Structure and biological activity of finback-whale (*Balaenoptera physalus* L.) heparin octasaccharide

Chemical, carbon-13 nuclear-magnetic-resonance, enzymic and biological studies

Noboru OTOTANI, Masaki KIKUCHI and Zensaku YOSIZAWA*

Department of Biochemistry, Tohoku University School of Medicine, Sendai, Miyagi 980, Japan

(Received 25 November 1981/Accepted 24 March 1982)

Finback-whale (*Balaenoptera physalus* L.) heparin was partially digested with a purified heparinase and an octasaccharide with high affinity for antithrombin III was isolated from the digest by gel filtration, followed by affinity chromatography on a column of antithrombin III immobilized on Sepharose 4B. This octasaccharide possessed high inhibitory activity for Factor Xa in the presence of antithrombin III, but was essentially inactive for thrombin–antithrombin III reaction. The anticoagulant activity determined by the activated-partial-thromboplastin-time method was very low (40–70 units/mg), although the initial whale heparin exhibited high activity (252 units/mg). On the basis of the results of chemical analyses, ^{13}C n.m.r. spectrum and enzymic studies with purified heparinase, heparitinases 1 and 2, the predominant structure of the octasaccharide was proposed as follows:

Comparing this structure with those of the heparin octasaccharides so far reported, the presence of the critical structural elements for binding to antithrombin III was suggested in the pentasaccharide region situated at the reducing end of this octasaccharide. Binding to antithrombin III of the critical structural elements alone would appear to elicit the acceleration of the Factor Xa–antithrombin III reaction. Additional structural elements required for the acceleration of the thrombin–antithrombin III reaction and for the manifestation of high anticoagulant activity are discussed.

Heparin has been shown to enhance the rate of inactivation of thrombin and other activated coagulation factors by antithrombin III (Rosenberg, 1977). Binding of heparin to antithrombin III appeared to be an essential part of the mechanism (Rosenberg, 1977; Nordenman *et al.*, 1978; Jordan *et al.*, 1980).

Abbreviations used: S, sulphate; GlcNAc(6S), 6-*O*-sulphated *N*-acetylglucosamine; GlcNS, *N*-sulphoglucosamine; GlcNS(6S), 6-*O*-sulphated *N*-sulphoglucosamine; GlcNS(3S), 3-*O*-sulphated *N*-sulphoglucosamine; GlcNS(3,6-diS), 3,6-di-*O*-sulphated *N*-sulphoglucosamine; IdUA, L-iduronic acid; IdUA(2S), 2-*O*-sulphated L-iduronic acid; GlcUA, D-glucuronic acid; ΔUA , Δ -4,5-hexuronic acid; $\Delta\text{UA}(2\text{S})$, 2-*O*-sulphated Δ -4,5-hexuronic acid; aMan, 2,5-anhydromannose; aMan(6S), 6-*O*-sulphated 2,5-anhydromannose; W-HAO, high-affinity octasaccharide for antithrombin III from whale heparin; P-HAO, high-affinity octasaccharide for antithrombin III from porcine heparin.

* To whom requests for reprints should be addressed.

As a critical structural element of heparin required for binding to antithrombin III, a tetrasaccharide:

was proposed by Rosenberg & Lam (1979). More recently, Lindahl *et al.* (1981*a,b*) suggested that the actual antithrombin III-binding region of the heparin molecule is contained within the pentasaccharide sequence 2–6 of their octasaccharide:

They reported also that the two *N*-sulphate groups at positions 4 and 6, the 6-*O*-sulphate group at position 2 and the 3-*O*-sulphate group at position 4 from the non-reducing end of the octasaccharide would all

appear to be involved in the binding to antithrombin III, although the roles of the *O*-sulphate groups in positions 5 and 6 remain to be evaluated.

On the other hand, Oosta *et al.* (1981) separated heparin fragments from the limited deamination products of a highly active fraction of porcine heparin and reported that hexa-, octa- and deca-saccharides can form a complex with antithrombin III and accelerate Factor Xa-antithrombin III, but not thrombin-antithrombin III, interactions. They reported, however, that large heparin fragments of about 14 and 16 residues are essential for approximating thrombin with antithrombin III or activating antithrombin III such that it can rapidly neutralize thrombin. Moreover, they claimed that the monosaccharide sequence responsible for activating antithrombin III with respect to thrombin neutralization and for approximating free enzyme with antithrombin III must lie on the non-reducing side of their critical tetrasaccharide. They proposed that heparin possesses multiple structural domains that modulate different functions of antithrombin III.

