Mechanism of Thrombin Binding to Endothelial Cells

By Pál I. Bauer, Raymund Machovich, Péter Arányi, Kálmán G. Bükí, Éva Csonka, and István Horváth

The interaction of human α-thrombin with mini-pig aortic endothelial cells was studied using 125I-labeled enzyme. Equilibrium between bound and free thrombin was attained within 1 min, and the Klotz-Hunston equations indicated two populations of binding sites. Approximately 30,000 sites/cell belonged to the high-affinity class with a KD of about 3 x 10^{-7} M. Modification of two lysine residues of thrombin with pyridoxal-5'-phosphate (PLP₂-thrombin) destroyed the high-affinity binding and about three-fourths of the low-affinity bindings. When the lysine residue of thrombin involved in heparin binding was protected with heparin against chemical modification (PLP₁-thrombin), the modified enzyme remained similar to the native one with respect to cellular binding, with some loss of low-affinity binding only. Heparin, in a tenfold molar excess to enzyme, inhibited the binding of the native as well as the PLP-thrombin, whereas it did not influence the interaction between PLP₁-thrombin and the cell. Since heparin might interfere with both the enzyme and the cell, the binding of heparin to endothelial cells was also examined. The results revealed that 1H-heparin also bound to cells. This binding was characterized by a KD of 3 x 10^{-7} M, approximately 10⁴ sites/cell. Furthermore, thrombin bound to endothelial cells was released by antithrombin III. On the basis of these and other data in the literature, a model is proposed for the mechanism of the binding of thrombin to endothelial cells.

THROMBIN, as a multifunctional enzyme, plays an important role in hemostasis and thrombosis. One of its functions is the binding to endothelial cells, resulting in several effects on these cells. There are changes in the synthesis and the release of prostacyclin and fibronectin, in the release of adenine nucleotides, protein C activation, mitogenesis, and plasminogen activator activity. The binding also influences the inactivation of thrombin by antithrombin III. The strong interaction between endothelial cells and thrombin has been well documented. After a rapid and reversible binding, however, a slower interaction of thrombin with endothelial cells takes place. The second step is irreversible, active-site specific, and associated with the formation of a covalent bond between thrombin and a 30,000 mol wt protein of endothelial cells. Under equilibrium conditions, thrombin binds to the cells with high and low affinity, and the active site of the enzyme is not involved in this initial interaction.

Thrombin also binds to heparin. This can be influenced by chemical modification of arginine or lysine residues of the protein. In addition to being essential for the mechanism of inactivation of thrombin, this interaction also provides a means to study the nature of binding of the enzyme to endothelial cells.

The aim of the present investigation was to elucidate some structural requirements of the binding of thrombin to endothelial cells, as well as the effects of heparin and antithrombin III on the interaction of thrombin with endothelial cells. For this purpose, the heparin binding site of thrombin was modified with pyridoxal-5'-phosphate, and its effect on endothelial cell binding was examined. Data show that modification of a lysine residue, involved in the heparin binding site of thrombin, results in a marked loss of ability of enzyme to bind to endothelial cells. Similar results were obtained in a study of the platelet–thrombin interaction. Thus, heparin prevents the interaction of thrombin with cells by blocking the high-affinity binding site. Furthermore, the enzyme bound to endothelial cells is available for antithrombin III.

MATERIALS AND METHODS

Fibrinogen (human, grade I), Sephadex G-25, and Sulphopropyl-Sephadex C-50 were purchased from Kabi AB, Stockholm, Sweden, and Pharmacia Fine Chemicals, Uppsala, Sweden, respectively. Heparin (bovine intestine, with a specific activity of 165 U/mg) was obtained from G. Richter Pharmaceuticals, Budapest, Hungary. Fetal calf serum was the product of Seromod G.m.b.H., F.R.G. 125I-Nal (tracer free) and 3H-NaBH₄, (with a specific activity of 210 TBq/mole) were purchased from the Isotope Institute of the Hungarian Academy of Sciences, Budapest, Hungary. Other chemicals were the products of Reanal Fine Chemicals, Budapest, Hungary.

Human thrombin was isolated from Cohn Fraction III, as published by Fenton et al. α-Thrombin was separated by chromatography on Sulphopropyl-Sephadex C-50 as described by Lundblad et al. Thrombin activity was assayed with fibrinogen substrate as described earlier. The specific activity of the α-thrombin obtained was approximately 3200 NIH units/mg of protein.

Antithrombin III, purified from human plasma, was the product of the American Red Cross Fractionation Center, Bethesda, Md. The final product of inhibitor protein, higher than 95% purity, was

From the 2nd Institute of Biochemistry and 2nd Institute of Pathology, Semmelweis University Medical School, Budapest, Hungary.

Supported in part by the Hungarian Ministry of Health Grant 17/1-10/349 and TPB 70.270/82. Antithrombin III used in these studies was provided by the American Red Cross National Fractionation Center under National Institutes of Health Grant HL 13881.

