (γ -thrombin) (K_i 79.1 μ M and 23.6 μ M respectively) than by human α -thrombin.

The binding of thrombin to fibrinogen is currently considered to involve two sites on the enzyme molecule [28], namely a hydrophilic region binding phenylalanine and other aromatic residues and a region that binds anionic ligands [29,30]. In the A α -chain of fibrinogen, residues on the C-terminal side of the Arg-16-Gly-17 bond are thought to be responsible for primary binding to thrombin. These residues occur on both E_{np} and E_{ip}. Since fragment E_{np} can interact with the anionic-ligand-binding site it can inhibit coagulation of fibrinogen by thrombin (Fig. 5) and also reverse the inhibition of the amidolytic activity of thrombin by fragment E_{ip} (Fig. 4). Fragment E_{ip} inhibits thrombin, presumably, by binding to the anionic-ligand-binding site of the enzyme and the residual FpA can block the binding of the tripeptide *p*-nitroanilide S2238 to the hydrophobic site.

Findings that discriminate two sites of interaction between the thrombin and fibrinogen (Asp-7 to Arg-16 and the as yet unidentified sequence on fibrinogen with affinity for the anionic-ligand-binding site), made by using a number of approaches, have been reported recently [31–34]. There is also evidence for the existence of two sites from a study with peptides synthesized to correspond to the C-terminus of hirudin [35]. The difference that we observed between K_i values observed with α -thrombin and γ -thrombin would arise from vitiation of the anionic-ligand-binding site in the degraded enzyme [36]. When the binding affinity for FpA was measured from inhibition of thrombin amidolysis of S2238, a K_i value of approx. 500 μ M was calculated (results not shown), of the same order as K_m values reported previously for hydrolysis of a series of P₆ to P₇' peptides [37]. The apparently higher affinity of the FpA sequence when present on the A α -chain remnant (as indicated by our results) or in peptide constructs of the A α -chain, residues 1–50 [31] may suggest the need for primary contact between thrombin and the A α -chain to occur on the C-terminal side of the Arg-16-Gly-17 bond before binding of the FpA sequence. It is interesting to note the recent report by Ni *et al.* [38], who demonstrated that the tight binding of the FpA sequence to the apolar site of thrombin arose from specific folding of the peptide on the enzyme surface.

The physiological significance of our findings will relate to the different anticoagulant properties of the two forms of fragment E and also perhaps to the physiological interaction of fragment E with or without FpA upon a number of cellular events,

including cell migration and neutrophil activation, in which fragment E heterogeneity may also be of importance [39,40]. This requires further investigation. Our findings should also be taken into account in consideration of recent reports that when thrombolytic agents (streptokinase, tissue plasminogen activator) are infused a 'coagulant state' is generated [41]. The evidence for this state is an observed increase in FpA concentrations in patients undergoing such therapy. A premise of this conclusion is the reported insensitivity of the Arg-16-Gly-17 bond to digestion by plasmin. Our findings show that this bond is susceptible to digestion in the early stages of fibrinogen digestion, contrary to previous reports, and that digestion by plasmin, rather than by thrombin or tissue plasminogen activator [42], may be responsible for the concentrations of FpA observed.

We thank Dr. Goran Claesson for most helpful discussion and advice and Eileen Bayford for her excellent assistance in the preparation of the manuscript.

