RELATIONSHIP BETWEEN ANTICOAGULANT ACTIVITY OF HEPARIN AND SUSCEPTIBILITY TO PERIODATE OXIDATION

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Received 25 September 1978

1. Introduction

Heparin and heparan sulphate chains have a common carbohydrate backbone composed of alternating β-D(or α-L) uronic acid and α-D-glucosamine residues joined 1 → 4. However, heparin is a potent anticoagulant while heparan sulphates have little or no activity [1]. The chemical distinction between the two glycans is based on differences in O- and N-sulphate content, in the ratio and distribution of N-acetyl and N-sulphate groups and in the L-iduronic acid to D-glucuronic acid ratio [2,3]. The anticoagulant activity of heparin appears to depend on chainlength, the distribution of N-sulphate groups and a high degree of O-sulphation [4]. The following requirements have been proposed:

(i) Molecular weight > 6000;
(ii) N-sulphate to N-acetyl ratio >1 provided that blocks of *N*-acetylglucosamine-uronic acid repeats are absent from the interior of the molecule,
(iii) O-Sulphate to hexosamine ratio >1,
(iv) L-Iduronic acid content >50% of total uronic acid.

It is generally assumed that O-sulphated L-iduronic acid residues are essential for biological activity [1]. These residues occur only in single sequences in heparan sulphate while heparin may contain up to 5 or 6 such units in consecutive order [2].

This work was initiated during a sabbatical (by L -Å F) at the Department of Biological Sciences, University of Lancaster, Lancaster, England

Heparin impedes blood coagulation by accelerating the reaction between antithrombin and serine proteases involved in the coagulation cascade [5]. With regard to their affinity for antithrombin commercial heparin preparations may be separated into high affinity (HA) and low affinity (LA) species with spec. act. ~20 and ~280 BP units/mg [6,7]. No apparent structural dissimilarity between the two heparin forms has been noted. An oligosaccharide fraction thought to represent the antithrombin-binding site of heparin was isolated after digestion of the HA-heparin–antithrombin complex with bacterial heparinase [8].

The gross composition of this fragment was similar to that of the rest of the molecule. More recently, a 'highly active' heparin preparation representing 8% of a commercial source and with spec. act. 360 BP units/mg has been isolated [9]. This material contains a tetrasaccharide sequence composed of equivalent amounts of L-iduronic acid, D-glucuronic acid, *N*-acetylglucosamine and N-sulphated glucosamine. Since this sequence is present in submolar proportions in 'relatively inactive' preparations it has been proposed that it represents a critical structural element required for anticoagulant activity.

Periodate oxidation of heparin-related molecules at low pH and temperature selectively oxidizes D-glucuronic acid associated with *N*-acetylglucosamine units [10] This report describes some preliminary studies using periodate oxidation as a probe for the elucidation of the structure–function relationships of heparin.

2. Materials and methods

The heparin used was of mucosal origin (Evans Medical Ltd, Liverpool, batch 5 H 488) and had an anticoagulant activity of 157 BP units/mg. Heparin by-products from beef lung were fractionated with cetylpyridinium (CP) chloride as in [11] CP-complexes obtained between 1.0 and 1.2 M NaCl were recovered.

Anticoagulant activity was measured by the Pritchard modification of the USP procedure [12].

Total uronic acid was determined with the carbazole [13] and orcinol methods [14]. L-Iduronic and D-glucuronic acid was estimated by ion-exchange chromatography [10] after acid hydrolysis, deaminative cleavage and rehydrolysis [15]

Periodate oxidation was carried out as in [10]. Two conditions were used: pH 3.0 and 4°C when D-glucuronic acid is selectively oxidized and pH 7.0 and 37°C when all non-sulphated uronic acids are oxidized. Oxyheparins were either reduced with sodium borohydride or cleaved by alkaline elimination [16].

