PLATELET ANTIHEPARIN PROTEINS AND ANTITHROMBIN III INTERACT WITH DIFFERENT BINDING SITES ON HEPARIN MOLECULE

Stefan NIEWIAROWSKI, Boguslaw RUCINSKI, Pranee JAMES and Ulf LINDAHL+

Specialized Center for Thrombosis Research, Department of Medicine and Physiology, Temple University Health Sciences Center, Philadelphia, PA 19140, USA and *+Department of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences, The Biomedical Center, S-75123 Uppsala, Sweden*

Received 4 April 1979

1. Introduction

Heparin acts as an anticoagulant by binding to antithrombin III and by accelerating the rate at which this protein inactivates thrombin and other serine proteases of the intrinsic clotting system [1]. Purified commercial preparations of heparin contain two species of the molecules that can be separated on the basis of their affinity to antithrombin III [2–4]. The high affinity heparin (HA-heparin) binds to antithrombin III in a 1:1 stoichometry with K_a 8×10^7 M [5]. Low-affinity heparin (LA-heparin) presumably binds to the same site of antithrombin III with an association constant 100-1000 times lower than that determined for the antithrombin III–HA-heparin binding [6]. The anticoagulant activity of LA-heparin is 10–100 times lower than that of HA-heparin, the ratio varying with the assay system used [7]. There is a good evidence that lysine may function as a heparin-binding site on antithrombin III molecule [1]. It was suggested [8] that a specific tetrasaccharide with a *N*-sulfated glucosamine at its reducing end and equivalent amounts of glucuronic and iduronic acid is a specific structural requirement for the interaction of heparin with antithrombin III. More recent data indicate that non-sulfated L-iduronic acid represents a structural feature that is essential for the anticoagulant activity of heparin (U.L., Bäckström, Höök, Thunberg, Fransson and Linker, unpublished data).

Stimulated platelets secrete two heparin binding proteins: platelet factor 4 (PF_4) and low affinity platelet factor 4 (LA-PF_4) [9]. Platelet factor 4 is one of the most potent agents neutralizing the anticoagulant activity of heparin. It is a single chain low molecular weight (7800) polypeptide [10–12], with a high affinity for heparin and a lower affinity to such glycosaminoglycans as heparan sulfate and dermatan sulfate [13]. The lysine residues clustered at the C-terminus of PF_4 are apparently involved in the interaction with heparin [14]. Evidence has been presented that PF_4 does not interfere with thrombin neutralization by antithrombin III [15].

The purpose of this study was to compare binding of platelet antiheparin proteins and of antithrombin III to LA- and HA-heparin. Both species of heparin as well as unfractionated pig mucosal heparin were attached to CNBr–Sepharose. Then adsorbtion of three proteins (antithrombin III, LA-PF_4 and PF_4) and their patterns of elution from columns containing the insolubilized heparins were compared. Our data indicate that antithrombin III and platelet antiheparin proteins bind to different sites on heparin molecule.

2. Materials and methods

Unfractionated heparin obtained from pig intestinal mucosa was supplied by Sigma Co, (St Louis, MO). This heparin contained 150 USP units/mg. Low affinity and high affinity heparins were obtained from pig mucosal heparin by affinity chromatography on antithrombin III–Sepharose as in [2]. The anticoagulant activities of HA-heparin and LA-heparin were 19 and 285 units/mg, respectively. The HA- and LA-heparin preparations were both polydisperse and overlapped on Sephadex G-100 gel chromatography [16]. The peak

K_{av} values were 0 16 and 0 26, respectively, corresponding to mol wt ~19 000–20 000 and 14 000–15 000 All three species of heparin were coupled to CNBr–Sepharose (Pharmacia, Uppsala) in the proportion of 10 mg heparin/1 g CNBr–Sepharose [17]

Washed platelets were prepared as in [18] and suspended in Tyrode–albumin solution Ionophore A 23187 was added to platelet suspension, 0 65 μg/10^9 cells After 3 min incubation at 37°C platelet aggregates were centrifuged at 2000 × *g* for 10 min The supernatant obtained from 4 × 10^9 platelets/1 ml contained about 1 3 mg total protein, 100 μg LA-PF_4 and 40 μg PF_4 per ml