Casu *et al.* (1981) prepared heparin oligosaccharides (essentially octasaccharides) having high affinity for antithrombin III and high anti-(Factor Xa) activity, but low activity of the activated partial thromboplastin time, by three independent approaches (extraction, partial deaminative cleavage with HNO_2 and partial depolymerization with bacterial heparinase) from porcine heparin and proposed their structures.

We isolated recently an octasaccharide with high affinity for antithrombin III from the products of partial digestion of porcine heparin with purified heparinase (Ototani & Yosizawa, 1981b). This octasaccharide (P-HAO) possessed high inhibitory activities for Factor Xa and thrombin in the presence of antithrombin III (Ototani & Yosizawa, 1981b). Moreover, P-HAO exhibited a high anticoagulant activity (240 units/mg) determined by the activated-partial-thromboplastin-time method. The proposed structure of P-HAO:

differed from that of the octasaccharide (heparinase-cleavage oligosaccharide) obtained from the heparinase digests of porcine heparin by Casu *et al.* (1981).

In order to ascertain critical structural elements of heparin required for anti-(Factor Xa) and antithrombin activities in the presence of antithrombin III, we isolated an octasaccharide (W-HAO) from the products of partial digestion of whale heparin with the purified heparinase. In the present paper we

report the structure and the biological activities of W-HAO and discuss the relationship between the structure and the biological activities of W-HAO, comparisons being made with the heparin octasaccharides so far reported. A preliminary report has been presented (Ototani & Yosizawa, 1981a).

Materials and methods

Materials

Finback-whale (*Balaenoptera physalus* L.) intestinal heparin (220 units/mg) was the preparation described previously (Ototani *et al.* 1981b). Porcine intestinal heparin (148 units/mg) and its octasaccharide (P-HAO) with high anticoagulant activity were those described previously (Ototani & Yosizawa, 1981b). Crude and purified heparinase (heparin lyase, EC 4.2.2.7) and purified heparinases 1 and 2 [heparinsulphate lyase, EC 4.2.2.8] were the preparations described previously (Ototani *et al.*, 1981a; Ototani & Yosizawa, 1981a). No sulphatase activity was detected towards GlcNS(6S), GlcNAc(6S), IdUA(2S) $\alpha 1 \rightarrow 4\text{aMan}(6\text{S})$, $\Delta\text{UA}(2\text{S})\alpha 1 \rightarrow 4\text{GlcNS}$ and P-HAO in these purified enzymes. $\Delta\text{UA}\alpha 1 \rightarrow 4\text{GlcNAc}$, $\Delta\text{UA}\alpha 1 \rightarrow 4\text{GlcNS}$, $\Delta\text{UA}(2\text{S})\alpha 1 \rightarrow 4\text{GlcNS}$ and $\Delta\text{UA}\alpha 1 \rightarrow 4\text{GlcNAc}(6\text{S})$ were the preparations described previously (Ototani & Yosizawa, 1974; Ototani *et al.*, 1974, 1981b). Human antithrombin III and antithrombin III immobilized on Sepharose 4B (antithrombin III-Sepharose) and other materials were those described previously (Ototani & Yosizawa, 1981b; Ototani *et al.*, 1981a).

Methods

Biological assays. The activities of heparin and its octasaccharide with respect to thrombin and Factor Xa inactivation were determined by the methods described previously (Ototani & Yosizawa, 1981b). The assay for anticoagulant activity by the activated-partial-thromboplastin-time method were performed as described previously (Ototani *et al.*, 1981b).

Determination of constituents. Hexuronic acid and Δ -4,5-hexuronic acid (Bitter & Muir, 1962), hexosamine (Gardell, 1953), sulphate (Antonopoulos, 1962), *N*-sulphoglucosamine (Dische & Borenfreund, 1950) and *N*-acetylglucosamine (Reissig *et al.*, 1955) were determined by the methods described in the cited references. Δ -4,5-Hexuronic acid was also determined by A_{235} (Hovingh & Linker, 1974).

