The Binding of Human and Bovine Thrombin to Human Platelets

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Human thrombin binds to specific receptors on the surface of human platelets in a manner analogous to bovine thrombin. Thus, two classes of binding are observed—high affinity with a dissociation constant (K_d) of 0.02 U/ml and low affinity with a K_d of 5 U/ml. Bovine and human thrombin bind to the same platelet receptors, although bovine thrombin binds with slightly greater affinity. When the amount of thrombin bound to platelets is related to the extent of 14C-serotonin release, bovine and human thrombin are equally effective. Antibodies to human and bovine thrombin were found to differ markedly in their ability to precipitate thrombin of the two species. Thus, antihuman thrombin precipitated eightfold more bovine thrombin than human thrombin, while antihuman thrombin precipitated tenfold more human thrombin than bovine thrombin. Similar differences were found in the ability of Fab fragments of these antibodies to block the interaction of thrombin of each species with human platelets. The finding that both species of thrombin, despite significant evolutionary differences in primary structure, retain essentially identical binding sites to platelets suggests that this part of the thrombin molecule is physiologically important and supports our hypothesis of a role for thrombin binding to platelets in platelet function and hemostasis.

We have previously reported the kinetics of binding of bovine thrombin to washed human platelets. At least two types of reversible binding appear to be present: one consisting of a small number of binding sites with high affinity and the other consisting of a large number of sites of lower thrombin affinity. Radioautography shows this binding to be present on the platelet surface. The displacement of 125I-thrombin bound to platelets by excess unlabeled thrombin also suggests surface binding. Our results as well as those of Detweiler and Feinman suggest the possibility of cooperative interaction between thrombin binding sites. In addition, our previous experiments have suggested that the site on thrombin which binds to the platelet receptor is distinct from the catalytic site, since thrombin blocked with diisopropyl fluorophosphate (DFP) binds to the thrombin receptor identically to native thrombin while acetylated thrombin which retains esterase activity does not bind to platelets. It remains to be established that the binding measured in these previous studies actually reflects a part of the physiologic effect of thrombin on platelets. Our previous studies were carried out only with bovine thrombin, and we now report similar binding experiments with human thrombin. In addition, we have compared the thrombin molecules from man and cow immunologically and have used antibovine thrombin Fab antibody fragments as a probe for defining the thrombin–thrombin receptor interaction.
MATERIALS AND METHODS

Isotopes were purchased from the following sources: carrier-free sodium $^{125}$I-sodium iodide, Mallinckrodt Nuclear, St. Louis, Mo.; 2-$^{14}$C-serotonin binoxalate (22.1 Ci/μmole), New England Nuclear, Boston, Mass. Bovine serum albumin (crystallized and lyophilized), papain, 2 x crystallized, and Australian Taipan snake venom were all obtained from Sigma Chemical Company, St. Louis, Mo. Membrane filters (Metricel GA-3, 1.2-μm pore size) were purchased from Gelman Instrument Co., Ann Arbor, Mich. Freund’s Complete Adjuvant was obtained from Miles Laboratories, Inc., Kankakee, Ill.

Preparation of Platelets

Platelets were obtained from volunteers and prepared as described previously. They were used within 6 hr from the time the blood was drawn.

Purification and Iodination of Thrombin

Bovine thrombin was purified from topical thrombin (Parke, Davis & Co., Detroit, Mich.) by the method of Glover and Shaw, yielding a fibrinogen clotting activity of 2000-2500 units/mg of protein. Clotting activity was determined by the method of Seegers, using purified bovine fibrinogen. Thrombin concentration was estimated by measuring ultraviolet absorbance at 280 nm, assuming 2.0 absorbance units equals 1 mg/ml of bovine thrombin. The molecular weight of bovine thrombin was assumed to be 36,000 based on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. These preparations contained 75%-95% α-thrombin, the remainder being products of thrombin autolysis. We found essentially identical binding and release data regardless of the preparation. This observation would indicate that “non-α”-thrombin has diminished or no reactivity with platelets.