A preliminary report of this work was presented at the 22nd Meeting of the Hungarian Biochemical Society, Debrecen. Acta Biochim Biophys Acad Sci Hung 17:66, 1982 (abstract).

Submitted June 22, 1982; accepted September 16, 1982.

Address reprint requests to Dr. Raymund Machovich, 2nd Institute of Biochemistry, Semmelweis University Medical School, Puskin 9, H-1444 Budapest, P.O. Box 262, Hungary.

e) 1983 by Grune & Stratton, Inc.

0006-4971/83/6102-0023$01.00/0

Blood, Vol. 61, No. 2 (February), 1983
freeze-dried, and before experimentation, dissolved in 0.15 M NaCl containing 0.01 M phosphate buffer, pH 7.0.

Chemical modification of protein was carried out with pyridoxal-5′-phosphate as detailed by Griffith.22 Briefly: 400 μg 125I-thrombin/ml was incubated either in the absence or in the presence of 25 μM heparin, with 0.5 mM pyridoxal-5′-phosphate in 0.05 M triethanolamine buffer, pH 8, containing 0.1% PEG 6000, for 120 min, at 25°C. After reduction by NaBH₄, the excess of the reactants, as well as heparin, were separated from thrombin by gel-filtration on a Sephadex G-75 column equilibrated with 0.5 M NaCl. Incorporation of pyridoxal-5′-phosphate was determined spectrophotometrically using ε₉₀₀ = 9000 M⁻¹ cm⁻¹.

Iodination of thrombin was carried out according to the chloramine-T method at 22°C for 30 sec in a volume of 1 ml containing 1 mg protein. The 125I-labeled thrombin was separated from free 125I by Sulphopropyl-Sephadex C-50. The specific radioactivity incorporated into the enzyme was approximately 1–7 x 10⁶ dpm/mg protein. The iodination technique caused an about 80% inactivation towards fibrinogen substrate, whereas the esterolytic activity of the enzyme was not impaired. Iodination was also carried out with lactoperoxidase, and the results were similar to the findings obtained by the chloramine-T method.

Protein concentrations were determined according to the method of Lowry et al.24 with bovine serum albumin as a standard.

Heparin was tritiated as described earlier.30 After reaction with 3H-NaBH₄, reduced heparin was dialyzed against H₂O (volume ratio of heparin:dialyzate 1:1000) at 4°C for 24 hr, followed by Sephadex G-100 gel-filtration. The specific activity of 3H-heparin obtained was approx. 1.4 x 10⁶ dpm/mg (0.35 MBq/mg). The radioactivity was measured in a Beckman LS 355 liquid scintillation spectrometer.

Molar concentrations for thrombin, antithrombin III, and heparin were calculated using the respective molecular weights of 36,000,26 65,000,41 and 11,000.12

Mini-pig aortic endothelial cells were cultured as described,33 using Dulbecco’s modified Eagle medium supplemented with 20% fetal calf serum.

Binding studies were carried out in a 0.5-ml reaction mixture of Dulbecco’s medium supplemented with 2.5 mg/ml albumin and 10 mM Hepes, pH 7.4, in a vial containing 2–8 x 10⁵ cells. After incubation of cells with 125I-labeled protein or 3H-labeled heparin at room temperature for various times at the concentration indicated in the legends to the figures, the cells were washed 3 times within 5–6 sec with cold phosphate-buffered saline, pH 7.4, containing 2.5 mg/ml albumin. Finally, 4 ml of Triton X-100-toluene cocktail (1:2 v/v) was applied to the vial for determination of radioactivity.

The binding data were calculated by use of the Klotz-Hunston equations.

**RESULTS**

**Binding of Lysine-Modified Thrombin to Endothelial Cells**

Thrombin was modified with pyridoxal-5′-phosphate in the presence or the absence of heparin, yielding either a heparin-protected phosphopyridoxylated-thrombin containing approximately 1 mole pyridoxal-phosphate/mole of thrombin (referred to as PLP-thrombin), or an unprotected one containing about 2 moles of pyridoxal-phosphate/mole of thrombin (PLP₂-thrombin).

Binding of thrombin to endothelial cells was determined as a function of enzyme concentration over a range of 0.05–50 nM. Incubations were carried out at 25°C for 7 min, since equilibrium between bound and free thrombin was attained within 1 min (Fig. 1, insert). Binding curves are shown in Fig. 1. The analysis of the binding data revealed two distinct classes of binding sites for native (control) and PLP-thrombin as well. The PLP₂-thrombin (thrombin modified in the absence of heparin) had only a single class of binding sites with a K₅₅ similar to the low affinity one of the native enzyme. These results are summarized in Table 1. Data show that control-thrombin and PLP-thrombin (with one lysine blocked) have similar binding sites with approximately the same K₅₅. On the other hand, thrombin modified in two lysine residues lost the high-affinity binding site. The data on the low-affinity binding sites (second phase of the curves in Fig. 1) are less reliable; therefore, they are not discussed here.