Electrophoresis. Electrophoresis on cellulose acetate membrane (Separax) was performed as described previously (Ototani *et al.*, 1981b). Paper electrophoresis was performed on Toyo no. 51A paper (40 cm length) in 0.05 M-ammonium acetate/acetic acid buffer, pH 5.0, at a potential gradient of 15 V/cm for 2 h (Suzuki *et al.*, 1968).

^{13}C n.m.r. measurement. The ^{13}C n.m.r. spectrum was obtained in $^2\text{H}_2\text{O}$ at 29°C with a JEOL SX-90-9 Fourier-transform spectrometer at 22.49 MHz. The chemical shifts were measured with reference to internal acetone. The chemical shift of acetone (30.3 p.p.m.) was determined with respect to internal tetramethylsilane in ^2H chloroform.

Preparation of octasaccharide with high affinity for antithrombin III. Whale heparin (250 mg) was incubated with purified heparinase (80 units) in 5 ml of 0.1 M-sodium acetate, pH 7.0, for 14 min at 30°C . The digest was then gel-filtered through a column (1.6 cm $\times$ 240 cm) of Sephadex G-50 with

Fig. 1. Gel-filtration profile of the fraction with high affinity for antithrombin III

The combined fraction with high affinity for antithrombin III obtained from four samples of the purified heparinase digestion was gel-filtered through a column of Sephadex G-50 as described in the text and the A_{235} of the fractions was plotted. V_0 is the void volume.

Fig. 2. Affinity chromatography of the octasaccharide fraction on a column of antithrombin III-Sepharose. Affinity chromatography of the octasaccharide fraction (peak 2 in Fig. 1) was performed on a column of antithrombin III-Sepharose as described in the text and the A_{235} of the fractions was plotted (●). The arrow shows the start of linear-gradient elution. Abbreviation used: HA, fractions with high affinity for antithrombin III. ---, NaCl concentration.

0.5 M-NaCl at a flow rate of 20 ml/h. Fractions (3 ml) were collected. The A_{235} of the fractions was determined. The fractions containing mainly octasaccharide were pooled and desalted with Sephadex G-25. The freeze-dried fraction was dissolved in 3 ml of 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.05 M-NaCl and then applied to a column (2.2 cm $\times$ 10 cm) of antithrombin III-Sepharose. After washing with the same buffer, linear gradient elution with 0.05–2.5 M-NaCl in the same buffer was performed at a flow rate of 20 ml/h. Fractions (5 ml) were collected and the A_{235} of the fractions were determined. The pooled fractions eluted with 0.5–2.0 M-NaCl were then desalted with Sephadex G-25. The desalted fractions obtained from four samples of the above-mentioned incubation mixture were combined and then gel-filtered through the same size of Sephadex G-50 column as described above (Fig. 1). The octasaccharide fraction (peak 2) was desalted with Sephadex G-25, followed by affinity chromatography on antithrombin III-Sepharose as described above (Fig. 2). An octasaccharide with high affinity for antithrombin III eluted with 0.8–1.6 M-NaCl was designated as 'W-HAO' (high-affinity octasaccharide for antithrombin III from whale heparin).

Results

Whale heparin octasaccharide with high affinity for antithrombin III

Whale heparin was partially digested with purified heparinase, followed by the above-mentioned treatments. The yield of W-HAO was 2% of the initial heparin.

Anticoagulant activity of W-HAO

The anticoagulant activity was determined by the activated-partial-thromboplastin-time method (Table 1). The results indicated that whale heparin possessed high anticoagulant activity, whereas W-HAO had relatively low activity. The semilogarithmic plot

Table 1. Anticoagulant activity of W-HAO, whale heparin and porcine heparin

The anticoagulant activity of the samples was determined by the activated-partial-thromboplastin-time method, with porcine heparin (148 units/mg) as a standard. Each activated partial thromboplastin time was an average for five experiments.

	Amount		Activated partial thromboplastin time (s)
	(μg)	(Units)	
W-HAO	3.2	—	67.3
	6.4	—	73.5
Whale heparin	1.5	—	94.5
	1.69	0.25	68.5
Porcine heparin	3.39	0.50	133.5
	5.07	0.75	226.6

of the activated partial thromboplastin time versus the activity of the standard porcine heparin gave a straight line from which the anticoagulant activity of the whale heparin and W-HAO were calculated to be 252 and 40–70 units/mg respectively.

