Biochem. J. (1987) 241, 111–119 (Printed in Great Britain)

Mechanism of inhibition of mammalian DNA topoisomerase I by heparin

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We have previously shown that heparin is a potent inhibitor of a mammalian DNA topoisomerase I. We have now investigated the mechanism of its inhibition. This was carried out first by scrutinizing the structural features of heparin molecules responsible for the inhibition. Commercial heparin preparation was fractionated by antithrombin III–Sepharose into non-adsorbed, low-affinity and high-affinity fractions, of which only the high-affinity fraction of heparin is known to contain a specific oligosaccharide sequence responsible for the binding to antithrombin III. These fractions all exhibited essentially similar inhibitory activities. Furthermore, when chemically sulphated to an extent comparable with or higher than heparin, otherwise inactive glycosaminoglycans such as heparan sulphate, chondroitin 4-sulphate, dermatan sulphate and neutral polysaccharides such as dextran and amylose were converted into potent inhibitors. Sulphated dermatan sulphate, one of the model compounds, was further shown to bind competitively to the same sites on the enzyme as heparin. These observations strongly suggested that topoisomerase inhibition by heparin is attributable primarily, if not entirely, to the highly sulphated polyanionic nature of the molecules. In a second series of experiments we examined whether heparin inhibits only one or both of the topoisomerase reactions, i.e. nicking and re-joining. It was demonstrated that both reactions were inhibited by heparin, but the nicking reaction was more severely affected than was the re-joining reaction.

INTRODUCTION

DNA topoisomerases are enzymes that introduce transient breaks in the DNA backbone and thereby participate in a number of genetic processes (for reviews see Cozzarelli, 1980; Gellert, 1981; Wang, 1981, 1985; Liu, 1983). Topoisomerases have been divided into two classes, distinguished by their reaction mechanisms. A type I enzyme, orginally known as ω protein, was first discovered in *Escherichia coli* by Wang (1971), and similar enzymes were subsequently found in various eukaryotic organisms, mostly in a chromatin-bound state (Wang, 1985). The type I topoisomerase is defined by its ability to break one DNA strand transiently, thus permitting interconversion of various topological isomers of DNA. It has become clear that covalent enzyme–DNA intermediates are characteristic of this reaction. The biological functions of type I topoisomerases have been a subject of speculation (Liu, 1983; Wang, 1985). On the basis of its catalytic activity *in vitro*, the enzyme has been implicated in a variety of genetic processes, such as DNA replication, RNA transcription, genetic recombination, chromosome condensation and decondensation. Viable *E. coli* mutants lacking this enzyme were isolated (Sternglanz *et al.*, 1981), but they were later found to have compensatory mutations in DNA gyrase genes (Pruss *et al.*, 1982; DiNardo *et al.*, 1982). In contrast, however, DNA topoisomerase I-deficient mutants of *Saccharomyces*, a lower eukaryote, were isolated and shown to be viable, although they possess normal amounts of type II topoisomerases (Thrash *et al.*, 1984; Uemura & Yanagida, 1984). Topoisomerase I was previously found associated with transcriptionally active chromatin (Weisbrod, 1982; Javaherian & Liu, 1983). Furthermore, it has been implicated to be catalytically active on transcriptionally active genes in *Drosophila* polytene chromosomes (Fleischmann *et al.*, 1984) as well as in nucleolus-associated ribosomal genes (Gocke *et al.*, 1983; Muller *et al.*, 1985). These experiments strongly suggest a role for topoisomerase I in transcriptional events by RNA polymerases I and II. With all these results taken together, topoisomerase I is implicated as being involved in various genetic processes. Specific inhibitors of this enzyme would potentially complement the mutant analyses and help dissect and establish the roles of this enzyme in DNA metabolism.

In a previous paper we reported that heparin is a potent inhibitor of a mammalian DNA topoisomerase I, and that this inhibition is unique for heparin, since other chemically related glycosaminoglycans are devoid of this activity (Ishii *et al.*, 1982). Heparin is a highly sulphated glycosaminoglycan that plays a well-characterized role in the inhibition of the proteolytic cascade mechanism of blood coagulation through an interaction with antithrombin III (Rosenberg, 1977; Björk & Lindahl, 1982). It was shown that commercially available heparin preparations are heterogeneous with respect to affinity towards antithrombin III, and the high-affinity fraction accounts for most of the anticoagulant activity of the original material (Björk & Lindahl, 1982; Lam *et al.*, 1976). Heparin has also been reported to inhibit the activity of several other enzymes, including *E. coli* DNA-dependent RNA polymerase (Walter *et al.*, 1967; Pfeffer *et al.*, 1977), human DNA polymerases, terminal

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deoxynucleotidyltransferase, Simian-sarcoma-virus reverse transcriptase (DiCioccio & Srivastava, 1978) and initiation and elongation factors of protein synthesis (Waldman & Goldstein, 1973; Slobin, 1976). In addition, it has been shown to have a wide variety of biological activities: release of lipases into the blood circulation (Olivecrona *et al.*, 1977; Bengtsson & Olivecrona, 1980; Jansen & Hulsmann, 1980), facilitated induction of luteotropin receptors (Nimrod & Lindner, 1980), induction of phagocytosis of mouse macrophages (vande Water *et al.*, 1981), stimulation of capillary endothelial-cell growth (Thornton *et al.*, 1983) and migration (Azizkhan *et al.*, 1980) and inhibition of growth of vascular smooth-muscle cells (Castellot *et al.*, 1981). These observations have led to the hypothesis that heparin is a multifunctional modulator in living organisms (Björk & Lindhal, 1982; Jaques, 1979).