Human thrombin was purified from Cohn fraction III paste (American Red Cross, National Fractionation Center) by the method of Fasco and Fenton, except that Australian Taipan snake venom was used to activate prothrombin. The thrombin was homogeneous based on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and had clotting activity of 2300-2700 units/mg of protein. Incubation of the human thrombin with $^{14}$C-diisopropyl fluorophosphate followed by SDS-polyacrylamide gel electrophoresis revealed 98.6% α-thrombin. Clotting activity was determined by the method of Fenton and Fasico. Thrombin concentration was estimated by measuring ultraviolet absorbance at 280 nm assuming 1.62 absorbance units equal 1.0 mg/ml of human thrombin. The molecular weight of human thrombin was assumed to be 36,000.

Thrombin was iodinated with $^{125}$I-sodium iodide using a modification of the chloramine-T method described by Hunter and previously published. Iodinated bovine thrombin was stored at -20°C and used within 7 days. Iodinated human thrombin was stored at -20°C and used within 4 days. Thrombin of high specific radioactivity (0.5-1.5 x 10⁶ cpm/unit) was used the same day that it was iodinated.

Binding of Thrombin to Platelets

Platelets (5 x 10⁷) were incubated with $^{125}$I-thrombin (2 x 10⁴-1.5 x 10⁶ cpm/unit) in a total volume of 0.5 ml of isotonic Tris-buffered saline (pH 7.5) containing 0.14 M sodium chloride, 0.015 M Tris-HCl, 0.0055 M glucose, and 5 mg/ml of bovine serum albumin (hereafter referred to as Tris-buffered saline) for 20 min at room temperature. Five milliliters of cold Tris-buffered saline were added, and the platelets were collected by a membrane filtration technique under reduced pressure. The filter was counted in a Packard autogamma counter. Plastic tubes (12 x 75 mm) and Millipore filters were soaked in 0.5%, albumin as described previously. A correction for nonspecific binding was made on all determinations of $^{125}$I-thrombin bound. Nonspecific binding was that binding of $^{125}$I-thrombin which could not be displaced from platelets by an excess (150 U/ml) of unlabeled thrombin. For all comparisons between human and bovine thrombin, experiments were performed on the same day with the same platelets. Small differences in binding parameters and $^{14}$C-serotonin release were seen with platelets obtained from different donors.
Measurement of \( ^{14} \text{C}-\text{Serotonin Release} \)

The technique has previously been published\(^1\) and involved incubation under similar conditions to those employed in the binding experiments except that platelets were previously loaded with \( ^{14} \text{C}-\text{serotonin} \).

Preparation of Antithrombin Antibodies and Fab Fragments

Previous investigators have commented on the inability to induce specific thrombin antibodies in animals.\(^1^5\) One possible explanation for this is that upon entry into the circulation thrombin is complexed with antithrombin and either removed from the circulation or rendered nonantigenic. Rosenberg and Damus have shown that DFP-modified thrombin does not compete with untreated thrombin for a binding site on antithrombin, suggesting that this inhibitor binds to thrombin at its active serine center.\(^1^6\) For this reason we treated thrombin with 0.01 \( M \) DFP for 30 min at room temperature before using it to immunize rabbits. The diisopropyl phosphoryl-thrombin (DIP-thrombin) was passed over a column of Sephadex G-25-80 to remove unreacted DFP. Rabbits were injected subcutaneously in the footpads with 1 mg DIP-thrombin in Freund's adjuvant. Four weeks after injection, the rabbits were boosted with 1 mg each of DIP-thrombin. Six weeks after primary immunization, blood was drawn for thrombin antibodies.

The IgG fraction of immune rabbit sera was obtained by DEAE-cellulose chromatography.\(^1^7\) A 5.0-ml solution of immune IgG in Tris-buffered saline to which was added 0.05 ml of 0.25 \( M \) EDTA, 0.10 ml of 0.5 \( M \) cysteine, and 0.005 ml of papain, 30 mg/ml, was incubated for 16 hr at 37\( ^\circ \)C under toluene. Then the papain was inactivated by adding 0.5 ml of N-ethylmaleimide (0.25 mg/ml) followed by incubation for 30 min at room temperature. The digest was dialyzed extensively against Tris-buffered saline at 4\( ^\circ \)C, and the precipitate that formed was removed by centrifugation. The conversion of antithrombin antibodies to Fab fragments was demonstrated by the failure of the Fab fragments to precipitate thrombin and by Sephadex G-150 chromatography. (Molecular weights of approximately 150,000 and 50,000 were obtained for the IgG and Fab fragments, respectively.)