Activity of W-HAO with respect to thrombin and Factor Xa inactivation

The activity of W-HAO with respect to thrombin and Factor Xa inactivation in the presence of antithrombin III was determined by the method described previously (Ototani & Yosizawa, 1981b). The results are plotted, together with those of the initial heparin, in Figs. 3 and 4. From these data, the amounts of the samples giving 50% inhibition for thrombin were calculated to be $\geq 20 \mu\text{g}$ and 19 ng for W-HAO and the initial heparin respectively. On the other hand, the amounts of the samples giving 50% inhibition for Factor Xa were calculated to be 20 and 6.8 ng for W-HAO and the initial heparin respectively. These observations indicated that,

Fig. 3. Activities of W-HAO and the initial heparin with respect to thrombin inactivation

The inhibitory activity of the samples was determined by the method described previously (Ototani & Yosizawa, 1981b). ●, W-HAO; ○, initial heparin.

Fig. 4. Activities of W-HAO and the initial heparin with respect to Factor Xa inactivation

The inhibitory activity of the samples was determined by the method described previously (Ototani & Yosizawa, 1981b). ●, W-HAO; ○, initial heparin.

although the initial heparin has high activities with respect to thrombin and Factor Xa inactivation in the presence of antithrombin III, W-HAO possesses only the anti-(Factor Xa) activity.

Analyses of W-HAO

W-HAO gave a single band both on Separax electrophoresis in pyridine/formic acid, pH 3.0, at 1 mA/cm for 20 min (a) and in 0.1 M-HCl at 5.0 V/cm for 1 h (b). The lower mobilities (4.2 cm in a and 1.6 cm in b) of W-HAO than those (4.4 cm in a and 1.9 cm in b) of P-HAO indicated that the former contained less sulphate than the latter.

The analytical data for W-HAO showed that the molar ratio total hexuronic acid/glucosamine/sulphate was 1.12:1.00:1.81 and also that the ratio of D-glucuronic acid/L-iduronic acid/ Δ -4,5-hexuronic acid was 1.00:1.89:0.91. Digestion of W-HAO with crude heparinase liberated 25% of hexosamine in total as N-acetylglucosamine, which was determined by the Morgan–Elson reaction (Reissig *et al.*, 1955). The remainder could be determined by the method of Dische & Borenfreund (1950), and was shown to be N-sulphoglucosamine.

The molecular size of W-HAO was confirmed by the following two methods: first, on the basis of a molar absorption coefficient of $5500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 235 nm for the unsaturated hexuronic acid, the molar ratio of the Δ -4,5-hexuronic acid to the total hexuronic acid was calculated to be 4.1:1; second, the hexosamine content of the W-HAO reduced with NaBH_4 was 74% of that of the intact molecule.

These observations indicated that W-HAO contained approx. three N-sulphoglucosamine residues, one N-acetylglucosamine residue, one D-glucuronic acid residue, two L-iduronic acid residues, one Δ -4,5-hexuronic acid residue and four O-sulphate groups.

^{13}C n.m.r. spectrum of W-HAO

^{13}C n.m.r. spectrum of W-HAO was obtained as described under 'Methods'. The profile of the signals of W-HAO was almost identical with that of the heparinase-cleavage oligosaccharide of Casu *et al.* (1981), except for the intensities of the signals, and for slightly (0.8–1.0 p.p.m.) upfield-shifted p.p.m. values of the all signals. The signals at 53.8 p.p.m., together with the signal at 22.4 p.p.m., confirmed the presence of an N-acetylglucosamine residue in this compound. Since the signals at 57.5 and 58.2 p.p.m. could be assigned to C-2 of the N-sulphoglucosamine residue at the reducing end and that located at the inner part (respectively) of the molecule, the signal at 56.9 p.p.m. may be ascribed to the β -shift of the C-2 signal of the N-sulphoglucosamine residue carrying the 3-O-sulphate group, as suggested by Casu *et al.* (1981) and Meyer *et al.* (1981). The ratio