PF_4 and LA-PF_4 were prepared as in [9] Both preparations were homogenous as tested in SDS–polyacrylamide gel electrophoresis

Antithrombin III purified by the method in [19] was a gift of Dr R Rosenberg (Harvard University, Boston, MA)

Radioiodination of LA-PF_4, PF_4 and antithrombin III was done with $Na^{125}I$ and chloramine T using a modification of the method of Hunter [9,20] The specific radioactivities of LA-PF_4, antithrombin III and PF_4 were 30 μCi/μg protein, 30 μCi/μg protein and 10 μCi/mg protein, respectively Before application on the columns all three labeled tracers were diluted by unlabeled proteins in the proportion of 1 4000

Protein was determined by the Lowry method [21]

3 Results and discussion

In the first series of experiments three radiolabeled proteins ([^{125}I]antithrombin III, [^{125}I]PF_4 and LA-[^{125}I]-PF_4) were applied on three types of heparin–Sepharose columns and their elution patterns were followed by measuring the ^{125}I radioactivity In the second series of experiments supernate from platelets aggregated by ionophore was applied on the same columns and the elution patterns of PF_4 and LA-PF_4 were followed by radioimmunoassay The results of these experiments are presented in the table 1 It can be seen that under these experimental conditions [^{125}I]antithrombin III was retained on unfractionated heparin–Sepharose and on HA-heparin–Sepharose, but it was not retained on LA-heparin–Sepharose Elution of [^{125}I]antithrombin III occurred at 0 2–0 75 M NaCl, with a 'peak' at 0 45–0 65 M LA-[^{125}I]PF_4 and [^{125}I]PF_4 were adsorbed on insolubilized unfractionated heparin, LA-heparin, and HA-heparin, respectively, and it was found that the elution pattern of either protein from these columns was independent of the type of heparin Similar results were obtained by applying the platelet supernate on the columns containing all three types of insolubilized heparin It should be pointed out, however, that LA-PF_4 in platelet supernate was completely retained in all three columns while in experiments with LA-[^{125}I]PF_4 ~30% of the radioactive material was not retained on heparin–agarose equilibrated with 0 15 M NaCl An excess of anti-LA-PF_4 antibody precipitated only 44% of this material which probably corresponded to partially denatured protein 95% material eluted with 0 4 M NaCl was precipitated in the same condition The major portion of either LA-[^{125}I]PF_4 or unlabeled LA-PF_4 was eluted at 0 4 M NaCl from all types of insolubilized heparins PF_4 showed much higher affinity to heparins, but again no differences between elution from LA-heparin and HA-heparin were observed It can be seen, however, that purification and iodination slightly decreased binding affinity of PF_4 to all three insolubilized heparins Figures 1 and 2 show representative experiments in which supernates from aggregated platelets were applied on LA-heparin–agarose and HA-heparin–agarose Both LA-PF_4 and PF_4 showed a distinct elution pattern, however, the type of heparin coupled to sepharose did not influence the binding affinity of any of the proteins

The above experiments demonstrated that LA-heparin and HA-heparin show equal ability to interact with the platelet antiheparin proteins LA-PF_4 and PF_4 Since the two types of heparin are differentiated solely on the basis of affinity for antithrombin III these results indicate that the specific structure required for interaction with antithrombin III differs from that required for interaction with platelet antiheparin proteins It appears that PF_4 and antithrombin III bind to different sites on the heparin molecule More recently Handin, Jordan and Rosenberg (personal communication) reached similar conclusion by measuring intrinsic fluorescence of antithrombin III interacting with heparin and PF_4

Our data are in agreement with the reports that LA-PF_4 [9] and βTG [22] show low affinity binding to heparin LA-PF_4 appears to be a precursor of

Table 1
Elution of antithrombin III, LA-PF_4 and PF_4 from heparin–Sepharose columns[a]