Ouchterlony Immunodiffusion and Quantitative Precipitation Reaction

These procedures were performed using standard techniques.\(^1^8,1^9\) Thrombin was incubated with rabbit antithrombin antisera or control sera in isotonic Tris-buffered saline for 20 hr at 4\( ^\circ \)C. The precipitate was washed with cold Tris-buffered saline and dissolved in Lowry reagent A. Protein determinations were by the method of Lowry.\(^2^0\) The amount of immune precipitate was determined by subtracting the amount of protein precipitated with nonimmune rabbit sera from the amount precipitated by immune rabbit sera.

Inhibition of Thrombin Clotting Activity

Clotting activity was determined as outlined by Seegers\(^4\) except that prior to addition of fibrinogen thrombin was incubated with either antithrombin Fab fragments or control, nonimmune Fab fragments for 5 min.

Inhibition of Thrombin Binding to Platelets and Thrombin Release Reaction

Binding and release experiments were performed as outlined except that thrombin was preincubated with antithrombin Fab fragments for 5 min prior to the addition of platelets.

RESULTS

In preliminary experiments we compared the binding of homogeneous human thrombin to platelets with that observed with homogeneous bovine thrombin. At low concentrations of thrombin (0.002–0.07 units/ml), binding appeared to be similar using either human or bovine thrombin (Table 1). The apparent dissociation constant (\( K_{\text{diss}} \)) as determined by the method of Steck and Wallach\(^2^1\)
Table 1. Binding of Human and Bovine Thrombin to Human Platelets

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<th>High-affinity Binding</th>
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<td>$K_d$ (units/ml)</td>
<td>Binding Sites per Platelet</td>
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<td>600</td>
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<td>Human thrombin</td>
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Platelets were incubated with $^{125}$I-thrombin in Tris-buffered saline (pH 7.4) and the amount of thrombin bound determined as described under Materials and Methods. Results were plotted by the method of Steck and Wallach and $K_d$ and number of binding sites determined from these plots.21

was 0.006 units/ml for bovine thrombin and 0.02 units/ml for human thrombin (Fig. 1). In addition, there were consistently greater numbers of high-affinity binding sites for bovine thrombin than human thrombin. The explanation for the slight but consistent difference seen remains obscure, but could reflect slight differences in the thrombin receptor binding site of the two thrombin species resulting in greater high-affinity binding of bovine thrombin at low thrombin concentration. Unlabeled thrombin of either species inhibited high affinity binding of either labeled thrombin. The inhibition was competitive, as shown by plotting the data according to Steck and Wallach (Fig. 1).21 The apparent inhibition constants22 for the unlabeled species were the same as the $K_d$ obtained using labeled thrombins. These experiments indicated that the thrombin of both species bound to the same high-affinity receptor sites on platelets and that iodination did not significantly affect the binding to platelets.

While it appears that human thrombin has less "high-affinity" binding, it is clear that the molecules of both species bind to the same sites, since human thrombin competes with bovine thrombin at all of the apparently greater number of high-affinity sites. This finding is most clearly seen in experiments where

![Graph showing binding of $^{125}$I-human thrombin to platelets. Results are plotted by the method of Steck and Wallach. Platelets and 0.002–0.17 units/ml of thrombin were incubated in isotonic Tris-buffered saline (pH 7.4) as described in Materials and Methods. Incubations were initiated by addition of platelets and after 20 min were terminated by membrane filtration. $\bullet$, $^{125}$I-human thrombin; $\circ$, $^{125}$I-human thrombin + 0.02 unit/ml human thrombin; $\triangle$, $^{125}$I-human thrombin + 0.02 unit/ml bovine thrombin. The intercept on the ordinate equals 1/thrombin bound at saturation per 5 x $10^9$ platelets. The intercept on the abscissa equals 1/thrombin free per 0.5 ml ($K_{ds}$) at which half-maximal binding occurs.](image-url)
a great excess of human thrombin completely blocks the binding of bovine thrombin to platelets (data not shown).