Solvolytic N-desulphation was carried out as in [17]. N-Acetylation and N-sulphation were performed by the methods in [18], [19], respectively

3. Results

Heparin was subjected to periodate oxidation at pH 3.0 and 4°C (D-glucuronic acid) and at pH 7.0 and 37°C (all non-sulphated uronic acids), respectively. As shown in fig.1a, oxidation under the former

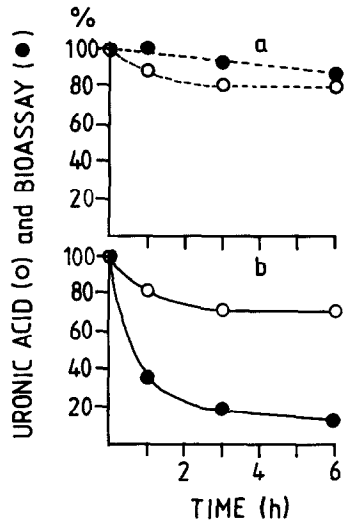

Fig.1 Uronic acid content (o) and anticoagulant activity (●) of heparin after (a) periodate oxidation at pH 3.0 and 4°C (- - - - -) and (b) periodate oxidation at pH 7.0 and 37°C (———) The destruction of uronic acids was monitored by carbazole measurements Oxyheparins were reduced prior to bioassay. The values are expressed as the percentage of zero-time controls

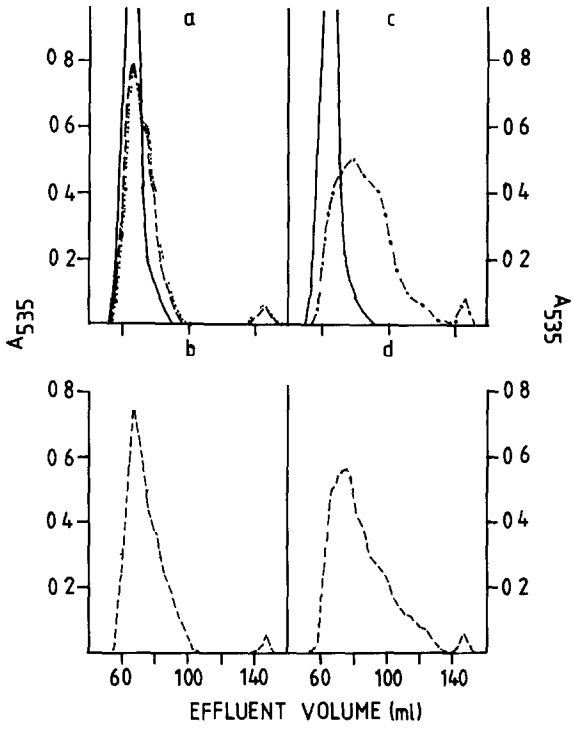

Fig 2. Gel chromatography on Sephadex G-50 of heparin and variously periodate-oxidized and degraded derivatives (20 mg each sample were applied) (a) heparin (———) and heparin that had been oxidized at pH 3 0 and 4°C for 3 h (- - - - - -) and 24 h (·), respectively, followed by alkaline cleavage, (b) N-desulphated and N-acetylated heparin that had been oxidized at pH 3.0 and 4°C for 24 h followed by alkaline cleavage, (c) heparin (———) and heparin that had been oxidized at pH 7.0 and 37°C for 6 h followed by alkaline cleavage (— —·—), (d) N-desulphated heparin that had been oxidized at pH 3.0 and 4°C followed by alkaline cleavage and N-acetylation Column Superfine grade, size, 12 mm × 180 cm, eluant, 0.2 M pyridine acetate (pH 5 0), elution rate, 10 ml/h, effluents were analyzed for uronic acid

Table 1
Uronic acid composition, anticoagulant activity and periodate susceptibility of heparins

