Species Differences in the Interaction of Thrombin and Fibrinogen*

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When thrombin from the blood plasma of one class of vertebrates is allowed to react with fibrinogen from the plasma of another vertebrate from another class, the ensuing clotting times are invariably longer than would be the case if the thrombin and fibrinogen had both been from closely related animals (1). Furthermore, the degree of interference imposed by this "species specificity" is apparently not predictable from a knowledge of the reciprocalcross. For example, it is claimed that opossum thrombin is specific for opossum fibrinogen, whereas opossum fibrinogen is readily clotted by a variety of thrombins (2). On the other hand, it has been reported that chicken fibrinogen is coagulated by mammalian thrombins at a very slow rate but that chicken thrombin clots mammalian fibrinogens efficiently (2). These generalizations are further complicated by the fact that the conditions in vitro of temperature and solution environment for the interaction of thrombin and fibrinogen and subsequent clot formation may vary from species to species (3, 4).

Immunoochemical studies on mammalian and avian fibrinogen preparations have generally shown that antibodies made against a mammalian fibrinogen cross react with fibrinogen preparations from other mammals but not with avian fibrinogens. The converse is true of antibodies made against avian fibrinogens (5-7). On the other hand, the amino acid composition of the fibrinopeptides cleaved from various mammalian fibrinogens by thrombin during the clotting process show distinct differences (8), in spite of the high degree of interchangeability in terms of speeds of clotting and the allegedly high percentage of antigenic cross reaction.

The present investigation was undertaken in an effort to characterize more clearly the meaning of "species specificity" in the thrombin-fibrinogen interaction. For this purpose, thrombin and fibrinogen were isolated from the blood plasma of the lamprey eel, an organism generally considered to be one of the most primitive vertebrates extant. Studies were made of the relative speed of clot formation when the lamprey thrombin and fibrinogen were allowed to react with each other and with thrombins and fibrinogens from mammalian plasmas. The lamprey eel thrombin was also compared with mammalian thrombin with regard to its ability to hydrolyze TAME.1 In addition, the by-products of the thrombin-fibrinogen interactions were isolated and the nature of the fibrinopeptide material determined.

EXPERIMENTAL PROCEDURE

Materials

Adult lamprey eels (Petromyzon marinus) were collected from streams in southern New Hampshire at the beginning of the spawning season. Siliconed 50-ml syringes fitted with No. 15 blunt needles were used to remove blood directly from the caudal vein after cutting off a section of tail with a razor blade. Nine volumes of the blood were mixed with 1 volume of 0.1 M sodium citrate or oxalate to prevent clotting.

Lamprey Eel Fibrinogen—Lamprey eel fibrinogen was isolated and purified with suitable modifications of the Cohn ethanol fractionation scheme (9). Two volumes of 35% ethanol were added to 5 volumes of citrated lamprey eel plasma at -3°. The preparation was left at this temperature for one hour with gentle stirring, during which time the conditions were 10% ethanol (volume per volume); pH 7.1; ionic strength, 0.1. The precipitate was centrifuged at 1000 X g and the supernatant fluid decanted. The precipitate (Fraction I) was either purified further immediately or dissolved in one third of the original plasma volume of 0.02 M sodium citrate and lyophilized. The dried powders were stable when stored at -20°. Purification of Fraction I was accomplished by dissolving the original precipitate or dried powder in one-half of the original plasma volume of 0.15 M ammonium acetate and reprecipitating the fibrinogen from 7.5% ethanol (volume per volume), other factors being kept the same as during the first precipitation. This second precipitation increased the percentage of clottable protein from 60 to 70% in Fraction I to greater than 90%. Alternatively, Fraction I could be further purified by the method of Laki (10) with the use of ammonium sulfate precipitation. The ammonium sulfate was removed by dialysis against 0.15 M ammonium acetate or by running the fibrinogen over a Sephadex column pre-treated with 0.15 M ammonium acetate. The homogeneity of the lamprey eel fibrinogen preparation was studied in a Spinco model E analytical ultracentrifuge at 59,780 r.p.m. The fibrinogen was previously dialyzed against 0.15 M NaCl containing 0.05 M imidazole buffer, pH 7.3.