In the present experiments we investigated the mechanism of inhibition of topoisomerase I by heparin. This was carried out from two approaches. First, what are the structural features of heparin molecules responsible for the inhibition? Secondly, what is the molecular mechanism of heparin inhibition of the topoisomerization reaction? More specifically, does it bind to DNA or enzyme? Does it inhibit the breakage reaction or the re-joining reaction, or both?

MATERIALS AND METHODS

Materials

Pig intestinal heparin (anticoagulant activity 166 U.S.P. units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine kidney heparan sulphate, supplied by Seikagaku Kogyo Co. (Tokyo, Japan), was purified by fractionation on a column of Dowex 1 (Cl^- form) resin. The fraction eluted with 1.25 M-NaCl was used in the inhibition study. A crude preparation of rooster comb dermatan sulphate was purified as described by Nagasawa *et al.* (1984), and the 20% (v/v) ethanol precipitate of the polysaccharide (M_r 41000 by viscosity measurement) was used in the present study. Sodium chondroitin 4-sulphate (M_r 64000 by the Nelson–Somogyi method) was obtained from Seikagaku Kogyo Co. Dextrans [D.P. (average degree of polymerization) 64 and 14, by the light-scattering method] were the product of Pharmacia Fine Chemicals (Uppsala, Sweden). Amylose (D.P. 100) was the product of Hayashibara Biochemical Laboratories (Okayama, Japan). Sepharose 6B, Sephadex G-100 and phenyl-Sepharose CL-4B gels were purchased from Pharmacia Fine Chemicals. Ultrogel AcA 44 agarose/polyacrylamide gel was purchased from LKB Produkter (Bromma, Sweden). Col E1 DNA was prepared from *E. coli* A745met$^-$thy$^-$ (col E1) as described by Sakakibara & Tomizawa (1974). Bacteriophage ϕX174 DNA was obtained from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.).

Preparation and assay of DNA topoisomerase I

Chromatin extract containing topoisomerase I was prepared from cultured mouse FM3A mammary carcinoma cells (Nakano, 1966) by the method of Germond *et al.* (1975). Proteins eluted from purified chromatin with 0.15 M-sodium phosphate buffer, pH 7.5, containing 1 mM-EDTA, 1 mM-dithiothreitol and 10% (v/v) glycerol were pooled as crude enzyme preparation. The enzyme was further purified by a modified version of the procedure reported by Ishii *et al.* (1983). Briefly, the chromatin extract was applied to a column of heparin–Sepharose. Topoisomerase was eluted stepwise between 0.5 M- and 0.7 M-NaCl in an elution buffer composed of 10 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-dithiothreitol, 0.5 mM-EDTA, 0.2 mM-phenylmethanesulphonyl fluoride and 10% (v/v) glycerol. The active fraction after dialysis against elution buffer was adjusted to 1 M-$(NH_4)_2SO_4$ and applied to a column of phenyl-Sepharose CL-4B. The enzyme was eluted stepwise between 0.8 M- and 0.49 M-$(NH_4)_2SO_4$. At this step the enzyme was found to be more than 80% pure, as judged by SDS/polyacrylamide-gel electrophoresis. Enzyme activity was assayed as previously described (Ishii *et al.*, 1982, 1983). In brief, standard reaction mixture (40 μl) contained 1 μg of supercoiled col E1 DNA (form I), 0.05 M-sucrose, 0.15 M-sodium phosphate buffer, pH 7.5, and various amounts of enzyme. Reaction mixture was incubated at 37 °C for 15 min. One unit of enzyme is defined as the activity that converts 1 μg of col E1 DNA (form I) into fully relaxed form Ir DNA under the conditions used. When an inhibitor was to be tested, the inhibitor and DNA were first mixed and the reaction was initiated by addition of the enzyme. The reaction product was characterized by electrophoresis on horizontal 1.2% (w/v) agarose slab gels with the buffer system described by Germond *et al.* (1975). After the loading of samples electrophoresis was carried out at 40 V for 15 h at room temperature. The gels were stained with 1 μg of ethidium bromide/ml in the same buffer for 30 min. Fluorescent DNA bands were detected by illuminating the gel with shortwave u.v. light and photographed. Amounts of DNA were quantified by densitometric scanning of photographic negatives. IC_{50}, the 50% inhibition dose, is defined conveniently as the concentration of inhibitor giving reaction products with varied superhelicity distributing evenly throughout the track between the starting substrate form I DNA and fully relaxed form Ir (see Fig. 1 for example).

Fractionation of heparin

The commercial preparation of heparin contained about 2.5% dermatan sulphate (calculated from the ratio of galactosamine to total hexosamine). A heparin preparation free from dermatan sulphate was prepared by the method of Rodén *et al.* (1972). Affinity chromatography of heparin on antithrombin III–Sepharose was performed essentially as described by Laurent *et al.* (1978). The non-adsorbed fraction, the low-affinity fraction and the high-affinity fractions (S-I, S-II and S-III) were pooled separately, concentrated *in vacuo* at 40 °C, extensively dialysed against distilled water and then freeze-dried.