Sample[a]	Uronic acid[b]		Anticoagulant activity	Destruction of uronic acid[c]	
	IdUA (%)	GlcUA (%)	(BP units/mg)	at pH 3, 4°C (%)	at pH 7, 37°C (%)
Heparin (H)	75 (80)	25 (20)	157	20	30
H-P13(Oh)-Red			120		
H-P13(3h)-Red			111		
H-P13(3h)-OH	(93)	(7)	100		
H-P13(6h)-Red			100		
H-P13(6h)-OH			86		
H-P13(24h)-Red	95	5	64		
H-P13(24h)-OH			30		
H-N-deSO$_3$			–[d]	25	60[e]
H-N-deSO$_3$-N-Ac	>95	< 5	–[d]	20	30
H-N-deSO$_3$-N-reSO$_3$			104	20	30
H-N-deSO$_3$-P13(3h)-OH-N-reSO$_3$			10		
HBP-(1.0–1.2)	50	50	88	30	55
HBP-(1.0–1 2)-P13(3h)-OH			15		

[a] *Abbreviations* P13 and P17, periodate oxidation at pH 3 0 and 4°C and pH 7 0 and 37°C, respectively (time of oxidation within parenthesis), Red, reduction with borohydride, OH, alkaline cleavage, N-deSO$_3$, N-desulphated, N-reSO$_3$, N-resulphated, N-Ac, N-acetylated, HBP (1 0–1.2), heparin by-products obtained as CP-complex between 1 0 and 1 2 M NaCl

[b] Uronic acid composition (IdUA, L-iduronic acid, GlcUA- D-glucuronic acid) was determined after hydrolysis, deaminative cleavage and rehydrolysis followed by ion-exchange chromatography. Values obtained in this way correlate well with the carbazole-to-orcinol ratios (L.-Å F., unpublished observations) Uronic acid contents calculated from *c/o* ratios are given within parenthesis The two methods are probably accurate within ± 5%

[c] Periodate oxidation was performed at pH 3 0 and 4°C (selective oxidation of D-glucuronic acid) and at pH 7 0 and 37°C (oxidation of all non-sulphated uronic acids) Destruction of uronic acid was monitored by carbazole measurements

[d] According to other reports [17] complete N-desulphation destroys the anticoagulant activity of heparin

[e] Periodate oxidation of N-desulphated heparin at elevated temperatures causes extensive erosion of the molecule by cleavage of both the C(2)–C(3) bond of glucosamine with concomitant release of NH_3 and the C(2)–C(3) glycol in non-sulphated uronic acids (L -Å. F , unpublished observations) Presumably, other periodate-sensitive sites appear after the initial attack

conditions caused negligible reduction of anticoagulant activity. When all of the susceptible residues had been oxidized (after 3 h) 90% of the activity was retained. In contrast, oxidation of all non-sulphated uronic acid residues (D-glucuronic as well as L-iduronic acid) completely abolished the anticoagulant activity (fig.1b). These results were obtained with samples that had been reduced with borohydride prior to assay. When oxidation at pH 3.0 and 4°C was followed by cleavage in alkaline medium the anticoagulant activity decreased slightly (table 1). However, prolonged oxidation (24 h) reduced the activity considerably.

Although oxidation of D-glucuronic acid at pH 3.0 and 4°C appears to be complete after 3–6 h (fig.1a) the possibility remains that a few critical residues were oxidized at a slower rate. However, oxidation for 3 h and 24 h, respectively, followed by scission in alkali yielded materials with identical elution profiles on Sephadex G-50 (fig.2a).

Periodate oxidation at pH 3.0 and 4°C selectively oxidizes D-glucuronic acid associated with N-acetyl-glucosamine while D-glucuronic acid associated with N-sulphated glucosamine may be resistant [10]. To investigate whether D-glucuronic acid in the latter combination in heparin was resistant to oxidation at low pH and temperature, N-desulphated and N-acetylated heparin was subjected to oxidation and

degradation. The extent of oxidation (~20%, table 1) and the elution profile after cleavage (fig.2b) were the same as those obtained for the original material. Thus, periodate-resistant D-glucuronic acid residues were not present in this heparin preparation. Estimations of the uronic acid composition of periodate-oxidized (pH 3.0 and 4°C) heparins also comfirmed that most, if not all, of the D-glucuronic acid residues were destroyed (table 1).