of the intensity of the signals in the 59.2–60.3 p.p.m. region to that at 66.4 p.p.m., which are attributable to the non-sulphated and sulphated C-6 signals of the glucosamine residues respectively (Casu *et al.*, 1981), was approx. 3:1, indicating that one of the four glucosamine residues had a 6-*O*-sulphate group. On the other hand, the signals at 99.4, 101.7 and 102.1 p.p.m. could be assigned to C-1 of 2-*O*-sulphated L-iduronic acid, L-iduronic acid and D-glucuronic acid residues respectively. The signals at 105.7 and 144.7 p.p.m. may be ascribed to C-4 and C-5 respectively of the Δ -4,5-hexuronic acid residue, since the signals at 107.6 and 144.7 p.p.m. were assigned to the corresponding ones of phenyl Δ -4,5-hexuronide (N. Ototani & Z. Yosizawa, unpublished work). The signal at 97.1 p.p.m. may be assigned to C-1 of the unsaturated hexuronic acid, together with that of the *N*-sulphoglucosamine residue, as indicated by Casu *et al.* (1981).

Enzymic studies on W-HAO

W-HAO (1 mg) was incubated with a mixture of purified heparinase (5 units), heparitinase 1 (4 units) and heparitinase 2 (4 units) for 15 h at 37°C and the digest was then subjected to paper electrophoresis. As shown in Fig. 5(a), two bands were detected. The compounds in these bands were designated as '1a' and '1b'. The ratio of 1a to 1b was 3:1, in terms of their A_{235} . The analytical data (Table 2) indicated that 1a and 1b were disulphated unsaturated disaccharide and monosulphated unsaturated disaccharide respectively. Authentic Δ UA(2S) α 1 $\rightarrow$ 4GlcNS migrated to the position corresponding to 1a, whereas authentic Δ UA α 1 $\rightarrow$ 4GlcNS and Δ UA α 1 $\rightarrow$ 4GlcNAc(6S) migrated to that of 1b. Although the mobility of 1b on paper chromatography on Toyo no. 51A paper, with butan-1-ol/acetic acid/water (10:3:6, by vol.) as solvent, coincided with that of Δ UA α 1 $\rightarrow$ 4GlcNAc(6S), it differed from that of Δ UA α 1 $\rightarrow$ 4GlcNS. On paper chromatography, 1a gave two spots, of which the mobility of the faster-moving spot coincided with that of Δ UA(2S) α 1 $\rightarrow$ 4GlcNS.

Subsequently, W-HAO (1 mg) was incubated with purified heparinase (5 units) for 15 h at 30°C, followed by the same treatments as above. The paper electrophoretogram (Fig. 5b) indicated the presence of four unsaturated oligosaccharides in the digests. The compounds in bands 2a, 2b, 2c and 2d were designated as '2a', '2b', '2c' and '2d' respectively. The analytical data and the polymerization index (Table 2) indicated that 2a, 2b and 2c were disulphated unsaturated disaccharide, monosulphated unsaturated disaccharide and trisulphated unsaturated tetrasaccharide respectively. The mobility of 2a on paper chromatography (see above) coincided with that of Δ UA(2S) α 1 $\rightarrow$ 4GlcNS. Although 2d was not studied further, it was thought to

Fig. 5. Paper electrophoretograms of the enzymic digests of W-HAO

W-HAO was separately digested with the purified heparinase plus purified heparitinases 1 and 2 (a), the purified heparinase (b) and the purified heparitinase 1 (c) as described in the text. Paper electrophoresis was performed as described in the text and the u.v.-absorbing compounds were located by a Mineral light (model S-2536). The paper strips were cut into appropriate sizes and the compound(s) in each strip were extracted with 2.0 ml of 0.1 M-HCl. The A_{235} and the constituents of each compound were determined. References I, II and III were Δ UA α 1 $\rightarrow$ 4GlcNAc, Δ UA α 1 $\rightarrow$ 4GlcNS and Δ UA(2S) α 1 $\rightarrow$ 4GlcNS.

consist of oligomer(s). The analytical data for 2c indicated the presence of one *N*-acetylglucosamine residue in the molecule. The ratio of 2a to 2c was found to be approx. 2 in terms of A_{235} . These findings indicated that the heparinase cleaved two bonds of W-HAO to give two molecules of the disulphated unsaturated disaccharide and one molecule of trisulphated unsaturated tetrasaccharide containing one *N*-acetylglucosamine residue, with

small proportions of the minor unsaturated oligosaccharides.

