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ANTITHROMBIN III BINDS TO HUMAN PLATELETS

Byung K. Kim, Manfred Steiner and Mario G. Baldini

Division of Hematologic Research, The Memorial Hospital
and Brown University, Pawtucket, Rhode Island 02860

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SUMMARY: The receptor site for antithrombin III (AT III) was investigated in normal human platelets. [^{125}I] iodinated AT III was utilized as tracer for the binding assay. Equilibrium of AT III binding was reached within 2 min. The binding capacity was pH-dependent with the optimum around pH 7.0. Binding specificity was demonstrated by inhibition of [^{125}I] AT III ligation using an excess amount of non-labeled AT III. The AT III•heparin complex did not suppress [^{125}I] AT III binding. Analysis of binding data by Scatchard plot revealed a single class of binding sites with K_d of 3.2 x 10^{-7} M and binding capacity of 3840 per platelet.

INTRODUCTION

Antithrombin III (AT III) is the major natural inhibitor of activated serine proteases of the blood coagulation cascade (1-5). In the absence of heparin the rate of AT III-mediated inhibition is slow but increases about 14-fold in its presence (6). Studies in our laboratory have shown that the neutralization of thrombin was also greatly accelerated in the presence of platelets (7). This observation suggested that platelets, analogously to heparin, were responsible for a conformational change of AT III. This in turn raised the possibility that the inhibitor bound to platelets. In these studies we have examined this conjecture and demonstrated that human platelets possess specific binding sites for AT III. Some of the ligand characteristics were also evaluated.

METHODS AND MATERIALS

Preparation of Platelet Suspension: Platelets were isolated from blood of normal volunteers as previously described (8). Platelets were washed twice with Ca^{++}-free Tyrode's buffer (pH 7.2) containing 15 vol % of acid-citrate-dextrose anticoagulant solution (ACD) and were finally suspended in Ca^{++}-free Tyrode's buffer at a concentration of 2-3 x 10^9/ml. All procedures were performed at 22°C.

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Purification of Antithrombin III: Procedures of purification from human plasma were essentially according to the method described by Rosenberg and Damus (6). Purified AT III had a specific activity of 5.2 U/mg protein, and was homogenous both by SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis. Crossed-immunoelectrophoresis showed no contamination with heparin-antithrombin III complex in our preparation (7). AT III was stored in aliquots of 0.1 ml (2.5 U/0.5 mg) at -85°C. Once the preparation was thawed for use it was kept at 4°C up to 4 weeks. During this period loss of activity was less than 5%. At -85°C there was no evidence of progressive loss in activity in the period of 8 months that we examined. One unit of AT III was defined as the thrombin neutralizing activity contained in 1 ml of defibrinated normal fresh pooled plasma (9). Protein amount was determined according to the method of Bradford (10) using a commercial assay kit (Bio-Rad Laboratories). For some experiments, commercially obtained AT III (specific activity 4.9 U/mg, Kabi Group Inc.) was utilized. This preparation was more than 95% pure as examined by SDS-gel electrophoresis and free of demonstrable contamination by AT III•heparin complex. Its binding behavior to platelets was comparable with our AT III preparation.

[^{125}I] Iodination of Antithrombin III: Purified AT III, 0.5 mg, was iodinated with 5 mCi of carrier-free Na[^{125}I] (Becton Dickinson Immunodiagnostics) by the lactoperoxidase (Worthington Biochemical Corp.) method essentially according to Morrison and Boyse (11). Labeling of the inhibitor protein was confirmed by radioscanning of SDS-polyacrylamide gel (Fig. 1) and by acid precipitation or gel filtration. Specific radioactivity of [^{125}I] iodinated AT III ranged between 2350 - 4500 cpm/pmol. The thrombin-neutralizing activity of AT III, measured by two-stage fibrinogen clotting assay was not modified by iodination. The specific activity of iodinated AT III was 5.15 U/mg, the same value as observed before the iodination procedure.

