HIGH AFFINITY BINDING OF THROMBIN TO PLATELETS. INHIBITION BY TETRANITROMETHANE AND HEPARIN[1]

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SUMMARY: [^{125}I] iodo-α-thrombin has been modified at the macromolecular substrate binding site in order to study the importance of this region in the platelet-thrombin interaction. Modification was effected by the nitration of tyrosine residues with tetranitromethane. This chemical modification abolished the ability of the enzyme to bind with a high affinity to the platelet surface but did not significantly alter low affinity binding. The presence of heparin was also found to inhibit high affinity binding. These results indicate that the high affinity binding site interacts with the fibrinogen binding region of the thrombin molecule and suggests that there are two distinct classes of binding sites for thrombin on the platelet membrane.

Thrombin binds to the surface of blood platelets (1) and causes them to aggregate and release intracellular materials. The binding phenomenon has been investigated in a number of laboratories with differing results (2-5). Recent investigations suggest the existence of either two or more classes of binding sites on the platelet surface with different affinities for thrombin (6, 7) or identical sites which exhibit negative cooperativity (8).

It is not clear from the above studies what features of the thrombin molecule are important for platelet binding, although it has been shown that the active site is not involved (2). Our approach to this question has been to utilize reagents that are known to interfere with the binding of macromolecular substrates to thrombin in an effort to perturb the platelet-thrombin interaction, and thereby indicate the nature of the platelet binding region on the thrombin molecule. Previous studies in this laboratory utilizing the modifying agents tetranitromethane (9), N-acetylimidazole (10), and N-butyrylimidazole (11), have demonstrated that the modification of tyrosine drastically alters the ability of thrombin to interact with fibrinogen. The macromolecular binding site of thrombin is also modified by the presence of heparin (12), drastically reducing the ability of the enzyme to clot fibrinogen and activate platelets

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(13). Therefore, the effect of nitration and heparin on the binding of thrombin to platelets and the subsequent activation of platelets by the nitrated enzyme were studied in order to assess the importance of the macromolecular substrate binding site of thrombin in the platelet-thrombin interaction.

The results suggest that the portion of the thrombin molecule which recognizes fibrinogen is also involved in the interaction of thrombin with platelets. The data are also consistent with the existence of at least two classes of binding sites on platelets, each having a different affinity for thrombin.

Materials and Methods. Thrombin purification and iodination. Bovine α-thrombin was purified according to methods developed in this laboratory (14). Discontinuous sodium dodecyl sulphate acrylamide gel electrophoresis (15), active site titration (16), and assays for fibrinogen clotting activity indicated that the enzyme preparations were 95% α-thrombin. The fibrinogen clotting activity was determined as previously described (14). Protein concentrations were determined by the ninhydrin reaction following alkaline hydrolysis (17). α-Thrombin was iodinated using the solid state lactoperoxidase method of David (18). Specific activities ranged from 20,000 to 40,000 cpm per N.I.H. Unit. As demonstrated previously (1), enzyme activities and platelet aggregating ability were not affected by this iodination procedure. Radioactivity was measured in a Packard Auto-Gamma Spectrometer.
Tetranitromethane modification. [^{125}I] iodo-α-thrombin was nitrated according to the method of Lundblad and Harrison (9). In all preparations 4-5 residues of tyrosine per molecule had been modified as determined by amino acid analysis on a Glenco 100-AS amino acid analyzer.
Platelet preparations. Platelets were isolated and enumerated as described previously (1). When appropriate, platelet-rich-plasma was incubated with [^{14}C]-serotonin (0.1 μCi/ml platelet-rich-plasma) for 30 minutes prior to platelet isolation. ^{14}C was measured in a Packard Tri-Card liquid scintillation counter.
Binding assays. Equilibrium binding experiments were carried out at ambient temperature in Ca^{2+} free Tyrodes solution, 10^{-4}M ethylenediameme tetraacetate, pH 7.4, containing bovine serum albumin at a final concentration of 5 mg/ml. Serial dilutions of the radiolabeled thrombin preparations were prepared in plastic counting tubes and their radioactivities measured. To each of these tubes was added 0.5 ml of the platelet suspension and after gentle agitation each was allowed to incubate for 30 minutes. Control samples were prepared in parallel fashion, but without platelets, in each experiment. After the incubation period 5 ml buffer (minus bovine serum albumin) was added to each incubation mixture and the platelets were collected by filtration on a Millipore Multisampling Device utilizing filters previously soaked for 2 hours in Tyrodes solution containing bovine serum albumin (5 mg/ml). Samples were subsequently washed with an additional 15 ml buffer after which the filters were removed and the associated radioactivity measured. The amount of radioactivity bound to control filters was calculated, plotted as a function of radioactivity added and substracted from the values obtained in the presence of platelets.
Measurement of aggregation and release. Aggregation and serotonin release experiments were carried out with platelets containing [^{14}C] serotonin. Aggregation was measured in a Payton Dual Channel Aggregometer. At zero time 0.1 ml of an appropriate thrombin preparation was added to 0.9 ml of a recalcified platelet suspension in a silconized aggregation cuvette. After complete aggregation or 5 minutes, the samples were transferred to plastic centrifuge tubes and centrifuged for 5 minutes at 1000 x g. The supernatant was analyzed for [^{14}C] serotonin content. Control samples containing no added thrombin were treated in a similar fashion to determine a zero release value.

