Biochem. J. (1981) **196**, 649–651
Printed in Great Britain

Dansyl (5-dimethylaminonaphthalene-1-sulphonyl)-heparin binds antithrombin III and platelet factor 4 at separate sites

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(*Received 13 February 1981/Accepted 3 March 1981*)

Antithrombin III binds to, and thereby augments the fluorescence of, dansyl-(5-dimethylaminonaphthalene-1-sulphonyl)-heparin; platelet factor 4 binding to the fluorescent heparin has little of this effect. Competition studies in which antithrombin III competes with platelet factor 4 for heparin binding demonstrate that heparin can simultaneously bind both proteins.

The major heparin-neutralizing factor of human blood is present in the α-granules of platelets and has been referred to as 'platelet factor 4'. It is a secretable low-molecular-weight (7800) protein (Hermodson *et al.*, 1977) with a high affinity for heparan (Handin & Cohen, 1976).

The exact molecular mechanism of the anti-heparin effect of platelet factor 4 has not been elucidated. Since heparin is known to act as an anti-coagulant by tightly binding to the natural proteinase inhibitor antithrombin III, and consequently accelerating the neutralization of thrombin and other serine proteinases involved in blood coagulation (Rosenberg, 1977), one plausible hypothesis is that platelet factor 4 competes with antithrombin III for the same binding site on heparin. Alternatively, in view of evidence suggesting that heparin potentiation of antithrombin III-mediated thrombin inhibition may require simultaneous binding of heparin to both molecules (Laurent *et al.*, 1978), it is possible that platelet factor 4 may prevent the binding of heparin to serine proteinases during the formation of heparin–antithrombin III–serine-proteinase ternary complexes.

Previously, Niewiarowski *et al.* (1979) demonstrated that platelet factor 4 binds equally well to heparin fractions of low or high affinity for antithrombin III, whereas antithrombin III preferentially binds to a heparin fraction (designated 'high-affinity heparin') representing approximately one-third of unfractionated heparin; those authors suggested that platelet factor 4 and antithrombin III have different binding sites on heparin. In the present paper I report competition experiments involving the two proteins and a fluorescent-labelled heparin. The data provide direct evidence that platelet factor 4 and antithrombin III bind to separate sites on heparin.

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Materials and methods

Porcine mucosal heparin was purchased from Sigma (lot 96C-0093; specified anticoagulant activity 158.7 U.S. Pharmacopeia units/mg). Heparin concentration was assayed by the carbazole reaction (Bitter & Muir, 1962) with D-glucuronolactone as the standard and by using a previously determined mean number-averaged molecular weight ($\bar{M}_n$) of 17400, and anticoagulant activity was determined by a thrombin amidolytic assay with a chromogenic substrate as previously described (Piepkorn *et al.*, 1980). Bovine antithrombin III (provided by Dr. G. Schmer of this Institution) was isolated by the method of Thaler & Schmer (1975), and the protein concentration was determined by absorbance measurements [$A_{280}^{1\%} = 6.0$ (Kurachi *et al.*, 1976)]. Human platelet factor 4 (gift from Dr. G. Schmer) was isolated by the method of Hermodson *et al.* (1977).

Heparin was partially *N*-desulphated (5.5% free amino groups) by solvolysis of the pyridinium salt and then labelled with dansyl (5-dimethylamino-naphthalene-1-sulphonyl) chloride (Pierce Chemical Co.) to the extent of 1.07 mol of dansyl groups/mol of heparin, as previously described (Piepkorn *et al.*, 1980). The labelled heparin (77 units/mg) retained about 50% of the anticoagulant activity of the starting material (158 units/mg).

0306-3283/81/050649-03$01.50/1

The previously reported enhancement of dansyl-heparin fluorescence on antithrombin III binding (Piepkorn *et al.*, 1980) and the minimal effects of platelet factor 4 alone on the dansyl-heparin spectrum (see the Results section) permitted competition experiments in which antithrombin III competed with platelet factor 4 for dansyl-heparin binding. Specifically, dansyl-heparin was added to separate mixtures containing either antithrombin III or platelet factor 4, or both, in standard buffer (0.05M-Tris/0.1M-glycine/0.03M-NaCl, pH 7.4). Portions (2ml) of each mixture (see Table 1 for concentrations) were then excited at the apparent observed maximum (340nm, 25±0.5°C) in a Baird-Atomic SF-100 fluorescence spectrophotometer and the emission spectra recorded over 480–560nm; measurements were made at the observed emission maxima.

Results and discussion

As previously reported (Piepkorn *et al.*, 1980), heparin, when partially *N*-desulphated and then labelled with the fluorescent dansyl group, has an observed absorption maximum at 340nm and an observed emission maximum at 520nm. On antithrombin III binding, there is a 2–2.5-fold saturable fluorescence augmentation, with a 10nm or greater blue spectral shift; this effect, which is presumably due to a protein-dependent hydrophobic perturbation of the microenvironment about the dansyl groups, can be abrogated by competition with excess unlabelled heparin (Piepkorn *et al.*, 1980). Fig. 1 and Table 1 (line 3) show, with non-saturating concentrations of antithrombin III, a 1.8-fold increase in dansyl-heparin fluorescence. When platelet factor 4 alone was added at molar ratios greater than 1:1 with respect to heparin (Fig. 1, and Table 1, line 2), little effect on the dansyl spectrum was apparent (less than 10% change in maximal fluorescence). Two explanations are possible for the different effects of the two proteins. First, the smaller size of the platelet factor 4 molecule (mol.wt. 7800) compared with antithrombin III (mol.wt. 56600; Kurachi *et al.*, 1976) may create a less hydrophobic microenvironment for vicinal dansyl groups. Alternatively, the dansyl labels may be clustered on the heparin molecule in the region of the antithrombin III binding site, at a region separate from the platelet factor 4-binding site.