REFERENCES

1. Doolittle, R. F. (1984) *Annu. Rev. Biochem.* **53**, 195–229
2. Bailey, K., Bettelheim, F. R., Lorand, L. & Middlebrook, W. R. (1951) *Nature (London)* **167**, 233–239
3. Mosesson, M. W., Finlayson, J. S., Umfleet, R. A. & Galanakis, D. (1972) *J. Biol. Chem.* **247**, 5210–5219
4. Pizzo, S. V., Schwartz, M. L., Hill, R. L. & McKee, P. A. (1973) *J. Biol. Chem.* **248**, 4574–4583
5. Koehn, J. A., Hurlet-Jensen, A., Nossel, H. L. & Canfield, R. E. (1983) *Anal. Biochem.* **133**, 502–510
6. Marder, V. J., Shulman, N. R. & Carroll, R. (1969) *J. Biol. Chem.* **244**, 2111–2119
7. Marder, V. J. (1970) *Thromb. Diath. Haemorrh. Suppl.* **39**, 187–195
8. Marder, V. J. & Shulman, N. R. (1969) *J. Biol. Chem.* **244**, 2120–2124
9. Kudryk, B. J., Collen, D., Woods, K. R. & Blombäck, B. (1974) *J. Biol. Chem.* **249**, 3322–3325
10. Olexa, S., Budzynski, A., Doolittle, R. F., Cottrell, B. A. & Greene, T. C. (1981) *Biochemistry* **20**, 6139–6145
11. Larrieu, M. J., Rigollot, C. & Marder, V. J. (1972) *Br. J. Haematol.* **22**, 719–733
12. Scully, M. F., Ellis, V., Watahiki, Y. & Kakkar, V. V. (1989) *Arch. Biochem. Biophys.* **268**, 438–446
13. Takagi, T. & Doolittle, R. F. (1973) *Biochemistry* **14**, 940–946
14. Nussenzweig, V., Seligmann, M., Pelimont, J. & Grabar, P. (1961) *Ann. Inst. Pasteur Paris* **100**, 377–389
15. Scully, M. F. & Kakkar, V. V. (1982) *Biochim. Biophys. Acta* **700**, 130–134
16. Doucet, J.-P. & Trifaró, J. M. (1988) *Anal. Biochem.* **168**, 265–271
17. Laurell, C. B. (1966) *Anal. Biochem.* **15**, 45–52
18. Southan, C., Thompson, E. & Lane, D. A. (1986) *Thromb. Res.* **43**, 195–204
19. Lundblad, R. L., Nesheim, M. E., Straight, D. L., Scott, S., Bowie, J., Jenzano, J. W., Roberts, J. D. & Mann, K. G. (1984) *J. Biol. Chem.* **259**, 6991–6995
20. Budzynski, A. Z., Marder, V. J. & Shainoff, J. R. (1974) *J. Biol. Chem.* **249**, 2294–2302
21. Hanna, L. S., Scheraga, H. A., Francis, C. W. & Marder, V. J. (1984) *Biochemistry* **23**, 4681–4687
22. Kowalska-Loth, B., Gardlund, B., Egberg, N. & Blombäck, B. (1973) *Thromb. Res.* **2**, 423–450
23. Gardlund, B. (1977) *Thromb. Res.* **10**, 689–702
24. Takagi, T. & Doolittle, R. F. (1975) *Thromb. Res.* **7**, 813–818
25. Henschen, A. (1983) *Thromb. Res. Suppl.* **5**, 27–39
26. Mihalyi, E., Weinberg, R. M., Towne, D. W. & Friedman, M. E. (1976) *Biochemistry* **15**, 5372–5381
27. Shrager, R. I., Mihalyi, E. & Towne, D. W. (1976) *Biochemistry* **15**, 5382–5386
28. Fenton, J. W., II (1989) *Semin. Thromb. Hemostasis* **15**, 265–268
29. Berliner, L. J., Sugawara, Y. & Fenton, J. W., II (1985) *Biochemistry* **24**, 7005–7009
30. Fenton, J. W., II, Olson, T. A., Zabinski, M. P. & Wilner, G. D. (1988) *Biochemistry* **27**, 7106–7112
31. Lord, S. T., Byrd, P.-A., Hede, K. L., Wei, C. & Colby, T. J. (1990) *J. Biol. Chem.* **265**, 838–843
32. Kaczmarek, E. & McDonagh, J. (1988) *J. Biol. Chem.* **263**, 13896–13900
33. Vali, Z. & Scheraga, H. A. (1988) *Biochemistry* **27**, 1956–1963
34. Naski, M. C. & Shafer, J. A. (1990) *J. Biol. Chem.* **265**, 1401–1407
35. Maraganore, J. M., Chao, B., Joseph, M. C., Zablonki, J. & Ramachandran, K. L. (1989) *J. Biol. Chem.* **264**, 8692–8698
36. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R. & Hofsteenge, J. (1989) *EMBO J.* **8**, 3467–3475
37. Marsh, H. C., Jr., Meinwald, Y. C., Lee, S. & Scheraga, H. A. (1982) *Biochemistry* **21**, 6167–6171
38. Ni, F., Meinwald, Y. C., Vasquez, M. & Scheraga, H. A. (1989) *Biochemistry* **28**, 3094–3105
39. Dejana, E. M., Vergara-Davden, G., Balconi, G., Pietra, G., Cherel, M. B., Conati, M. B., Larrieu, M. & Marguerie, G. (1984) *Eur. J. Biochem.* **139**, 657–662
40. Kazura, J. W., Wenzel, J. D., Salata, R. A., Budzynski, A. Z. & Goldsmith, G. H. (1989) *J. Clin. Invest.* **83**, 1916–1924
41. Owen, J., Friedmann, K. D., Grossman, B. A., Wilkins, C., Berke, A. D. & Powers, E. R. (1988) *Blood* **72**, 616–620
42. Wetz, J., Cruikshank, M. K., Thong, B., Leslie, B., Levine, M. N., Ginsberg, J. & Eckhardt, T. (1988) *J. Clin. Invest.* **82**, 1700–1707

Received 7 February 1991/21 June 1991; accepted 1 July 1991