Lamprey Eel Thrombin—The lamprey eel thrombin was prepared from its prothrombin precursor which was obtained by

1 The abbreviation used is: TAME, p-tosyl-L-arginine methyl ester.
adoption of oxalated lamprey eel plasma on barium sulfate (Baker, 100 mg per ml). The barium sulfate was washed with 0.15 M sodium chloride, 0.06 M sodium oxalate, and finally with 0.006 M sodium citrate. After the final wash, the prothrombin was eluted by suspending the barium sulfate in one-tenth of the original plasma volume of 0.1 M sodium citrate. The barium sulfate was removed by centrifugation followed by filtration. Prothrombin activity was precipitated from the eluate under conditions of 20% ethanol (volume per volume); pH 5.4; temperature, -5°C. After standing at -5°C for 17 hours, the precipitate was removed by centrifugation at 1000 × g. It was redissolved in one-third of an original plasma volume of 0.05 M sodium citrate and lyophilized. The tan powder was stored at -20°C. Thrombin could be prepared from this powder by dissolving it in 0.15 M sodium chloride (5 mg of powder per ml) and exposing the solution to lamprey eel white cells or lamprey tissue extract in the presence of calcium (final concentration = 0.025 M). The generation of this “biothrombin” was monitored by assaying the thrombin on lamprey fibrinogen. When the conversion of prothrombin to thrombin was completed, the white cells or tissue factor were removed by centrifugation and the calcium by dialysis. Alternatively, thrombin was prepared from the prothrombin by dissolving the powder in 25% sodium citrate. The solution was allowed to stand in the cold for 17 hours, after which time the sodium citrate was removed by dialysis in the cold. The “citrate thrombin” generally yielded more thrombin activity per milligram of prothrombin powder than the biothrombin. Both thrombin solutions were stable for months when frozen.

**Mammalian Thrombins and Fibrinogens—**Armour bovine Fraction I was further purified by the method of Laki (10). Fraction I preparations from other mammals (dog and sheep) were prepared and purified under conditions similar to those described above for the lamprey eel. Human thrombin was supplied by Dr. J. T. Tripp of the National Institutes of Health. The material contained 21.7 units per mg of dry powder. Bovine thrombin was purchased from Mann Research Laboratories, New York, New York.

**Methods**

**Thrombin Assays**—The clotting times of the various thrombin-fibrinogen mixtures were taken to be the time of the first appearance of a fibrin web in a gently shaken tube. The assays were performed in 8.5 × 75-mm clotting tubes at 25°C in 0.05 M imidazole buffer, pH 7.3. The TAME esterase activity of thrombin preparations was determined with the use of a modification of a trypsin spot plate determination (11). A thrombin dilution, 0.1 ml, was added to 0.1 ml of a reaction mixture on a porcelain spot plate. The reaction mixture contained 0.086 M TAME, 0.15 M NaCl, 0.028 M Tris, pH 7.6, and 10⁻⁴ M phenol red. The plates were covered with Saranwrap (Dow) to prevent evaporation, and the time necessary for the color to change from red to yellow was recorded. Standard color changes were obtained by adding appropriate amounts of acid to the reaction mixture. The tests were run at room temperature and thrombin dilutions were used which would effect the color change within four hours.

**Isolation and Characterization of Fibrinopeptides**—Purified fibrinogen preparations were dissolved in 0.15 M ammonium acetate and clotted with small amounts of the thrombin of choice. The reaction was carried out for a period 10 times longer than the time necessary for gel formation. The clots were removed mechanically on a glass rod and the clot liquors lyophilized. The lyophilized powders were dissolved in a minimal amount of distilled water and subjected to paper electrophoresis in a 0.05 M sodium acetate buffer, pH 5.0 (12). The lamprey eel and bovine fibrinopeptides were isolated by preparative electrophoresis, hydrolyzed in constant boiling HCl for 24 hours under reduced pressure at 110°C, and their amino acid compositions estimated by one-dimensional paper chromatography (13).