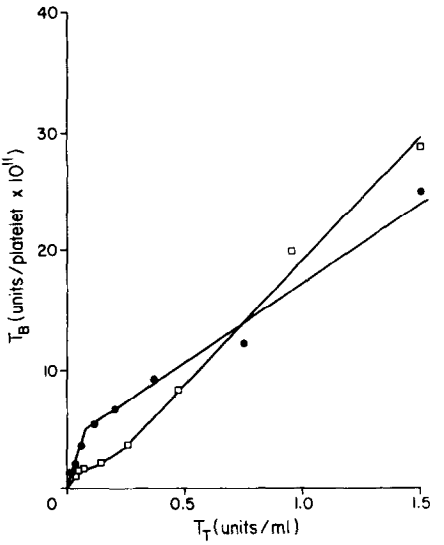

Figure 1. The binding of native [^{125}I] iodo-α-thrombin (●–●) and nitrated [^{125}I] iodo-α-thrombin (□–□) to platelets. T_B and T_T refer to thrombin bound and thrombin added, respectively. Each platelet incubation mixture contained 2 x 10^8 platelets per ml. Binding assays were carried out under ambient conditions as described in Materials and Methods.

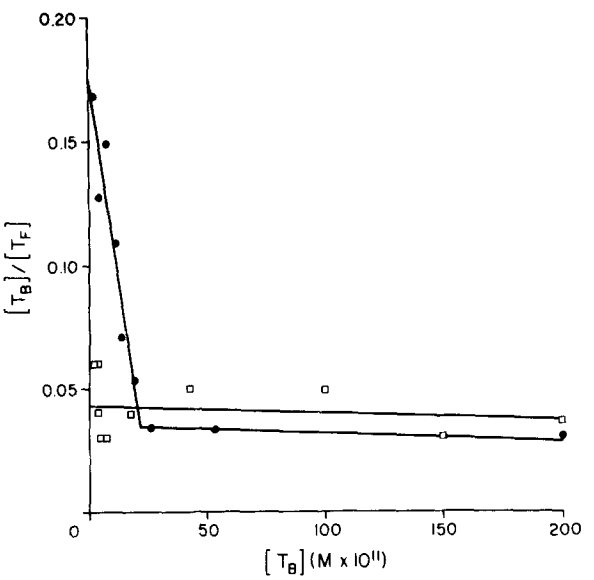

Figure 2. Scatchard plot comparing the binding of native (●–●) and nitrated (□–□) [^{125}I] iodo-α-thrombin to platelets. $[T_B]$ and $[T_F]$ represent the concentration of thrombin bound and free, respectively.