Preparation of sulphated polysaccharides

Glycosaminoglycans (heparan sulphate, dermatan sulphate and chondroitin 4-sulphate) and neutral polysaccharides (amylose and dextran) were sulphated according to a procedure developed by K. Nagasawa & H. Uchiyama (unpublished work). The outline of this procedure is as follows. To a solution of glycosaminoglycan (tri-n-butylammonium salt) dissolved in dimethylformamide or of neutral polysaccharide dissolved in dimethyl sulphoxide, pyridine/SO_3 (10 mol/mol equiva-

lent of available hydroxy group) was added, and the mixture was kept at 25 °C for 2 h (glycosaminoglycan) or 22 h (neutral polysaccharide). The reaction was terminated by the addition of water, and the pH of the solution was adjusted to 9 with 5 M-NaOH. The sodium salts of sulphated polysaccharides were isolated by the conventional procedure, except for sulphated amylose, which was divided into eight fractions on Sepharose 6B and Sephadex G-100 because of the broad distribution of molecular size. The analytical data of the products are given in Table 2.

Preparation of heparin–Sepharose 4B and sulphated dermatan sulphate–Sepharose 4B

Amino-Sepharose 4B, which was prepared by coupling epoxy-activated Sepharose 4B with ammonia, was bound to heparin or sulphated dermatan sulphate by using the procedure of Funahashi *et al.* (1982). The amount of ligand bound to the gel was estimated by the method described in the same reference. The degrees of substitution of ligand were as follows: heparin–Sepharose 4B, 3.6 mg/ml of gel; sulphated dermatan sulphate–Sepharose 4B, 11.6 mg/ml of gel.

Affinity chromatography of topoisomerase I on DNA–cellulose

A 20 μg portion of purified topoisomerase I in 600 μl of elution buffer used in the purification procedure described above was applied to a column of DNA–cellulose [100 μl of gel containing 114 μg of native calf thymus DNA packed over a cotton plug in a plastic Gilson tip P200 (Gilson Frans, Villiers le Bel, France)] equilibrated with the same buffer. After the column had been washed the enzyme was eluted with 600 μl portions of elution buffer containing increasing concentrations of heparin as described in the legend to Fig. 2. Eluates were precipitated with 10% (w/v) trichloroacetic acid and subjected to SDS/polyacrylamide-gel electrophoresis.

Affinity chromatography of topoisomerase I on heparin–Sepharose 4B and sulphated dermatan sulphate–Sepharose 4B

Portions (4 ml each) of chromatin extract containing 200 μg of protein/ml were applied to a column (0.7 cm × 1.1 cm) of heparin–Sepharose 4B and sulphated dermatan sulphate–Sepharose 4B with a bed volume of 0.5 ml each. After the columns had been washed with 0.15 M-sodium phosphate in the elution buffer described above, proteins were eluted with 5 ml each of 0.4 M-, 0.6 M-, 0.8 M- and 1.0 M-NaCl in elution buffer. Portions (2–8 μl) from each pooled eluate were taken for the measurement of enzymic activity, and 1.2 ml each after precipitation with 10% trichloroacetic acid for the analysis of protein composition by SDS/15%-polyacrylamide-gel electrophoresis.

Experiments of competitive elution of the enzyme from heparin–Sepharose 4B with heparin or sulphated dermatan sulphate were carried out as follows. Portions (13 μg each) of purified topoisomerase in 400 μl of elution buffer were applied to two parallel columns of heparin–Sepharose 4B (50 μl of beads packed over a cotton plug in a plastic Gilson tip P200). After the columns had been washed and equilibrated with 0.15 M-sodium phosphate in elution buffer, the enzyme was eluted with 300 μl portions of elution buffer containing increasing concentrations of heparin or sulphated dermatan sulphate as described in the legends to Figures. Eluates were precipitated with 10% (w/v) trichloroacetic acid and subjected to SDS/15%-polyacrylamide-gel electrophoresis.

Separation of breakage and re-joining reactions of topoisomerase I by using a single-stranded circular bacteriophage ϕX174 DNA

For measuring the breakage and the re-joining reactions separately, we followed the conditions described by Been & Champoux (1981). In brief, the breakage reaction was started by mixing bacteriophage ϕX174 single-stranded DNA (25 μg/ml) with excess of purified topoisomerase (6.8 μg/ml) in a molar ratio of 1:4 in reaction buffer composed of 10 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-KCl, 1 mM-EDTA, 0.25 mM-dithiothreitol and 50% (v/v) glycerol. Note that no Mg^{2+} was contained in the buffer. After incubation at 37 °C for 20 min, 0.1 vol. of 0.11 M-$MgCl_2$ was added to the reaction mixture to start the re-joining reaction. At various time intervals samples were taken and subjected to electrophoresis on agarose gels as described by Been & Champoux (1981).

Analytical methods

Gel-filtration chromatography of heparin samples was performed on Ultrogel AcA 44 agarose/polyacrylamide gel or Sepharose 6B gel. Anticoagulant activity of heparin samples was assayed by the whole-blood assay method of the U.S. Pharmacopeia (1970), and the activity is expressed as units/mg. Total sulphate contents of heparin and sulphated polysaccharide samples were analysed by a turbidimetric method (Dodgson & Price, 1962).

RESULTS

Fig. 1 shows a typical profile of topoisomerase I inhibition by heparin, the IC_{50} being about 1.8 μg/ml. This inhibition seemed to be specific for heparin, as other natural glycosaminoglycans, including heparan sulphate, dermatan sulphate, chondroitin 4- and 6-sulphates and hyaluronic acid, were all without effect (Ishii *et al.*, 1982; see also Table 2 in the present paper). It may be taken for granted that the heparin inhibition of topoisomerase is due to its binding to enzyme by occupying the DNA-binding domain of the enzyme. This was tested by first binding the enzyme to a DNA–cellulose column followed by elution with increasing concentrations of heparin. Fig. 2 shows that the enzyme was primarily eluted at a heparin concentration of 10 μg/ml. Furthermore, in a previous paper (Ishii *et al.*, 1982) it was shown that the heparin inhibition was overcome by increasing either the substrate DNA or the enzyme protein. These results suggested that heparin inhibition was due to its competition with DNA. In the following sections we further investigated the molecular mechanism of the topoisomerase inhibition by heparin from two aspects: structure–activity relationship of heparin molecules in the inhibition, and additionally which of the breakage or the re-joining components of the topoisomerization reaction was more affected by heparin.