At higher concentrations of thrombin, 0.2–2.0 units/ml, binding of human and bovine thrombin were similar (Table I). The apparent dissociation constant for human thrombin was 4.6 units/ml and for bovine thrombin, 1 unit/ml. Unlabeled thrombin of either species inhibited the binding of either labeled thrombin as described above. With these higher concentrations of thrombin, the same number of receptor sites were observed for both thrombins (30,000 per cell). This result is expected if both molecules can bind to the same sites. While these binding studies as presented demonstrate two apparent classes of binding sites, we do not know whether distinctly different thrombin binding sites exist. It is possible that there is a single receptor site with negatively cooperative binding.\(^1,^2\)

**Thrombin-induced Release of \(^1^4\)C-Serotonin From Platelets**

We next compared human and bovine thrombin with respect to platelet \(^1^4\)C-serotonin release. A consistent difference between the amount of human and bovine thrombin necessary to induce the release of \(^1^4\)C-serotonin was observed (Fig. 2A). Fifty per cent of maximal serotonin release was obtained with 0.02 units/ml of bovine thrombin, and 0.04 units/ml with human thrombin. Although the amount of thrombin necessary to induce release varied from day to day, a consistent two- to four-fold difference was observed between the amount of bovine and human thrombin necessary to induce half-maximal release.

We next determined whether the increased release of serotonin seen with bovine thrombin was due to the increased binding of bovine thrombin or to increased functional activity of bound bovine thrombin. When the amount of either of the two species of thrombin bound to platelets was compared to the extent of serotonin release, human thrombin was as effective or slightly better than bovine thrombin (Fig. 2B). This result was obtained by preincubating platelets with \(^1^4\)C-serotonin then adding \(^1^2\)I-labeled human or bovine thrombin and measuring both the extent of release (\(^1^4\)C-serotonin retained) and the amount of thrombin bound to the platelets.

**Quantitative Precipitation Reactions and Ouchterlony Gels**

We next explored the use of thrombin antibodies to compare the structures of the thrombin molecules of the two species and as a possible probe to study the thrombin–platelet interaction. Using rabbit antiovine thrombin antisera, a marked difference between human and bovine thrombin precipitation was seen when quantitative precipitin curves were compared (Fig. 3A). At the equivalence points of these reactions, there was 0.165 mg of protein precipitated using bovine thrombin, and 0.02 mg of protein was precipitated using human thrombin. The Ouchterlony gel reflected both the quantitative difference and in addition a qualitative difference as a spur of partial identity was seen between bovine and human thrombin (Fig. 4A). With a similarly prepared rabbit antihuman thrombin antiserum a strong precipitin line was formed against human thrombin; however, a much weaker one formed against bovine thrombin (Fig. 4B). As in the case of rabbit antibovine thrombin antiseraums, a spur of partial
Thrombin-induced release of $^{14}$C-serotonin from platelets. Platelets were loaded with $^{14}$C-serotonin as described. Ten-minute incubations were performed as described under Materials and Methods. Release is expressed as per cent of serotonin retained compared to control incubations without thrombin. The point of 50% of maximal serotonin released is defined as the thrombin concentrations which give 60% serotonin retained. $\left(\frac{100\% - 20\%}{2} + 20\%\right)$ o, bovine thrombin; $\circ$, human thrombin. (B) Effect of thrombin bound on $^{14}$C-serotonin release from platelets. Incubations were performed as in (A) except that thrombin was labeled with $^{125}$I-sodium iodide. Samples were counted in a liquid scintillation counter for $^{14}$C-serotonin and in a gamma scintillation counter for $^{125}$I-thrombin bound. The specific activity of $^{125}$I-thrombin was chosen so that the correction for $^{125}$I counting in the liquid scintillation counter was always less than 20% of the $^{14}$C cpm. $\circ$, $^{125}$I-bovine thrombin; $\circ$, $^{125}$I-human thrombin.