Table I. Binding Parameters for Native α-Thrombin, Nitrated α-Thrombin, and Native α-Thrombin in the Presence of Heparin

	Sites/Platelet		Association Constant	
	High Affinity	Low Affinity	High Affinity	Low Affinity
native α-thrombin	790	30,000	$6.7 \times 10^{8} M^{-1}$	$3.6 \times 10^{6} M^{-1}$
Tetranitromethane Treated α-thrombin	-	41,000	-	$3.1 \times 10^{6} M^{-1}$
native α-thrombin (minus heparin)	900	37,000	$8.7 \times 10^{8} M^{-1}$	$3.2 \times 10^{6} M^{-1}$
native α-thrombin (plus 1 U/ml heparin)	300	39,000	$9.0 \times 10^{8} M^{-1}$	$2.7 \times 10^{6} M^{-1}$

Results. Effect of tyrosine modification. The results of a typical binding study using tetranitromethane treated [^{125}I] iodo-α-thrombin and native [^{125}I] iodo-α-thrombin are shown in Figure 1. It is observed that the binding of nitrated α-thrombin is markedly reduced at low concentrations of ligand when compared with native α-thrombin, while little difference between the two species is seen at higher ligand concentrations. As a result of this difference in affinity at low ligand concentration, the biphasic binding pattern observed with native α-thrombin is not seen with nitrated α-thrombin. A Scatchard Plot (19) of these data is shown in Figure 2. The data obtained with native α-thrombin appears to be characteristic of a system demonstrating two classes of binding sites, differing in both number and association constant. In contrast, the data obtained with nitrated α-thrombin indicate the presence of only low affinity binding sites. Approximations as to the number of binding sites and the magnitude of the association constants may be calculated from these plots and are presented in Table I. With native α-thrombin there are striking differences between high and low affinity binding; high affinity binding is two orders of magnitude tighter than low affinity binding and there are approximately 40 times less high affinity sites than low affinity sites. It appears that low affinity binding of the tetranitromethane treated α-thrombin is similar to that of the native material with respect to both the number of binding sites per platelet and the value of the association constant.

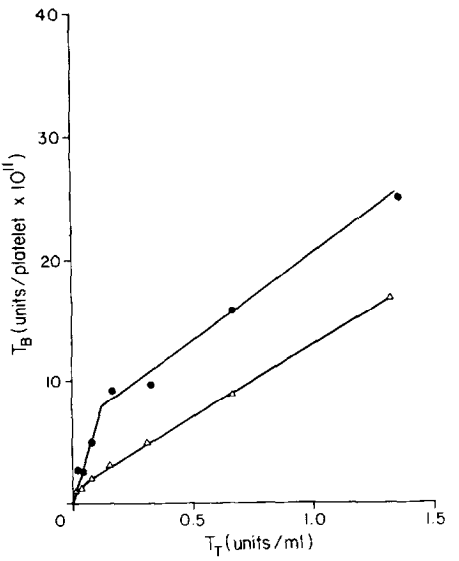

Figure 3. The binding of [^{125}I] iodo-α-thrombin to platelets in the presence (Δ - Δ) and absence (●-●) of heparin (1 Unit per ml). Heparin (The Upjohn Co.) was incubated with each thrombin dilution for 30 min. prior to incubation with platelets (2 x 10^8 platelets per ml). T_B and T_T represent thrombin bound and total thrombin added, respectively. Binding assays were carried out under ambient conditions as described in Materials and Methods.

The nitrated material could be readily displaced from platelets by the addition of an excess of unlabeled, native α-thrombin, as could the radiolabeled, unmodified material. In contrast, the addition of large concentrations of bovine serum albumin (50-100 mg/ml) did not displace either the native or modified preparations.

Aggregation and release of [^{14}C] serotonin were effected by the native material in a concentration range corresponding to high affinity binding (i.e. less than 0.1 U/ml). Tetranitromethane treatment, however, impaired the ability of α-thrombin to effect aggregation and release of [^{14}C] serotonin. On a per molecule basis, the modified material was ten times less effective in platelet activation than the native α-thrombin but with respect to fibrinogen clotting activity was more effective in causing aggregation and release.