**RESULTS**

The physico-chemical properties of lamprey eel fibrinogen appear to be very similar to those of mammalian fibrinogens. Isolation techniques such as the Cohn ethanol fractionation scheme and Laki’s ammonium sulfate purification step can be used with only minor modifications. The purified lamprey eel fibrinogen preparations contained more than 90% clottable protein and were homogeneous in the ultracentrifuge. The estimated sedimentation coefficient (S₂₀,₅₀ = 8 S) was similar to those of mammalian fibrinogens. The lamprey fibrinogen did not react with rabbit antibodies prepared against human fibrinogen.

The human and bovine thrombins clotted the lamprey eel fibrinogen at approximately half the rate that they clotted bovine fibrinogen, although the actual ratio of clotting activities varied somewhat with different preparations and with the concentration of fibrinogen used. Since the ratios were essentially similar for the two mammalian thrombins, a unit of human or bovine thrombin was arbitrarily defined as that dilution which would clot bovine fibrinogen in 15 ± 1 seconds at 25°C.

Lamprey eel thrombin clotted lamprey fibrinogen approximately 30 times faster than it clotted bovine fibrinogen preparations (Fig. 1). Accordingly, a unit of lamprey thrombin was defined as that dilution which would clot lamprey fibrinogen in...

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2 The authors are grateful to E. H. Hazen for his gift of dog Fraction I and to E. Buck for making available sheep blood.

3 The immunology was performed by Drs. H. Hamashima and J. Harder.
When bovine fibrinogen preparations were clotted with human thrombin, two negatively charged peptides were detected (Fibrinopeptides A and B). Sheep fibrinogen yielded two peptides which were electrophoretically similar to the bovine fibrinopeptides, but dog fibrinogen released one peptide corresponding to bovine Fibrinopeptide B and another slower moving component which was isoelectric at approximately pH 4.0 (Fig. 3). All the above were clotted with human thrombin. When the bovine fibrinogen was clotted with lamprey eel thrombin, only Fibrinopeptide A was detected (Fig. 4), in spite of the fact that more than 90% of the protein was incorporated into the clot (Fig. 1).

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* Known composition of bovine fibrinopeptide controls (14).
† Based on spectrophotometry before acid hydrolysis. All other amino acids estimated from paper chromatography.
Lamprey eel and human thrombins both degraded lamprey eel fibrinogen to yield the same products (Fig. 4). At pH 5.0, only one negatively charged peptide was detected. The material stained with ninhydrin and gave a positive Sakaguchi color test, indicating the presence of arginine. The peptide had a mobility slightly greater than that of bovine Fibrinopeptide A. A second peptide was also detected in some lamprey eel clot liquors after longer exposures to either thrombin. This second peptide was positively charged and moved toward the cathode at pH 5.0.

Both lamprey peptides and the two bovine peptides (as controls) were isolated and their amino acid compositions estimated from paper chromatography after acid hydrolysis (Table I). The negatively charged (major) lamprey peptide contained a disproportionately large number of acidic amino acid residues. Arginine was the only basic amino acid present. All the amino acids detected also occur in at least some mammalian A fibrinopeptides (8). The second lamprey peptide (minor), which may not necessarily be a primary degradation product, is unique in that it is positively charged. Although histidine is not present, there appears to be at least twice as much lysine as arginine.

**DISCUSSION**

The fact that mammalian thrombins clot fibrinogen from as primitive an animal as the lamprey eel suggests that the fundamental nature of the thrombin-fibrinogen clotting process persists throughout the vertebrates. The relative rapidity with which the mammalian thrombins clot the foreign fibrinogen further suggests that the same type of peptide linkage is being cleaved in both lamprey eel and mammalian fibrinogens. The major lamprey fibrinopeptide contains only one basic amino acid, an arginine residue which is almost assuredly COOH-terminal or the human thrombin could not have cleaved the peptide (14). Such an hypothesis is further borne out by the similar TAME esterase activities of lamprey thrombin and human thrombin. Evidently the thrombins have retained an arginine specificity in the face of deep seated modifications elsewhere in the molecule indicated by the very slow interaction of lamprey thrombin and bovine fibrinogen.

The major lamprey eel fibrinopeptide maintains the over-all negatively charged character found in all mammalian fibrinopeptides previously examined. Such a finding lends support to the idea that the function of the fibrinopeptides is to keep fibrinogen molecules apart before degradative attack by thrombin. Although differences from the bovine fibrinopeptide amino acid composition are apparent, the discrepancies are superficially (i.e. without knowing the amino acid sequence) no greater than the reported differences among the peptides of various mammalian species (8).