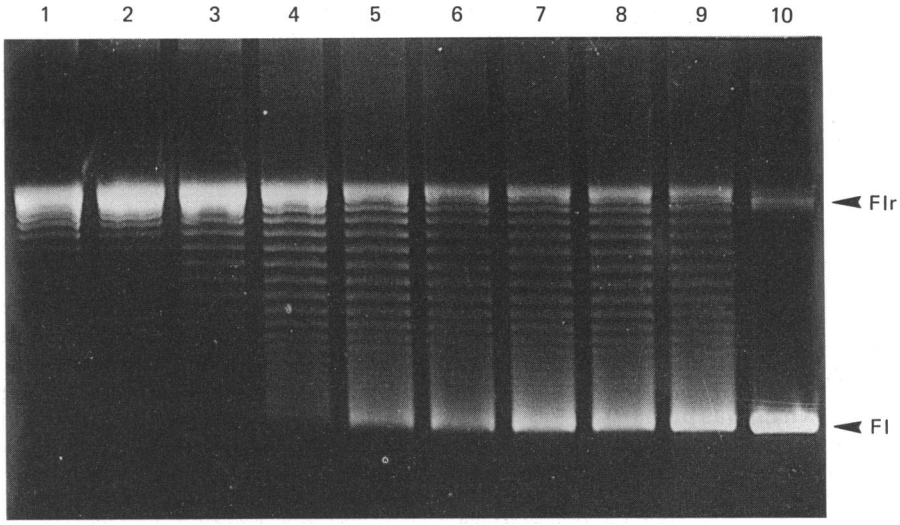

Fig. 1. Inhibition of topoisomerase I by heparin

Topoisomerase I was incubated with various amount of heparin and the products were analysed by agarose-gel electrophoresis. Concentrations of heparin were 0 μg/ml (tube 1), 0.25 μg/ml (tube 2), 0.75 μg/ml (tube 3), 1.25 μg/ml (tube 4), 1.75 μg/ml (tube 5), 2.50 μg/ml (tube 6), 3.25 μg/ml (tube 7), 3.75 μg/ml (tube 8) and 5.0 μg/ml (tube 9). No enzyme was added to reaction tube 10. FI and FIr denote the positions of the substrate supercoiled form I DNA and product relaxed form Ir DNA respectively. Electrophoresis is from above to below.

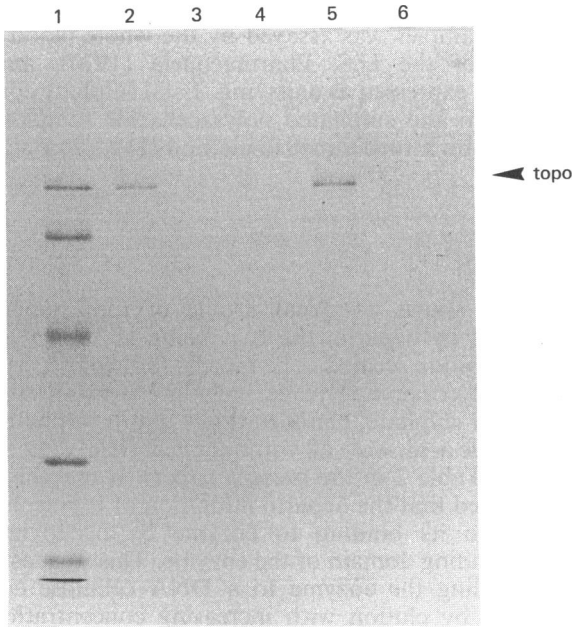

Fig. 2. Competitive binding of heparin to topoisomerase I

A preparation of purified topoisomerase I was applied to a column of DNA–cellulose. The enzyme (topo) was eluted stepwise with increasing concentrations of heparin. Eluates were analysed by SDS/polyacrylamide-gel electrophoresis. Lane 2, purified topoisomerase I; lanes 3–6, flowthrough, eluates with 0.001 μg, 0.1 μg, 10 μg and 1000 μg of heparin/ml respectively. Lane 1, marker proteins: from above, phosphorylase *b*, M_r 94000; bovine serum albumin, M_r 67000; ovalbumin, M_r 43000; carbonic anhydrase, M_r 30000; soya-bean trypsin inhibitor, M_r 20000; α-lactalbumin, M_r 14400.

What are the structural features of heparin molecules responsible for the inhibition?

Heparin is a highly sulphated glycosaminoglycan and is known to work as a multifunctional modulator through binding with a variety of proteins, including enzymes of nucleic acid metabolism (Walter *et al.*, 1967; Waldman & Goldstein, 1973; Slobin, 1976; Pfeffer *et al.*, 1977; DiCioccio & Srivastava, 1978), fibronectin (Stathakis & Mossesson, 1977; Rollins *et al.*, 1982), capillary endothelial-cell growth factor (Shing *et al.*, 1984) and antithrombin III (Björk & Lindahl, 1982). Heparin preparations are known to consist of heterogeneous molecules with differing degrees of affinity towards antithrombin III (Björk & Lindahl, 1982) and towards hydrophobic groups such as phenyl-Sepharose (Ogamo *et al.*, 1980). In order to obtain some insight into the structural features of heparin molecules responsible for the topoisomerase inhibition, we first fractionated a pig mucosal heparin preparation on an antithrombin III–Sepharose column into three fractions, namely non-adsorbed (NA), low-affinity (LA) and high-affinity (HA) fractions, the last being subdivided into three subfractions, S-I, S-II and S-III. We then asked whether these heparin fractions with differing degrees of affinity towards antithrombin III inhibit topoisomerase equally well or differentially according to their affinity towards antithrombin III. Table 1 gives the results of such an experiment in terms of IC_{50} together with their anticoagulant activity and some analytical data. The Table clearly shows that NA and LA fractions were completely inactive as anticoagulant, this activity being confined to HA fractions, in accordance with previous reports that only those heparin molecules contained in HA fractions possessed the unique oligosaccharide sequence responsible for the binding to antithrombin III