Ligand	Molar concentrations of NaCl required for ligand elution[b]		
	Unfractionated heparin–Sepharose	LA-heparin–Sepharose	HA-heparin–Sepharose
[^{125}I]Antithrombin III	0.45 (0.2–0.75)	0.15	0.65
LA-[^{125}I]PF_4[c]	0.35 (0.2–0.4)	0.35 (0.25–0.5)	0.4 (0.25–0.45)
[^{125}I]PF_4	1.2 (0.95–1.25)	1.05 (0.8–1.2)	1.15 (0.85–1.25)
LA-PF_4[d]	0.4 (0.25–0.6)	0.4 (0.2–0.55)	0.35 (0.25–0.6)
PF_4[d]	1.35 (1.25–1.40)	1.4 (1.25–1.5)	1.4 (1.2–1.45)

[a] Samples were applied to 3 ml heparin–agarose columns. All columns were equilibrated with 0.15 M NaCl–0.05 M Tris (pH 8.0) except for columns on which [^{125}I]PF_4 was applied. Those columns were equilibrated with 0.4 M NaCl–0.05 M Tris (pH 5.0). The material not retained on the columns was washed out with this buffer. Then elution of bound proteins was achieved by applying a continuous gradient of NaCl. Each collected sample was 1.4 ml. The concentration of NaCl was determined by measurement of conductivity. In experiments with radiolabeled proteins, the ^{125}I radioactivity of each sample was measured in an Intertechnique gamma counter. In all other experiments the levels of PF_4 and LA-PF_4 were determined by specific radioimmunoassay [9]

[b] The values in brackets refer to the ranges of NaCl concentrations at which the specific activity was eluted. The single values indicate NaCl concentration at which 'peak' activity was eluted

[c] In all experiments with LA-[^{125}I]PF_4, ~30% of the radioactive material failed to bind to heparin–Sepharose equilibrated with 0.15 M NaCl

[d] Supernate released from washed platelets aggregated by ionophore A23187 was used as a source of LA-PF_4 and PF_4

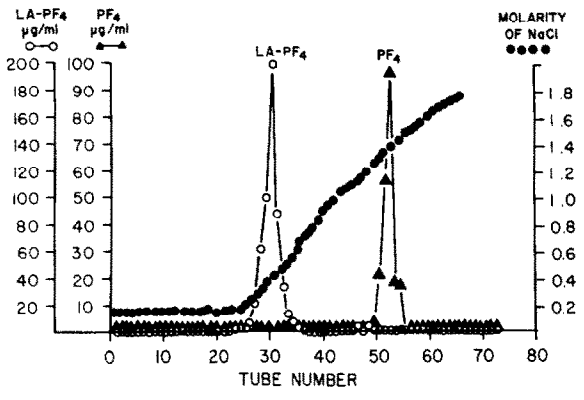

Fig.1. Elution of LA-PF_4 and PF_4 from LA-heparin–Sepharose. The volume of the sample (supernate after platelet aggregation) applied on the column was 12.0 ml.

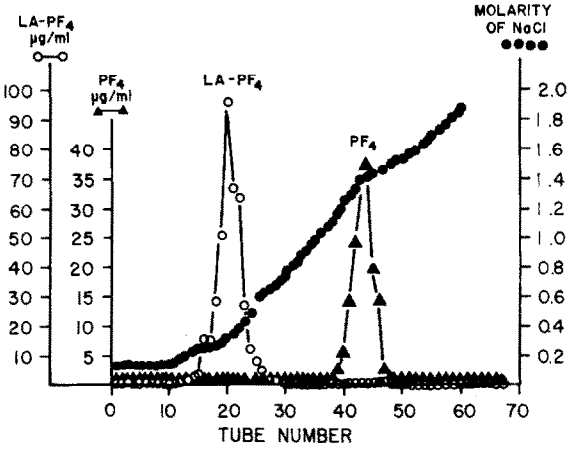

Fig.2. Elution of LA-PF_4 and PF_4 from LA-heparin–Sepharose. The volume of the sample (supernate after platelet aggregation) applied on the column was 12.0 ml.