Effect of heparin on binding. The effect of heparin on the binding of α-thrombin to platelets is depicted in Figure 3. As seen with tetranitromethane treatment, heparin also affects binding only at the lower ligand concentrations. Scatchard analysis (19) of these data (Table I) also suggests that only high affinity binding was affected and that the effect was to lower only the number of molecules bound and not the association constant.

Discussion. In this study, modification of α-thrombin either by treatment with tetranitromethane or by the presence of heparin produced a marked decrease in the ability of α-thrombin to bind with high affinity to platelets, but did not appreciably affect low affinity binding. Since these modifications are known to affect the interaction of α-thrombin with fibrinogen (9, 12), the results are interpreted to indicate that the macromolecular or fibrinogen binding site on the thrombin molecule is also involved in high affinity binding of the enzyme to platelets. This concept is consistent both with the results obtained by Ganguly (7) who found that hirudin, an inhibitor of fibrinogen clotting activity, decreased the binding of α-thrombin to platelets and with the apparent non-involvement of the active site in binding to platelets (2).

Several alternative explanations of the results are possible, but the most plausible is that both the nitration of tyrosine and the binding of heparin occur at sites other than the fibrinogen binding region and cause gross conformational changes which affect both the fibrinogen binding site and an as yet undefined platelet binding site on the thrombin molecule. A generalized conformational change seems unlikely, however, since low affinity binding was essentially unaffected by either nitration or the presence of heparin. Furthermore, the ability of nitrated α-thrombin to hydrolyze low molecular weight substrates was not significantly impaired (9), and, when only one tyrosine per molecule of thrombin was modified (11), fibrinogen clotting activity was still drastically reduced. This explanation is also not consistent with the available literature which suggests that heparin binds at the fibrinogen binding site on the thrombin molecule (12) and apparently interacts in a 1:1 molar fashion (21). Thus, it appears likely that these modifications affect the fibrinogen binding site and that this region is also involved in high affinity binding to platelets.

The finding that treatment of thrombin with tetranitromethane or the additior of heparin to the incubation mixture inhibited high affinity binding but did not affect low affinity binding is most consistent with the existance of distinct high and low affinity binding sites on the platelet surface. To explain these results in terms of the existance of identical sites, initially of high affinity, exhibiting negative cooperativity (8) is difficult in view of the following arguments. First, if the sites are homogeneous, then the affinity of the modified thrombin for these sites must be fortuitously reduced to the same values as seen with the low affinity binding of native α-thrombin. Furthermore, negative cooperativity must not exist in the case of nitrated thrombin or with native α-thrombin in the presence of heparin. Finally, the fact that the tetranitromethane modified material was able to activate platelets, although at higher than normal concentrations, suggests first, that it is binding at the high affinity site with a decreased affinity and, second that at some con-

centration of modified thrombin negative cooperativity should have been evident. Thus, a more reasonable explanation of these results is that modification of thrombin at the fibrinogen binding site lowers the affinity of the enzyme for a distinct population of high affinity sites on the platelet surface but does not affect its affinity for the low affinity binding sites.

We have recently found that native α-thrombin binds with both high and low affinity to formaldehyde fixed platelets (20), which also argues against the occurrence of conformational changes and negative cooperativity upon thrombin binding. This data will be reported elsewhere (Workman et al., manuscript in preparation).

The role of low affinity binding in the platelet-thrombin interaction is uncertain. The question certainly arises as to whether or not low affinity binding is specific since the physiological response is produced at α-thrombin concentrations less than 0.1 U/ml, the range in which high affinity binding occurs. Although it may well be impossible to provide an unequivocal answer, the fact that an excess of unlabeled α-thrombin is able to displace both the modified and native enzyme, while bovine serum albumin is not, is at least consistant with a specific interaction. The present data suggest that low affinity binding involves a region of the thrombin molecule different from the high affinity region, since low affinity binding was not affected by either modification. Thus, we view the interaction of thrombin with platelets as involving heterogenous sites on the platelet surface as well as multiple sites on the thrombin molecule. Low affinity binding may well reflect specific binding of thrombin to platelets as a mechanism for concentrating thrombin at sites of vascular injury.

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