The disparate effects of the two proteins on dansyl-heparin binding proved exploitable in the competition experiments presented in Table 1, in which platelet factor 4 and antithrombin III were simultaneously mixed with heparin and thereby given an equal chance of heparin interaction. Mixtures 4–12 demonstrate that increasing platelet

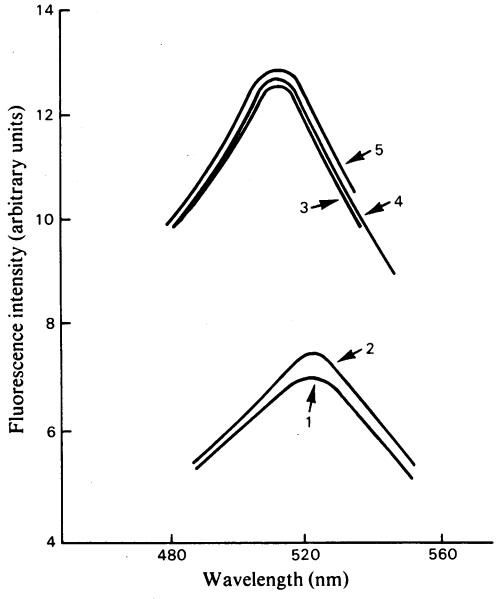

Fig. 1. *Emission spectra of dansyl-heparin on competitive binding of antithrombin III with platelet factor 4*
The numbered spectra correspond to the mixtures given in Table 1 with the specified excitation wavelength and exit slitwidths.

Table 1. *Competition of platelet factor 4 with antithrombin III for dansyl-heparin binding*

Mixture	[Reactant] (μmol/litre) Dansyl-heparin	Antithrombin III	Platelet factor 4	Relative fluorescence enhancement*
1	2.04	—	—	—
2	2.10	—	2.24	1.06
3	2.00	1.38	—	1.80
4	1.98	1.37	0.22	1.82
5	1.97	1.36	0.45	1.83
6	1.96	1.36	0.67	1.85
7	1.95	1.35	0.89	1.86
8	1.94	1.34	1.10	1.86
9	1.93	1.34	1.32	1.86
10	1.92	1.33	1.53	1.86
11	1.92	1.32	1.74	1.86
12	1.91	1.32	1.94	1.86

* F/F_0, where F is the fluorescence of dansyl-heparin in the presence of either or both proteins, and F_0 is the fluorescence of dansyl-heparin alone; means for two experiments are given. Emission intensity was scanned over the 520nm maxima, the excitation monochromator was set at 340nm, and exit slits were 6nm.

factor 4 concentrations, even at molar excess with respect to antithrombin III, resulted in less than a 5% change in the magnitude of the antithrombin III-dependent augmentation of dansyl-heparin fluorescence. Thus platelet factor 4 does not interfere with the heparin binding of antithrombin III when both proteins are simultaneously mixed with heparin. It is concluded that platelet factor 4 does not compete with antithrombin III for the same binding site, but rather must bind to a separate region of the heparin molecule. The alternative interpretation, that antithrombin III prevents platelet factor 4 binding to heparin, is unlikely, because of the higher affinity of platelet factor 4 than of antithrombin III for heparin, as evidenced by the greater salt molarities required for eluting platelet factor 4 compared with antithrombin III from columns of immobilized heparin (Niewiarowski *et al.*, 1979).

The present paper extends the observations of Niewiarowski *et al.* (1979), who showed that the salt molarities required for platelet factor 4 elution from separate columns of immobilized unfractionated heparin, immobilized heparin of low affinity for antithrombin III (designated 'low affinity heparin') and immobilized heparin of high affinity for antithrombin III ('high affinity heparin') are similar; antithrombin III, however, required greater salt concentrations for elution from the high-affinity-heparin column than from the low-affinity-heparin column. Separate binding sites on heparin for antithrombin III and platelet factor 4 are implied by these results and are directly confirmed by the present studies.

This information has importance in suggesting a molecular mechanism for the heparin-neutralizing effect of platelet factor 4 and indirectly for the anticoagulant effect of heparin. Although it is generally accepted that heparin anticoagulant potency correlates with its affinity for antithrombin III, and that heparin accelerates the serine-proteinase-neutralizing function of antithrombin III (Rosenberg, 1977), other evidence indicates that heparin must also bind the serine proteinase, within a ternary complex, for anticoagulant effect (Laurent *et al.*, 1978). Potentially, platelet factor 4 could act at three sites to achieve its antiheparin effect. First, it could directly compete with antithrombin III for heparin binding; the present results and those of Niewiarowski *et al.* (1979) make this possibility unlikely. Secondly, platelet factor 4 could block the formation of the stable antithrombin III–serine-proteinase complex, but Niewiarowski *et al.* (1965) have presented evidence that this does not occur. Finally, platelet factor 4 may competitively block the binding of heparin to the serine proteinase in the heparin–antithrombin III–serine-proteinase complex. Available evidence indicates that the latter is the likely mechanism.

This investigation was supported by Public Health Service Award no. IT 21-Am-07019-04. I thank Dr. Gottfried Schmer and Dr. David Lagunoff for helpful suggestions.

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