β-thromboglobulin (βTG), another platelet secretory protein βTG shows deletion of 4 amino acids (Asn–Leu–Ala–Lys) at the N-terminal end of LA-PF_4 The remaining amino acid sequences of LA-PF_4 and βTG appear to be identical [9] The complete amino acid sequence of βTG (mol wt 8800) shows ~50% homology with the sequence of PF_4 [23] Since both proteins show typical clusters of lysine at their C-terminal ends, it can be suggested that lysine is also involved in the interaction of LA-PF_4 or βTG with heparin Preliminary experiments performed in our laboratories indicate that LA-PF_4 also binds to heparan sulfate at physiological ionic strength

It was demonstrated [24] that HA- and LA-heparin have equal ability to interact with lipoprotein lipase and that the specific heparin structure required for interaction with antithrombin III differs from that required for interaction with lipoprotein lipase The relationship between lipoprotein lipase and PF_4 binding sites on the heparin molecule remains to be determined

Acknowledgements

This work has been supported by grants from National Institutes of Health (HL-14227, HL-15226, HL-05976), from the Swedish Medical Research Council (grant no 2309) and from AB Kabi, Stockholm The technical assistance of Mr R Piroschak is gratefully acknowledged

References

[1] Rosenberg, R D (1977) Sem Hematol 14, 427–440
[2] Hook, M , Bjork, I , Hopwood, J and Lindahl, U (1976) FEBS Lett 66, 90–93
[3] Lam, L H , Silbert, J E and Rosenberg, R D (1978) Biochem Biophys Commun 69, 570–577
[4] Andersson, L O , Barrowcliffe, T W , Holmer, E , Johnson, E A and Sims, G E C (1976) Thromb Res 9, 575–583
[5] Nordenman, B , Danielson, A and Bjork, I (1978) Eur J Biochem 90, 1–6
[6] Danielson, A and Bjork, I (1978) Eur J Biochem 90, 7–12
[7] Teien, A N , Abildgaard, U , Hook, M and Lindahl, U (1977) Thromb Res 11, 107–117
[8] Rosenberg, R D , Armand, G and Lam, L (1978) Proc Natl Acad Sci USA 75, 3065–3069
[9] Rucinski, B , Niewiarowski, S , James, P , Walz, D A and Budzynski, A Z (1979) Blood 53, 47–62
[10] Deuel, T F , Keim, P S , Farmar, M and Heinrikson, R L (1977) Proc Natl Acad Sci USA 74, 2256–2258
[11] Hermodson, M , Schmer, G and Kurachi, K (1977) J Biol Chem 252, 6276–6279
[12] Walz, D A , Wu, V Y , De Lamo, R and McCoy, L E (1977) Thromb Res 11, 893–898
[13] Barber, A J , Kaser-Glausmann, R , Jakabova, M and Luscher, E F (1977) Biochim Biophys Acta 286, 312–329
[14] Handin, R I and Cohen, H J (1976) J Biol Chem 251, 4273–4282
[15] Niewiarowski, S , Fabriszewski, R and Poplawski, A (1965) Thromb Diath Haem 141, 490–499
[16] Laurent, T C , Tengblad, A , Thunberg, L , Hook, M and Lindahl, V (1978) Biochem J 175, 691–701
[17] Axen, R , Porath, J and Ernback, S (1967) Nature 214, 1302–1304
[18] Mustard, J F , Perry, D W , Ardlie, N C and Packham, M A (1972) Br J Haematol 22, 193–204
[19] Rosenberg, R D and Damus, P S (1973) J Biol Chem 248, 6490–6505
[20] Hunter, W M (1973) in Handbook of Experimental Immunology, (Weir, D M ed) pp 17 1–17.36, Blackwell, Oxford
[21] Lowry, O H , Rosebrough, N J , Farr, A L , Randall, R J (1951) J Biol Chem 193, 265–275
[22] Moore, S and Pepper, D S (1976) in Platelet Physiology, (Gordon, J L ed) pp 293–311, Elsevier/North-Holland Biomedical Press, Amsterdam, New York
[23] Begg, S , Pepper, D S , Chesterman, C N and Morgan, F Y (1978) Biochemistry 17, 1739–1744
[24] Bengtsson, G , Olivecrona, T , Hook, M and Lindahl, U (1977) FEBS Lett 79, 59–63