On the other hand, W-HAO (1 mg) was incubated with purified heparitinase 1 (4 units) for 15 h at 37°C, followed by the same treatments as those described above. The paper electrophoretogram indicated the presence of one major and three minor unsaturated oligosaccharides in the digests (Fig. 5c). The compounds in the bands in this Figure were designated as '3a' '3b' '3c' and '3d'. The major one (3b) was 90% in total of the digests in terms of A_{235} . The hexosamine content of 3b reduced with NaBH_4 was 51% of that of the intact molecule. This finding, together with the polymerization index and the analytical data (Table 2), indicated that 3b consisted of sulphated unsaturated tetrasaccharide(s). Since the molar ratio of sulphate to total hexuronic acid of 3b was 1.78 (Table 2), we suggest that 3b may be a mixture of trisulphated and tetrasulphated unsaturated tetrasaccharides. These observations indicated that the heparitinase 1 cleaved W-HAO at the middle of the octasaccharide chain to give tri- and tetra-sulphated unsaturated tetrasaccharides. Of the minor products, 3c was indicated to be disulphated unsaturated tetrasaccharide containing one *N*-acetylglucosamine residue by the polymerization index and the analytical data (Table 2). Although 3a and 3d were not studied further, they were thought to consist of disulphated unsaturated disaccharide and oligomer(s) respectively.

As judged from the observations, the proposed predominant structure of W-HAO was as shown in Fig. 6. The linkages susceptible to the purified heparinase, heparitinases 1 and 2 and the sulphated oligosaccharide regions producing the corresponding compounds in the bands in Fig. 5 are also indicated in this Figure. The presence of 2b (monosulphated unsaturated disaccharide) as a minor component in the digest of W-HAO with the purified heparinase suggested that a part of either the sulphated unsaturated hexuronic acid residue or the sulphated hexuronic acid residue located at the adjacent position of the reducing terminus might be replaced by its non-sulphated counterpart. The presence of 3c (disulphated unsaturated tetrasaccharide containing *N*-acetylglucosamine) as a minor component in the digest with the purified heparitinase 1 supported the presence of a small portion of the non-sulphated unsaturated hexuronic acid at the non-reducing end of W-HAO.

Discussion

An octasaccharide (W-HAO) isolated from the products of partial digestion of finback-whale heparin with the purified heparinase possessed high affinity for antithrombin III and high inhibitory activity towards Factor Xa in the presence of

Fig. 6. Predominant structure of W-HAO

Sites A, B, and C are indicated to be the linkages susceptible to the purified heparinase, heparitinases, and heparinase respectively. 1a, 1b, 2a, 2c and 3b are the sulphated oligosaccharide regions producing the corresponding compounds in the bands in Fig. 5.

Table 2. *Analytical data for the compounds separated from the enzymic digests of W-HAO*

W-HAO was separately digested with the purified heparinase plus purified heparitinases 1 and 2 (a), the purified heparinase (b) and the purified heparitinase 1 (c). Each digest was subjected to paper electrophoresis and treated as described in Fig. 5. The constituents of each compound were determined and expressed as the molar ratio to total hexuronic acid.

Digestion	Compound	Yield*	HexN†	UA‡	S§	GlcNAc	PI
(a)	1a	75	0.87	1.00	2.02	0	2
	1b	25	0.91	1.00	1.25	0.84	2
(b)	2a	61	0.90	1.00	1.96	0	2
	2b	6	NS¶	1.00	0.96	NS	2
	2c	30	0.89	1.00	1.48	0.42	4
	2d	3	NS	NS	NS	NS	NS
(c)	3a	3	NS	NS	NS	NS	NS
	3b	90	0.96	1.00	1.78	0.20	4
	3c	5	NS	1.00	0.90	0.40	4
	3d	2	NS	NS	NS	NS	NS

* Expressed as percentage in total of the A_{235} of the digest.

† Total hexosamine.

‡ Total hexuronic acid.

§ Sulphate.

|| PI, polymerization index; this was calculated from the value of the total hexuronic acid determined by the method of Bitter & Muir (1962) and unsaturated hexuronic acid determined by A_{235} based on a molar absorption coefficient of $5500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

¶ NS, not studied.

antithrombin III, but low activity of the activated partial thromboplastin time.