Table 1. Inhibitory activities of heparin fractions with differing degrees of affinity for antithrombin III on topoisomerase I

Pig intestinal heparin was fractionated on antithrombin III–Sepharose and the separated fractions were assayed for topoisomerase I inhibition and anticoagulant activities as described in the Materials and methods section. IC_{50}, the concentration giving 50% inhibition, is given as the average of two determinations that coincide within an error of 20%. $K_{av.}$ was calculated from the elution position from Ultrogel AcA 44 filtration.

Heparin fraction	IC_{50} (μg/ml)	Anticoagulant activity (U.S.P. units/mg)	$K_{av.}$	Total sulphate* (%)	Total sulphate* (mol/mol of of uronic acid)
Starting material	1.8	167	0.34	12.20	2.27
Non-adsorbed fraction (NA)	100.0	2	0.54	10.94	1.91
Low-affinity fraction (LA)	3.8	7	0.34	11.54	2.08
High-affinity fraction (HA)					
Subfraction S-I	2.3	214	0.32	12.48	2.36
Subfraction S-II	2.5	244	0.34	12.35	2.32
Subfraction S-III	1.3	299	0.28	12.60	2.40

* Includes *O*-sulphate and *N*-sulphate

Table 2. Effect of sulphation of various glycosaminoglycans and neutral polysaccharides on the inhibition of topoisomerase I

Various glycosaminoglycans and neutral polysaccharides were sulphated and assayed for topoisomerase I inhibition. $K_{av.}$ was calculated from the elution position from Sepharose 6B gel filtration.

Polysaccharide	Total sulphate (%)	Total sulphate (mol/mol of disaccharide unit)	$K_{av.}$	IC_{50} (μg/ml)
Heparin	12.20	2.27	0.51	5.0
Heparan sulphate	6.34	0.94	0.43	~ 300
Sulphated heparan sulphate	14.60	3.24	0.42	0.15
Chondroitin 4-sulphate	6.51	0.94	0.41	> 1000
Sulphated chondroitin 4-sulphate	14.20	2.98	0.43	0.20
Dermatan sulphate	6.73	0.98	0.26	> 1000
Sulphated dermatan sulphate	14.30	3.02	0.30	0.15
Sulphated dextran (D.P. 14)	17.30	3.90	0.78	5.0
Sulphated dextran (D.P. 64)	17.10	3.85	0.58	0.35
Sulphated amylose	17.30	3.66	0.54	0.35

(Björk & Lindahl, 1982). In contrast, however, all fractions except the NA fraction were active in topoisomerase inhibition. LA fraction is exceptional in that, although it is inactive as anticoagulant, it is almost as active as HA fractions in topoisomerase inhibition. It may be relevant that inactive NA fraction contained low-M_r heparin compared with LA and HA fractions, as evidenced by its high $K_{av.}$ value. This problem was pursued by subjecting the higher-M_r portion of the NA fraction to rechromatography on Ultrogel and examining its inhibitory activity. This high-M_r NA fraction with $K_{av.}$ 0.35 possessed much higher specific activity than that of the original NA fraction, IC_{50} being 10.0 μg/ml, one-tenth that of the NA fraction. Taken all together, these data can be summarized as follows. In contrast with the anticoagulant activity, i.e. irrespective of the affinity towards antithrombin III, heparin molecules with a certain degree of polymerization possess inhibitory activity on topoisomerase. This in turn suggests that the highly sulphated polyanionic nature of heparin rather than a specific oligosaccharide sequence is of critical importance in the inhibition; furthermore, it may well be speculated that no sugar-specificity is required for the polymer as long as it is sulphated to a certain degree. This postulate was tested in the following experiment, where we examined various artificially sulphated preparations of glycosaminoglycans and neutral polysaccharides for topoisomerase inhibition. The results are summarized in Table 2. Heparan sulphate, chondroitin 4-sulphate and dermatan sulphate were all inactive, whereas when sulphated to an extent of 3.0 or more sulphate groups per disaccharide unit these glycosaminoglycans were converted into potent inhibitors, as shown by a dramatic decrease in IC_{50} values for topoisomerase. Furthermore, the same goes with neutral polysaccharides such as dextran and amylose: surprisingly, glucans such as these were changed into strong inhibitors on sulphation. It is also to be noted in Table 2 that sulphated dextran with a higher degree of polymerization (D.P. 64) was more than 10 times as active as that with a lower degree of polymerization (D.P. 14). These observations lend strong support to the contention that the structural feature of