Binding Studies: Binding experiments were performed immediately after preparation of platelet suspensions and were completed within 3 hr from the collection of blood. The binding procedure was essentially the same as that previously described for serotonin (8). Aliquots, 1 ml in volume, of platelet suspensions containing 2-3 x 10^9 cells/ml were incubated at 22°C with 10 µl of AT III of the desired concentration. A trace amount of [^{125}I] iodinated AT III was added. In general, incubations were terminated after 4 min by rapid centrifugation (6000 x g for 1 min) of 0.8 ml portions of the samples. Platelet pellets were resuspended in 1 ml of Tyrode's buffer containing 2 mM EDTA and were then

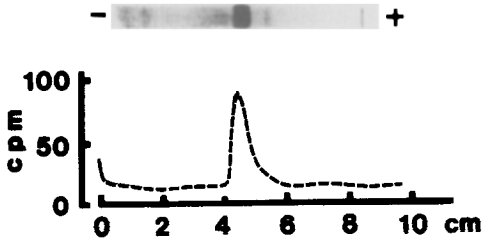
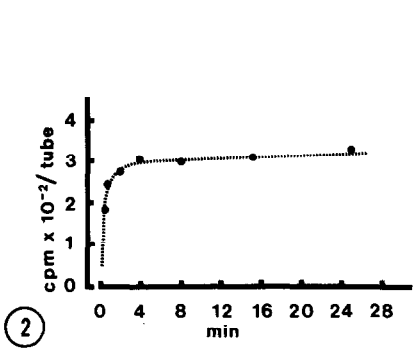


Figure 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis and radioactivity scan of [^{125}I] AT III. Gels of 7.5% with 0.1% sodium dodecyl sulfate were prepared (12). Samples containing 40 µg AT III with a trace amount of [^{125}I] AT III were applied. After fixation and Coomassie blue staining the gels were sliced into 2 mm sections and the [^{125}I] radioactivity counted.



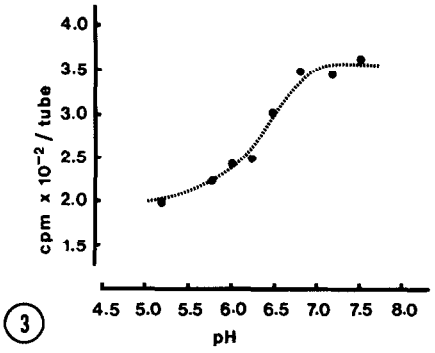


Figure 2: Equilibrium binding of AT III by platelets. Platelet suspensions containing 3 x 10^9 cells/ml were incubated with 50 nM AT III plus a trace amount of [^{125}I] AT III at 22°C. Aliquots, 1 ml in volume, were withdrawn at the indicated times and platelet-bound AT III measured.

Figure 3: Effect of pH on AT III binding. Incubation media of 8 different pH ranging from 5.2 to 7.5 were tested. The other conditions of assay were as described in legend to figure 2. Incubations were performed at 22°C for 4 min.

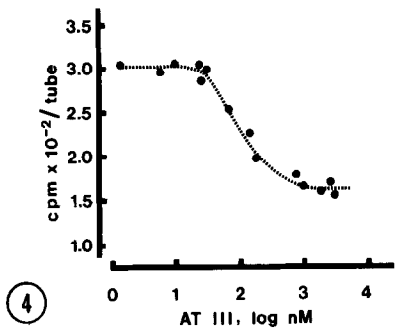
centrifuged at 6000 x g for 1.5 min. The radioactivities of platelet pellets and of 0.1 ml fractions of incubation mixture, representing total activity, were determined in a well-type scintillation counter.

Materials: All chemicals used in this study were of the highest purity commercially available.

RESULTS AND DISCUSSION

Time Course and pH Dependence of Binding: The binding activity showed a gradua increase up to pH 6.8 at which point binding was optimal and remained so up to pH 7.5 (Fig. 2). Based on this result all other experiments were performed at pH 7.2. AT III binding at a concentration of 50 nM reached equilibrium within 2 min (Fig. 3). A certain degree of reequilibration during the washing process, even though it is rapid, is unavoidable. Thus the above time estimate represents a maximal value which in actuality is certainly lower.