**Effect of Heparin on Binding to Endothelial Cells of Native and Modified Thrombins**

Native (control) as well as modified thrombins were preincubated in the presence of various concentrations of heparin. Thereafter, their binding to endothelial cells was determined. The results are demonstrated in Fig. 2. The binding of the native enzyme as well as of

![Graph showing binding of 125I-phosphopyridoxylated thrombin to endothelial cells.](https://example.com/graph.png)
PLP-thrombin was inhibited by heparin, whereas the interaction of the PLP2-thrombin with endothelial cells was not altered by heparin.

**Binding of Heparin to Endothelial Cells**

Since heparin might influence the interaction between thrombin and endothelial cells by binding to thrombin or endothelial cells, we studied the binding of 3H-labeled heparin to endothelial cells. Incubations were carried out at 25°C for 300 min and heparin concentrations varied between 0 and 50 μM. We demonstrated that endothelial cells bind 3H-heparin. The cells had about 1.4 x 10^6 heparin binding sites, with a K_d of 3 x 10^{-7} M as calculated from the Scatchard plot (Fig. 3). However, this interaction developed very slowly; it took hours rather than minutes at 25°C to reach equilibrium. Therefore, we suggest that it is the thrombin–heparin and not the cell–heparin interaction that interferes with the binding of thrombin to these cells.

**Antithrombin III Releases Thrombin Bound to Endothelial Cells**

Endothelial cells were incubated for 10 min at 25°C with 0.25 nM ^125^I-thrombin; thereafter, cells were washed 3 times with albumin containing PBS, and 0.5 ml of incubation medium or incubation medium supplemented with antithrombin III was added. At various intervals, as indicated in Fig. 4, the medium was removed with suction, and cell-bound radioactivity was determined. Dissociation of bound thrombin had a half-time of 1 min, and antithrombin III, in an excess molar ratio to thrombin, increased the dissociation rate of thrombin 1.6-fold. Previous modification of the active center of thrombin by phenylmethylsulfonyl fluoride abolished the effect of antithrombin III on the dissociation rate (data not shown).

**DISCUSSION**

Several previous studies used chemically modified thrombins to probe the structure of the enzyme neces-
necessary to interact with fibrinogen, platelet, and/or heparin. Thrombin, modified at the active center, binds to heparin, indicating that the active site of thrombin is not required for binding. Modification of arginine or lysine residues of thrombin, on the other hand, resulted in loss of fibrinogen and/or heparin binding of the enzyme, as well as of the interaction with platelets.

According to our present findings, one lysine residue, involved in the heparin binding site of thrombin, also participates in the binding of the enzyme to endothelial cells. Both interactions are tight, i.e., the $K_d$ for thrombin–endothelial cell is approximately $3 \times 10^{-8} \text{M}$ under our experimental conditions, whereas that for the thrombin–heparin interaction is $2 \times 10^{-9} \text{M}$. Heparin binds to endothelial cells also, with a $K_d$ of $3 \times 10^{-7} \text{M}$. The effect of heparin on the thrombin–endothelial cell interaction seems to be a consequence of the binding of heparin to thrombin, rather than to the cells (see Results).

The difference between high and low affinity binding of thrombin is not clear at present in terms of endothelial cell receptor(s). The results obtained here and elsewhere suggest a model, however, for the heparin binding site of thrombin and its relation to other binding sites, e.g., fibrinogen, platelets, and endothelial cells. The fibrinogen binding site extends over a large portion of the surface of the enzyme, which overlaps with the low-affinity platelet binding site. The high-affinity platelet binding site is overlapping with the heparin binding site, which contains a specific lysine residue. The same lysine is necessary for the high-affinity endothelial binding site as well. In both the fibrinogen and heparin binding sites, arginine residues are also involved. As to the localization of the low-affinity endothelial binding site, no reliable data are available at present. Its proposed function, namely the irreversible, active-site-specific, and covalent linkage of thrombin to a 30,000 mol wt protein on endothelial cells, does not seem to play a physiologic role.

Thrombin, inactivated with diisopropylfluorophosphate, binds to endothelial cells with an affinity similar to that of the active one. Thus, the enzyme function of the thrombin bound to cells is preserved, enabling it to participate in biologic reactions, like clot formation. Our finding, however, suggests that the active site of the bound enzyme is available for antithrombin III in circulating blood, since antithrombin III increases the release of endothelial-cell-bound thrombin.

It is interesting that this increased dissociation rate could be observed clearly within half a minute of incubation with antithrombin III, i.e., faster than it could be anticipated on the basis of the rate of interaction of antithrombin III with purified thrombin in the absence of cells. The latter is effectively accelerated by heparin. Thus, we suggest that the endothelial cell membrane, similarly to the mechanism described for heparin, induces a conformational change of thrombin resulting in a rate-enhancement of inactivation of the enzyme by antithrombin III. This hypothesis is in agreement with the data of Lollar and Owen.

ACKNOWLEDGMENT

We thank Vera Pichler for technical assistance.

REFERENCES

Mechanism of thrombin binding to endothelial cells

PT Bauer, R Machovich, P Aranyi, KG Buki, E Csonka and I Horvath