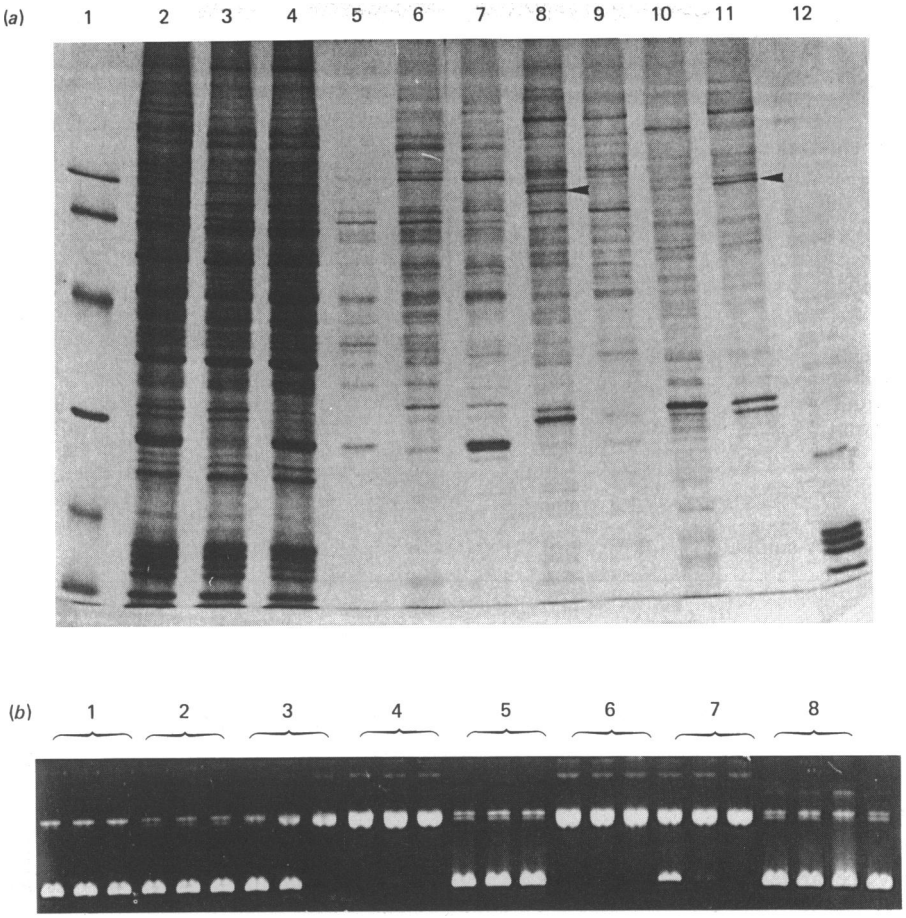

Fig. 3. Binding of heparin and sulphated dermatan sulphate to topoisomerase I

Chromatin extract was applied to columns of heparin–Sepharose 4B and sulphated dermatan sulphate–Sepharose 4B, and eluted stepwise with 0.4 M-, 0.6 M-, 0.8 M- and 1.0 M-NaCl. Eluates were assayed for topoisomerase activity (*b*) and for protein composition by SDS/polyacrylamide-gel electrophoresis (*a*). (*a*) Lane 2, chromatin extract; lanes 3, 5, 7, 9 and 11, flow through, 0.4 M-, 0.6 M-, 0.8 M- and 1.0 M-NaCl eluates respectively, from sulphated dermatan sulphate–Sepharose 4B; lanes 4, 6, 8, 10 and 12, flowthrough, 0.4 M-, 0.6 M-, 0.8 M- and 1.0 M-NaCl eluates respectively, from heparin–Sepharose 4B. Arrowheads indicate double bands of topoisomerase I. Lane 1, marker proteins as described in the legend to Fig. 2. (*b*) Sets 1–4, 0.4 M-, 0.6 M-, 0.8 M- and 1.0 M-NaCl eluates respectively from sulphated dermatan sulphate–Sepharose 4B; sets 5–8, 0.4 M-, 0.6 M-, 0.8 M- and 1.0 M-NaCl eluates respectively from heparin–Sepharose 4B. Within each set from left to right 2 μl, 4 μl and 8 μl portions of the respective eluate were analysed. The rightmost lane was for substrate col E1 DNA.

heparin molecules responsible for topoisomerase inhibition is that of a strong polyanionic nature, i.e. no saccharide specificity is required for the polymer to be a potent inhibitor as long as it is sulphated to a certain degree, and furthermore that it has a certain degree of polymerization.

The foregoing discussion is only pertinent provided that the artificially sulphated polysaccharides inhibit the enzyme by the same mechanism as heparin, i.e. by binding to the same site(s) on the same enzyme molecule as heparin. This assumption was tested by binding the enzyme preparation to heparin–Sepharose and to sulphated dermatan sulphate–Sepharose and eluting the enzyme with increasing concentrations of salts or sulphated glycosaminoglycans. First, chromatin extract prepared as described in the Materials and methods section was applied to a heparin–Sepharose 4B or sulphated dermatan sulphate–Sepharose 4B column and eluted with 0.4 M-, 0.6 M-, 0.8 M-, and 1.0 M-NaCl. Eluates were analysed for proteins by SDS/polyacrylamide-gel electrophoresis and for enzyme activity. The results are given in Fig. 3. As shown in Fig. 3(*b*), the main topoisomerase activity was eluted with 0.6 M-NaCl from heparin–Sepharose, whereas it is eluted from sulphated dermatan sulphate–Sepharose with 1.0 M-NaCl. In parallel with this observation, topoisomerase protein bands were mainly seen in the corresponding fractions (arrowheads in Fig. 3*a*). These observations clearly indicate that both heparin and sulphated dermatan sulphate bind to the enzyme, sulphated dermatan sulphate having a higher affinity than heparin for the enzyme. This is quite in accord with the sulphated dermatan sulphate being a more potent inhibitor than heparin (see Table 2). In a second series of experiment we applied a purified enzyme preparation to a column of heparin–Sepharose 4B and the column was eluted with either heparin or sulphated dermatan sulphate. The results given in Fig. 4 show that the enzyme bound to heparin was primarily eluted by free heparin at concentrations of 1.0 mg/ml or more, whereas it is eluted