Identity was seen. When a precipitin reaction was carried out using antihuman thrombin antisera, at equivalence, 0.26 mg of protein was precipitated with human thrombin and 0.025 mg with bovine thrombin (Fig. 3B).

Effect of Rabbit Antibovine Thrombin Fab Fragments on Binding of Thrombin to Platelets and Release of $^{14}$C-Serotonin

The immunologic data outlined above indicated that there were considerable structural differences between the thrombins of the two species. Since the binding and release studies suggested that the platelet binding sites on the two thrombin molecules were very similar, we hoped that the cross-reacting antibodies
Fig. 3. Quantitative precipitation reaction of thrombin and rabbit antithrombin antiserums. Reaction mixtures contained thrombin, 0.04 ml of antiserums, and isotonic Tris-saline in a total volume of 0.5 ml. Incubations were at 0°C for 16 hr. Precipitates were washed once with cold incubation buffer at 4°C, and the protein was determined. Controls consisted of normal rabbit sera. (A) Rabbit antibovine thrombin antiserums. ●, human thrombin; ○, bovine thrombin. (B) Rabbit antihuman thrombin antiserums. ●, human thrombin; ○, bovine thrombin.

might include antibodies directed against regions of the platelet binding site common to both molecules. For these experiments Fab fragments to antibovine thrombin were used, since intact antibody molecules may cause platelet aggregation and release. In preliminary experiments we observed marked differences in the ability of antibovine thrombin Fab to block binding of human and bovine thrombin to platelets. Thus there was marked inhibition of the binding of bovine thrombin to platelets with relatively much less inhibition of human thrombin binding (data not shown). Inasmuch as we have shown that there is a close correlation between the amount of thrombin bound to platelets and the extent of serotonin release, we were not surprised to find similar inhibition
of $^{14}$C-serotonin release. For these experiments, bovine thrombin was preincubated with antibovine Fab fragments prior to addition to $^{14}$C-serotonin-loaded platelets. Marked inhibition of $^{14}$C-serotonin release was observed under these conditions (Fig. 5A). When $^{14}$C-serotonin release induced by human thrombin was measured in the presence of antibovine thrombin fragments, there was much less inhibition (Fig. 5B). The ability of antithrombin Fab fragments to block thrombin activity was estimated by determining the shift in the apparent concentration of thrombin which caused 50% of maximal serotonin release. Approximately 25 units of bovine thrombin were inhibited by 1 ml of Fab fragments, whereas only 1.1 units of human thrombin were inhibited by
Fig. 5. Effect of rabbit antibovine thrombin Fab fragments on thrombin-induced release of
14C-serotonin. Conditions were the same as in Fig. 3 except that thrombin was preincubated with
antithrombin Fab fragments for 5 min. Control Fab fragments had no effect on release. (A) Bovine
thrombin, ○○, bovine thrombin; ••, bovine thrombin + 0.005 ml Fab fragments; □□, bovine
thrombin + 0.01 ml Fab fragments. (B) Human thrombin, ■, human thrombin; ●, human throm-
bin + 0.025 ml Fab fragments; □, human thrombin + 0.05 ml Fab fragments.

1 ml of Fab fragments. As in the case of the binding of both thrombins to plate-
lets, antibovine thrombin Fab fragments had a marked effect on serotonin re-
lease induced by bovine thrombin and very little effect on human thrombin. In
other experiments the antibovine thrombin Fab fragments were shown to in-
hbit clotting of fibrinogen by bovine thrombin with relatively little effect on
clotting of fibrinogen by human thrombin.

We then passed the antibovine thrombin Fab fragments over a column of
human thrombin attached to Sepharose-4B in an attempt to remove cross-
reacting antibodies that might be specifically directed against the platelet-
receptor portion of the thrombin molecule. However, the antibovine Fab frag-
ments continued to inhibit binding and serotonin release induced by bovine
thrombin. These results suggested that the heterogeneous antibodies in the
antibovine Fab preparation reacted with different antigenic determinants on
human and bovine thrombin to block its action on platelets. Thus, with our
thrombin antibody preparation, we were unable to demonstrate a common anti-
genic site that was unique to the platelet receptor (or fibrinogen clotting) por-
tion of the thrombin molecule. It was not possible to determine whether the antibodies inhibited binding to platelets by interacting directly with some component of the platelet binding site or by a steric effect due to binding at some distant sites. The latter explanation seems likely in view of the parallel inhibition of clotting and binding to platelets produced by the antibody. In an analogous series of experiments, antihuman thrombin Fab fragments gave similar results, i.e., blocking of binding and \(^{14}\)C-serotonin release induced by human thrombin much greater than inhibition of those parameters induced by bovine thrombin.