Although the monosaccharide sequence of the proposed structure of W-HAO and its biological activities were essentially identical with those of the octasaccharide (heparinase-cleavage oligosaccharide) described by Casu *et al.* (1981), the former contained less 6-*O*-sulphate groups than the latter. Moreover, the pentasaccharide region located at the reducing portion of W-HAO was identical with the pentasaccharide sequence 2–6 from the non-reducing end of the octasaccharide described by Lindahl *et al.* (1981a,b), except for the above 6-*O*-sulphate groups. This pentasaccharide sequence was suggested to contain the actual antithrombin III-binding region of the heparin molecule (Lindahl *et al.*, 1981a,b). It is suggested, therefore, that the abovementioned 6-*O*-sulphate groups missing from W-HAO are non-essential for the binding to antithrombin III and also for high anti-(Factor Xa) activity.

As reported in our previous paper (Ototani & Yosizawa, 1981b), an octasaccharide (P-HAO) isolated from the products of partial digestion of porcine heparin with the purified heparinase possessed high affinity for antithrombin III, high inhibitory activities towards Factor Xa and thrombin in the presence of antithrombin III, and high activity of the activated partial thromboplastin time. The proposed structure of P-HAO, except for both the terminal monosaccharides, is identical with that of the octasaccharides isolated from the limited deamination products of the same heparin by

Lindahl *et al.* (1981a,b) and Casu *et al.* (1981). The octasaccharide (deaminative-cleavage oligosaccharide) described by Casu *et al.* (1981) was reported to possess high affinity for antithrombin III and high anti-(Factor Xa) activity, but low activity of the activated partial thromboplastin time. Since the non-reducing terminus of P-HAO was unsaturated hexuronic acid, it is suggested that the L-iduronic acid residue located at the non-reducing end of the above-mentioned octasaccharides described by Lindahl *et al.* (1981a,b) and Casu *et al.* (1981) is non-essential for the antithrombin III-binding and anti-(Factor Xa) activities, as suggested by Lindahl *et al.* (1981a,b) for the antithrombin III-binding activity.

Although P-HAO was eluted with 1.5–2.0M NaCl from the antithrombin III–Sepharose column (Ototani & Yosizawa, 1981b), W-HAO was eluted with 0.8–1.6M NaCl. It therefore appeared that the binding of the former to antithrombin III was significantly stronger than that of the latter. The strong binding to antithrombin III of the former appears to be on account of the presence of the disaccharide unit:

at the reducing end, in addition to the adjacent pentasaccharide sequence, which is suggested to contain the actual antithrombin III-binding region of the octasaccharide described by Lindahl *et al.* (1981a,b).

As judged from these observations, it is conceivable that the strong binding to antithrombin III of

the heptasaccharide sequence of P-HAO may elicit an acceleration of the thrombin-antithrombin III reaction and also the manifestation of high anticoagulant activity in the activated-partial-thromboplastin-time system. As W-HAO lacks the above-mentioned disaccharide region, it is thought to be inactive for the thrombin-antithrombin III reaction. It is suggested also that binding to antithrombin III of the critical structural elements in the pentasaccharide region of the octasaccharides elicit the acceleration of the Factor Xa-antithrombin III reaction. In addition, the octasaccharides with high anti-(Factor Xa) activity appear to be unable to manifest high anticoagulant activity in the activated-partial-thromboplastin-time system unless they possess high inhibitory activity for thrombin in the presence of antithrombin III.

Although W-HAO and P-HAO were isolated by essentially identical procedures from the whale heparin and porcine heparin respectively, they differed in structure and biological activity. Whale heparin has been shown to contain a higher proportion of *N*-acetylglucosamine residues and lower sulphate content than porcine heparin (Kotoku *et al.*, 1967). Also, the content of 6-*O*-sulphates on the glucosamine residues in the former was lower than that in the latter (Kosakai & Yosizawa, 1979, 1981). The content of the trisaccharide sequence:

in the former was higher than that in the latter, and the cluster of the disaccharide sequence:

was suggested to be smaller in the former than that in the latter (Kosakai & Yosizawa, 1981). These structural differences between these heparins may elicit different susceptibilities of their linkages to the partial digestion with the purified heparinase, resulting in the production of the different octasaccharides.