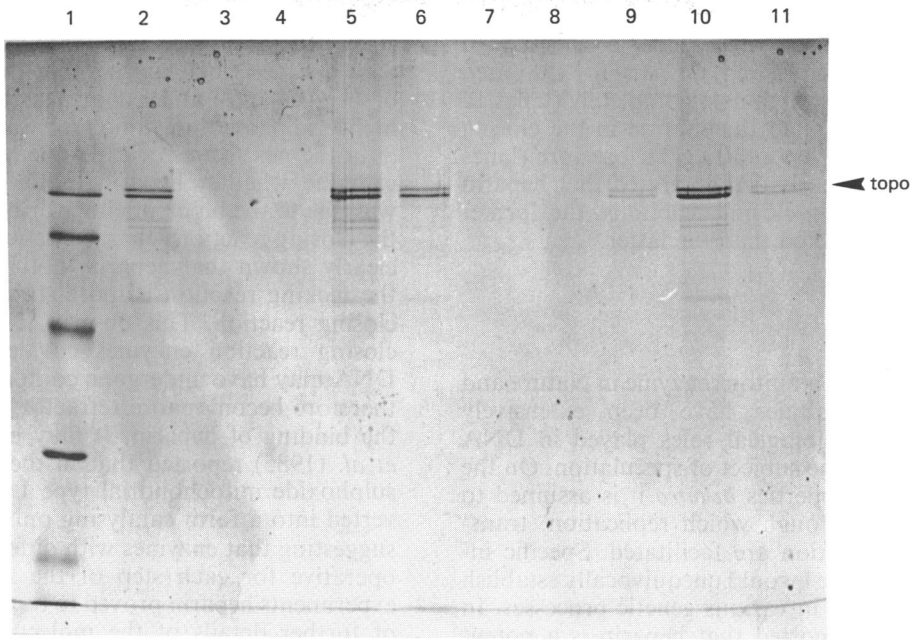

Fig. 4. Competitive binding of heparin and sulphated dermatan sulphate to topoisomerase I

Purified preparations of topoisomerase were applied to two parallel columns of heparin–Sepharose 4B. The enzyme (topo) was eluted stepwise with increasing concentrations of either heparin or sulphated dermatan sulphate. Eluates were analysed by SDS/15%-polyacrylamide-gel electrophoresis. Lane 2, purified topoisomerase I; lanes 3–6, flowthrough, eluates with 0.1 mg, 1.0 mg and 10.0 mg of heparin/ml respectively; lanes 7–11, flowthrough, eluates with 0.001 mg, 0.01 mg, 0.1 mg and 1.0 mg of sulphated dermatan sulphate/ml respectively. Lane 1, marker proteins as described in the legend to Fig. 2.

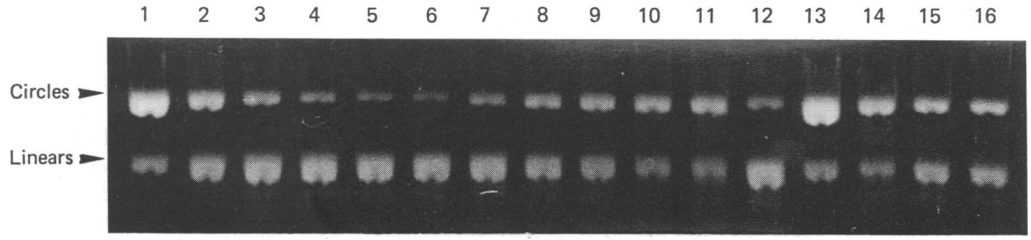

Fig. 5. Inhibition by heparin of the breaking and the re-joining reactions of topoisomerase I

The breaking reaction was started by mixing bacteriophage ϕX174 single-stranded circular DNA with excess of purified topoisomerase I in molar ratio of 1:4 in buffer without Mg^{2+}, and incubated at 37 °C. Mg^{2+} was added at 11 mM to the reaction mixture at 20 min to start the re-joining reaction, and the mixture was further incubated at 37 °C. At 0 min and at 20 min heparin was added at concentrations of 0.1, 10.0 and 20.0 μg/ml. Lanes 1–6, samples taken at 0, 2, 5, 10, 20 and 120 min respectively after the start of nicking reactions; lanes 7–11, samples taken at 1, 5, 10, 30 and 100 min respectively after the start of the re-joining reaction by the addition of Mg^{2+}; lanes 12 and 13, samples taken at 120 min after the initiation of the nicking reaction in the presence of heparin at 0.1 and 10.0 μg/ml respectively; lanes 14, 15 and 16, samples taken at 120 min after the initiation of the re-joining reaction in the presence of heparin at 0.1, 10.0 and 20.0 μg/ml respectively.

by a much lower concentration of sulphated dermatan sulphate, 0.1 mg/ml. These data seem to indicate that the sulphated dermatan sulphate binds competitively to the same site(s) on the enzyme molecules as heparin.

Does heparin inhibit the breakage and/or the re-joining reactions?