DISCUSSION

Previous studies have suggested that bovine thrombin binds to the platelet surface by a site on the thrombin molecule distinct from the catalytic site.\(^{1,25}\) Numerous other studies indicate that human and bovine thrombin are functionally very similar in regard to the clotting of fibrinogen. The data presented here indicate that human thrombin contains a very similar platelet binding site to bovine thrombin. Binding is saturable, and \(^{125}\)I-thrombin can be displaced by an excess of unlabeled thrombin. In addition, at least two types of binding sites for human thrombin are present, as with bovine thrombin. One population of low-affinity sites with a \(K_{\text{diss}}\) of about 5 units/ml and one of high-affinity sites with a \(K_{\text{diss}}\) of 0.02 units/ml. Bovine and human thrombin appear to bind to the same sites as competitive inhibition is demonstrated at both high- and low-affinity sites. It is of interest that the affinity of bovine thrombin for human platelets is consistently two- to four-fold greater than human thrombin. It is unlikely that this is a result of either impurity or diminished activity of the human thrombin as the specific activity was high (2700 units/ml), and it was homogeneous in SDS gel electrophoresis, and 98.6% was \(\alpha\)-thrombin. The reason for this difference in binding is unclear but could reflect slight variation in the affinity of thrombin for the thrombin receptor. This difference has not been reflected in differences in the ability of the thrombin to induce \(^{14}\)C-serotonin release, as approximately the same degree of release is obtained with both (Fig. 4B). Thus both species of thrombins are functionally nearly identical.

Partial amino acid sequencing of human and bovine thrombin reveals significant differences. Walz and Seegers report that the A-chain of human thrombin is 13 residues shorter from the amino terminus than that of bovine thrombin.\(^{26}\) In addition, they have found differences in eight residues of the 34 identified. Thompson, Ericsen, and Enfield have found that in the first 50 residues of the human B-chain, there are differences in only three residues from the corresponding bovine B-chain.\(^{27}\) Their analysis of the first 16 residues of the human A-chain and corresponding bovine A-chain is identical to that of Walz and Seegers.

The marked antigenic differences observed here are consistent with the primary structural differences noted above. Using the data of Prager and Wilson\(^{28}\) which relate antigenic differences to amino acid sequence differences with proteins of known sequence, we would estimate that human and bovine thrombin differ in amino acid sequence at between 10% and 25% of amino acids. Despite these relatively large differences, functionally the two molecules appear to be
nearly identical with respect both to clotting of fibrinogen and binding to platelets and induction of the release reaction. Our attempts to use antithrombin antibodies as a probe for studying the interaction of thrombin with its receptor on the platelet membrane have been unsuccessful as we could not demonstrate specific antibody activity against the platelet-binding portion of the thrombin molecule.

The fact that both species of thrombin, despite significant evolutionary differences in primary structure, retain essentially identical binding sites to platelets suggests that this part of the thrombin molecule is important physiologically and supports our hypothesis for a role for thrombin binding to platelets in platelet function and hemostasis.

In other recent experiments where thrombin binding was perturbed by anions, further support for this hypothesis was obtained. Thus, changes in thrombin binding exactly paralleled changes in the release of 14C-serotonin.24 Further, only thrombin bound to the platelet receptor, as neither prothrombin nor any of the intermediates of prothrombin activation would bind to the thrombin receptor.29

ACKNOWLEDGMENT
We thank Dr. J. W. Fenton II, Division of Laboratories and Research, New York State Department of Health, for determining the α-thrombin content of our human thrombin.

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The binding of human and bovine thrombin to human platelets

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