On the other hand, Casu *et al.* (1981) isolated an octasaccharide (heparinase-cleavage oligosaccharide) essentially identical, except for 6-*O*-sulphate groups, with W-HAO from porcine heparin after exhaustive digestion with heparinase. The structure and the biological activities of their octasaccharide differed from those of our P-HAO isolated from the same source after partial digestion with the purified heparinase (Ototani & Yosizawa, 1981b). Although the heparinase preparations and the incubation conditions in these studies differed from each other, the mechanism of the production of the different octasaccharides from the same source is obscure at present.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science

and Culture of Japan. We thank Dr. T. Okuyama for his helpful discussions.

References

- Antonopoulos, C. A. (1962) *Acta Chem. Scand.* **16**, 1521-1522
- Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330-334
- Casu, B., Oreste, P., Torri, G., Zopetti, G., Choay, J., Lormeau, J.-C., Petitou, M. & Sinaÿ, P. (1981) *Biochem. J.* **197**, 599-609
- Dische, Z. & Borenfreund, E. (1950) *J. Biol. Chem.* **184**, 517-522
- Gardell, S. (1953) *Acta Chem. Scand.* **7**, 207-215
- Hovingh, P. & Linker, A. (1974) *Carbohydr. Res.* **37**, 181-192
- Jordan, R. E., Oosta, G. M., Gardner, W. T. & Rosenberg, R. D. (1980) *J. Biol. Chem.* **255**, 10081-10090
- Kosakai, M. & Yosizawa, Z. (1979) *J. Biochem. (Tokyo)* **86**, 147-153
- Kosakai, M. & Yosizawa, Z. (1981) *J. Biochem. (Tokyo)* **89**, 1933-1944
- Kotoku, T., Yosizawa, Z. & Yamauchi, F. (1967) *Arch. Biochem. Biophys.* **120**, 553-562
- Lindahl, U., Thunberg, L. & Bäckström, G. (1981a) *Glycoconjugates (Proc. Int. Symp. Glycoconjugates 6th, Tokyo, Japan)* (Yamakawa, T., Osawa, T. & Handa, S., eds.), pp. 362-363, Japan Scientific Societies Press, Tokyo
- Lindahl, U., Thunberg, L., Bäckström, G. & Riesenfeld, J. (1981b) *Biochem. Soc. Trans.* **9**, 499-501
- Meyer, B., Thunberg, L., Lindahl, U., Larm, O. & Leder, I. G. (1981) *Carbohydr. Res.* **88**, C1-C4
- Nordenman, B., Danielsson, Å. & Björk, I. (1978) *Eur. J. Biochem.* **90**, 1-6
- Oosta, G. M., Gardner, W. T., Beeler, D. L. & Rosenberg, R. D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 829-833
- Ototani, N. & Yosizawa, Z. (1974) *J. Biochem. (Tokyo)* **76**, 545-551
- Ototani, N. & Yosizawa, Z. (1981a) *Glycoconjugates (Proc. Int. Symp. Glycoconjugates 6th, Tokyo, Japan)* (Yamakawa, T., Osawa, T. & Handa, S., eds.), pp. 411-412, Japan Scientific Societies Press, Tokyo
- Ototani, N. & Yosizawa, Z. (1981b) *J. Biochem. (Tokyo)* **90**, 1553-1556
- Ototani, N., Nakamura, K. & Yosizawa, Z. (1974) *J. Biochem. (Tokyo)* **75**, 1283-1289
- Ototani, N., Kikuchi, M. & Yosizawa, Z. (1981a) *Carbohydr. Res.* **88**, 291-303
- Ototani, N., Kikuchi, M. & Yosizawa, Z. (1981b) *J. Biochem. (Tokyo)* **90**, 241-246
- Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955) *J. Biol. Chem.* **217**, 959-966
- Rosenberg, R. D. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 10-18.
- Rosenberg, R. D. & Lam, L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1218-1222
- Suzuki, S., Saito, H., Yamagata, T., Anno, K., Seno, N., Kawai, Y. & Furuhashi, T. (1968) *J. Biol. Chem.* **243**, 1543-1550