Specificity of Binding: Strict linearity between cell number and AT III bound to platelets was observed in the range from 1 to 4.3 x 10^9 cells/ml. Specificity of the platelet receptors for AT III was demonstrated by the suppressive effect of a large excess of non-labeled AT III on the binding of [^{125}I] AT III



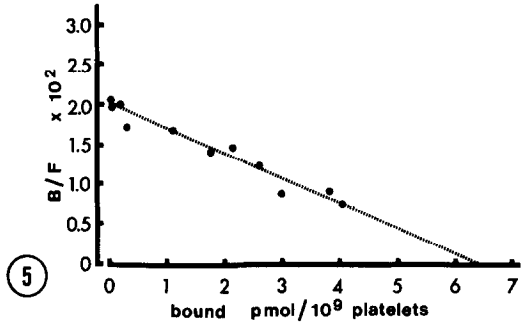


Figure 4: Suppression of [^{125}I] AT III binding by an excess of non-labeled AT III. Incubation temperature, time and medium pH were standard. Concentrations of non-labeled AT III varied from 2 - 1500 times that of [^{125}I] AT III.

Figure 5: Scatchard plot of antithrombin III binding data. Experimental conditions were the same as those of figure 4. The ratios of bound/free [^{125}I] AT III (B/F) were plotted as a function of [^{125}I] AT III bound per 10^9 platelets. Each value represents specifically bound AT III (nonspecifically bound and trapped free AT III were subtracted).

(Fig. 4). In our system 45 - 50% of the platelet-bound radioactivity was non-displaceable indicating mostly nonspecific binding. One washing step, immediately following separation of platelets from the medium, eliminated an average of 98% of the trapped free ligand with a minimal loss of bound one. The fraction of bound ligand which is nondisplaceable can vary considerably. When a similar method was used to study serotonin binding (8), 12 - 19% were found to be nondisplaceable while 40% of thrombin was bound "nonspecifically" (13). Methods like these are predicated on the identical behavior of labeled and unlabeled ligand. Although we do not know whether iodination altered the binding activity of AT III, we were able to demonstrate that the labeled inhibitor was as active as unlabeled protein in neutralizing thrombin.

Evaluation of specific AT III binding to platelets according to Scatchard (14) revealed a linear plot indicative of a single class of receptor sites (Fig. 5). The apparent dissociation constant (K_d) was 3.2 ± 2.1 x 10^{-7} M (mean ± SE) and the number of binding sites 3840 ± 1760 per platelet (mean ± SE).

TABLE I

Effect of Heparin on Antithrombin III Binding

	Amount of Heparin, nM			
	0	60	180	300
	% bound [^{125}I] AT III per 10^9 platelets			
without cold AT III heparin added later	1.82	1.79	1.71	1.75
cold AT III (500 nM) preincubated with heparin	0.94	1.26	1.54	1.89

Heparin greatly accelerates the complex formation between AT III and activated serine proteases (6). The binding of heparin to AT III is associated with a change in the conformation of the inhibitor protein (15,16,17). To determine whether induction of a conformational change in the inhibitor protein will alter its binding to platelets, we measured the ligand activity of AT III preincubated with 3 different concentrations of heparin (Table I). Platelets incubated for 2 min with 1.6 nM [^{125}I] AT III before addition of heparin showed no change in binding activity. Unlabeled AT III at a concentration of 500 nM produced a 50% reduction in the binding of [^{125}I] AT III. However, preincubation of the nonradioactive AT III for 10 min with heparin diminished its ability to suppress [^{125}I] AT III binding. This effect was strictly dose-dependent. After 10 min incubation of AT III with heparin at a molecular ratio of 1, approximately 90% of AT III was identified as AT III·heparin complex. We interpret this result as further evidence for the specificity of the binding process.

Our studies have shown the existence of specific receptor sites for AT III on the platelet surface. In methodology and interpretation of results we have adhered to established procedures for the binding of labeled protein to cells. Although the problems which such methods entail can hardly ever be completely

resolved, nonspecific binding as well as preservation of a functional active protein after the labeling process were determined. The problem most resistant to corrective measures is the perturbing influence that the method of separating bound from free ligand exerts on the binding process. The method we have used included one washing step of the labeled cells. This makes interpretation of equilibrium conditions of binding difficult.

The physiological significance of the binding of AT III to platelets is not clear yet. In preliminary experiments we have obtained evidence for accelerated neutralization of thrombin by platelet-bound AT III.

ACKNOWLEDGEMENT

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