Establishing the structural feature of heparin molecules responsible for the inhibition, we next examined whether heparin affects the breakage and/or the re-joining reactions of topoisomerization. In order to carry out these experiments we took advantage of a system described by Been & Champoux (1981), where nicking and closing reactions of the enzyme can be separately measured, i.e. reaction of the enzyme on bacteriophage ϕX174 single-stranded circular DNA in the absence of Mg^{2+} results in a linear molecule with the enzymes covalently linked to the 3′-phospho end of the DNA (nicking reaction); addition of Mg^{2+} recircularizes the linear complex (closing reaction). This can be quantitatively measured by electrophoresis on agarose gels of the DNA samples after proteolysis. Thus we added various amounts of heparin to these reactions. The results are given in Fig. 5. The nicking reaction is completed in

about 20 min (lanes 1–6), and the closing reaction proceeds much faster (lanes 7–11). Heparin when added to the nicking reaction at 0.1 μg/ml partially inhibited the enzyme and at 10 μg/ml almost completely (lanes 12 and 13), whereas it inhibited the enzyme in the closing reaction only partially even at 10 μg/ml or more (lanes 14–16). These results clearly demonstrated that heparin inhibits both nicking and closing reactions, the former being more severely affected than the latter.

DISCUSSION

Topoisomerase I is a ubiquitous enzyme in Nature and its enzymological properties have been extensively studied. However, its biological roles played in DNA metabolism have been the subject of speculation. On the basis of its catalytic properties *in vitro* it is assumed to form a swivel point through which replication, transcription and recombination are facilitated. Specific inhibitors of topoisomerase I would unequivocally establish the roles of this enzyme in various genetic processes. In a previous paper we reported that heparin is a potent inhibitor of a mammalian DNA topoisomerase I (Ishii *et al.*, 1982). Heparin is a well-known inhibitor of the proteolytic cascade of blood coagulation through interaction with antithrombin III (Rosenberg, 1977; Björk & Lindahl, 1982). Heparin preparations are heterogeneous with respect to their affinity towards antithrombin III, and a specific oligosaccharide sequence responsible for the antithrombin III binding was determined and found only in the antithrombin-III-binding species. Heparin has also been reported to inhibit the activity of several other enzymes, including those involved in nucleic acid metabolism. However, no detailed mechanism of inhibition has been elucidated. In the present paper we undertook to determine the mechanism of topoisomerase inhibition by heparin. In the initial experiment we found that heparin fractions that did not bind to antithrombin III inhibited the enzyme at an efficiency comparable with that shown by the antithrombin-III-binding species. This observation led us to postulate that the highly sulphated polyanionic nature of heparin rather than a specific oligosaccharide sequence within it is of importance in the inhibition. Comparative studies with model compounds such as sulphated heparan sulphate, sulphated dermatan sulphate etc. revealed that they competitively bind to the same site(s) on the enzyme as heparin and thus led to the conclusion that the aforementioned hypothesis was correct. This mechanism of heparin inhibition of topoisomerase is quite in contrast with the situation of antithrombin III binding, where a specific oligosaccharide sequence plays a critical role. In a previous investigation we carried out similar mechanistic studies of heparin binding to fibronectin (Ogamo *et al.*, 1985), and reached the same conclusion that regardless of constituent saccharide the degree of sulphation and polymer size of sulphated polysaccharides are both critical factors determining the degree of binding to fibronectin. Heparin has been implicated to be a multifunctional modulator in living organisms by interacting with a variety of enzymes and proteins (Walter *et al.*, 1967; Waldman & Goldstein, 1973; Lam *et al.*, 1976; Slobin, 1976; Rosenberg, 1977; Pfeffer *et al.*, 1977; Stathakis & Mossesson, 1977; DiCioccio & Srivastava, 1978; Ishii *et al.*, 1982; Björk & Lindahl, 1982; Rollins *et al.*, 1982; Shing *et al.*, 1984; Ogamo *et al.*, 1985). The data just described suggest that these pleiotropic effects of heparin may at least in part be attributable to the highly sulphated polyanionic nature of the molecule.

In the next series of experiment we further set out to examine whether heparin inhibition of topoisomerase was due to the block of either or both of the nicking and the closing reactions characteristic of the enzyme. It was clearly shown that heparin inhibits both reactions, but the nicking reaction is more strongly affected than the closing reaction. This observation suggests that in the closing reaction enzymes covalently attached to the DNA may have undergone conformational change, and therefore become more refractory than free enzymes to the binding of heparin. It may be noted that Fairfield *et al.* (1985) reported that in the presence of dimethyl sulphoxide mitochondrial type I topoisomerase is converted into a form catalysing only the nicking reaction, suggesting that enzymes with different conformation are operative for each step of the reactions. From these experiments heparin proved to be a good tool for analysis of further details of the molecular mechanism of the topoisomerase reaction *in vitro*. However, it cannot be used as a specific inhibitor of the enzyme *in vivo*, because, as mentioned above, it inhibits a variety of enzymes non-specifically, and furthermore it is of high molecular mass and seemingly unable to enter a living cell. The possibility still remains that heparin exerts its regulatory effects on nuclear enzymes through endocytosis and penetration into the nucleus; this possibility is worth being tested in view of the fact that heparin plays an important role as modulator in various cell systems as described above.

The cytotoxic alkaloid camptothecin, which has strong anti-tumour activity against a wide range of experimental tumours, has been found to be an inhibitor of mammalian DNA topoisomerase I (Hsiang *et al.*, 1985). We have also found a series of low-M_r inhibitors of a mammalian DNA topoisomerase I as secondary microbial metabolites (K. Ishii, Y. Ikegami & T. Andoh, unpublished work). These substances may prove to be useful tools in the elucidation of the roles of the enzyme *in vivo* in various aspects of DNA metabolism.

This work was supported in part by the Japan Private School Promotion Foundation.

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Received 12 May 1986/24 July 1986; accepted 9 September 1986