Since all thrombins investigated to date clot their own (homologous) fibrinogens at a rate faster than they clot fibrinogens from distantly related animals, it is difficult to avoid the conclusion that the thrombin and fibrinogen molecules of a given species (or group) are mutually adapted to yield as effective a clotting reaction in terms of rate as is possible under the circumstances. Point mutations which affect amino acid substitutions in the neighborhood of the attachment sites can result in sterically and electronically changes in either the thrombin or fibrinogen molecules which will decrease the effectiveness of their interaction. In order for the over-all effectiveness of the interaction to be maintained, a complementary mutation must occur in the other reactant. In order for "species specificity" to have come about, a large number of mutations must have persisted, over sizable periods of time, which were indeed detrimental to the interaction of thrombin and fibrinogen and to the organism as a whole. Other point mutations must have occurred which did not persist. For example, the loss of an arginine residue at the fibrinopeptide-fibrinogen junction would not persist, and the occurrence of the (presumably) COOH-terminal arginine in the fibrinopeptides of fish and mammals alike exemplifies a primary restriction in the evolution of a protein molecule. The necessity of an over-all negatively charged character for the fibrinopeptides imposes a secondary type of restriction. Unlike the essential arginine residue, the over-all negative charge distribution can be effected by a variety of amino acid compositions. This is borne out by the fact that various mammalian fibrinopeptides vary in electrophoretic mobility and isoelectric point (8). The fibrinogens of the ox and the sheep, which are quite closely related animals, yield fibrinopeptides which are electrophoretically indistinguishable (Fig. 3), even though end-group analysis (15) has shown the NH2-terminal residues of both sheep fibrinopeptides to be different from the NH2-terminal residues of the two corresponding bovine peptides. In addition to evolutionary restrictions concerning the nature of the linkage cleavage, the attachment sites, and the nature of the peptides cleaved, other restraints on the nature of amino acid substitutions in other parts of the fibrinogen molecule must have been imposed in order to preserve the ability of the fibrin monomers to polymerize. The result of the sum total of these restrictions is that the overall physical and chemical properties of fibrinogens from distantly related organisms are very similar.

In light of these observations the following general propositions are extended to explain the occurrence of "species specificity" in protein-protein (or polypeptide-polypeptide) interactions. Mutations can result in amino acid substitutions in either of the protein reactants which may be (a) helpful, (b) without effect, (c) slightly harmful, or (d) harmful. All of these judgments are made in terms of the rate of reaction between the two proteins. Substitutions of the last type would not persist, but the fact that slightly harmful point mutations must persist long enough for selection pressures to realize complementary (helpful) mutations in the other protein reactant is the crux of a "species specificity" based on a rate of reaction criterion. Category (b) embraces those mutations which result in amino acid changes which do not distort the ionic or electronic conditions involved in the particular protein-protein interaction under study. Such changes result in what might be termed a "secondary species specificity," one which can be detected physically or chemically but which does not influence the physiological rate of reaction.

**SUMMARY**

1. Purified preparations of lamprey eel fibrinogen were prepared, reacted with lamprey eel and human thrombins, and the clotting times compared with those obtained with purified bovine fibrinogen.

2. Mammalian thrombins clot lamprey eel fibrinogen relatively efficiently, whereas lamprey thrombin does not clot bovine fibrinogen very well at all. Unit preparations of lamprey eel and human thrombins both exhibited approximately the same degree of esterase activity toward the artificial substrate p-tosyl L-arginine methyl ester.

3. Only one negatively charged peptide is cleaved from lamprey eel fibrinogen by lamprey eel or human thrombins.
A second peptide, bearing a positive charge at pH 5.0, was also detected in preparations that had stood longer. The amino acid composition of the two lamprey peptides was estimated from paper chromatography. When lamprey eel thrombin clotted bovine fibrinogen, only Fibrinopeptide A was released in detectable amounts.

4. A general explanation of the evolution of “species specificity” of protein protein interactions is introduced which includes as a necessary corollary the persistence of slightly harmful mutations over extended periods of time.

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