Oxidation and cleavage of D-glucuronic acid residues in heparin resulted in a slight decrease in molecular size (fig.1a) but retention of biological activity. In contrast, oxidation and cleavage of all non-sulphated uronic acids (including L-iduronic acid, table 1) which destroyed the anticoagulant activity produced a more pronounced fragmentation of the molecule (fig.2c). However, in both cases the bulk of the fragments are estimated to be larger than dodecasaccharide.

In a study of L-iduronate ring conformations in desulphated heparins it was discovered that L-iduronic acid in the $^4C_1$ and $^1S_3$ conformers were susceptible to periodate at pH 3.0 and 4°C provided adjacent glucosamine residues had free amino groups, while L-iduronic acid residues in the $^1C_4$ chair were resistant [20]. In completely N-desulphated heparin which has lost its anticoagulant activity [17] 25% of the uronic acid residues were susceptible to oxidation (table 1). After further cleavage in alkali and subsequent N-resulphation the activity remained low. In a control preparation of heparin that was N-desulphated and N-resulphated activity was restored. The fragments obtained after oxidation and cleavage of N-desulphated heparin were considerable smaller (fig.2d) than were those obtained after degradation of intact heparin (fig.1a). In fact, the elution profile was similar to that observed with heparin that had been oxidized at pH 7.0 and 37°C followed by alkaline elimination (fig.2c).

A heparin by-product with appreciable anticoagulant activity (88 BP units/mg) and an L-iduronic acid-to-D-glucuronic acid ratio of 1.1 was also oxidized at low pH and temperature for 3 h followed by alkaline cleavage. In this case the activity was reduced to 15 BP units/mg.

4. Discussion

The results of the present study show that most, if not all, of the D-glucuronic acid residues of heparin can be oxidized without appreciable loss of anticoagulant activity. In contrast, oxidation of nonsulphated L-iduronic acid residues destroys the activity. Oxidation and subsequent reduction does not affect the chain length. Therefore it may be proposed that nonsulphated L-iduronic acid residues are critical structural elements of an 'active site' in heparin.

After oxidation and cleavage of D-glucuronic acid residues the heparin fragments obtained still retained high anticoagulant activity. However, in certain heparin by-products with a high D-glucuronic acid content the activity was lost after fragmentation of the molecule. This suggests that although D-glucuronic acid residues are not essential elements of 'active sites' they may be intercalated between various sites (e.g., sites for thrombin and antithrombin III).

Non-sulphated L-iduronic acid in heparin is likely to adopt 2 or 3 different ring conformations [20]. D-Glucuronic acid-like conformers ($^4C_1$ and $^1S_3$) of L-iduronic acid seem to be part of an 'active site' in heparin since oxidation and cleavage of such residues destroys the anticoagulant activity. The collective experience in the field [1–9] has suggested a participation of both sulphated and non-sulphated L-iduronic acid residues in the heparin–antithrombin binding site. The present results strongly favour this idea. Since non-sulphated L-iduronic acid residues are conformationally versatile [20] the possibility exists that the catalytic function of heparin in the antithrombin–thrombin reaction is exerted via alterations in the ring shape of L-iduronic acid.

Prolonged oxidation (24 h) of heparin at low pH and temperature markedly reduced the anticoagulant potency. It is possible that side-reactions of periodate (products of periodate and citrate, J. E. Scott, personal communication) or alterations of L-iduronate ring shapes are causing this effect.

Acknowledgements

Grants were received from Wellcome Trust (Visiting Scientist Fellowship to L.-Å. F.) and Swedish MRC (B76-13X-00139-12C). Professor C. F. Phelps, Dr I. A. Nieduszynski and Dr H. G. Hind provided valuable advice and Mrs B. Havsmark and Mr J. Savage skilfull technical assistance, which is gratefully acknowledged.

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