Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 541-547

INHIBITION OF TOPOISOMERASE I BY HEPARIN

Kazuyuki Ishii[1], Atsuko Katase[1], Toshiwo Andoh[1] and Nobuko Seno[2]

Department of Hygienic Chemistry, Meiji College of Pharmacy, Yato 1-22-1 Tanashi, Tokyo 188[1] and Department of Chemistry, Ochanomizu University, Ohtsuka 2-1-1, Bunkyo-ku, Tokyo 112[2], Japan

Received November 17, 1981

DNA topoisomerase I isolated from mouse mammary cacinoma cells was shown to be inhibited by heparin, the dose giving 50% inhibition (IC_{50}) being 0.20 μg/ml. Other chemically related acid mucopolysaccharides including heparan sulfate, dermatan sulfate etc. were more than 500 times less active than heparin. When the amount of enzyme was doubled relative to the substrate the inhibition was reversed. Addition of heparin to assay mixtures after the initiation of the reaction immediately inhibited the enzyme reaction

INTRODUCTION

DNA topoisomerase I which catalizes the conversion of superhelical DNA to a relaxed convalently closed form have been found in a variety of prokaryotic and eukaryotic cells (1,2). The enzyme was reported to be localized in the nuclei largely in association with chromatin (3,4) and the activity increases in paralell with DNA synthesis in S phase in synchronized sea urchin embryonic cells (4). Recently, the enzyme was found to catalyze the catenation of double-stranded DNA rings (5). These observations together with the enzyme being capable of relaxing topological constraint of DNA led to suggestions that it might be involved in processes requiring such events, including replication, transcription, recombination and condensation and decondensation of DNA. We describe here the potent inhibition of the mammalian topoisomerase I by heparin. Heparin is a sulfated mucopolysaccharide which plays a well characterized role in inhibition of the proteo-

*Please address correspondence to Dr. Andoh.

0006-291X/82/020541-07$01.00/0

lytic cascade mechanism of coagulation through an interaction with antithrombin III (6). In addition it has been shown to inhibit the activity of several other mammalian enzymes, including DNA-dependent RNA polymerase (7,8), DNA polymerases and reverse transcriptase (8), and initiation and elongation factors (10,11). These observations have led to the hypothesis that heparin is a multifunctional molecule (12). Our results characterize the interaction of topoisomerase I with heparin as one of the most sensitive yet reported and may provide opportunity to explore physiological role of this enzyme in the metabolism of DNA.

MATERIALS AND METHODS

Topoisomerase I was prepared from cultured mouse mammary carcinoma cell FM3A (13) by the method of Germond *et al.* (14). One µl of the enzyme preparation converted 2 µg of native superhelical col E1 DNA to relaxed closed circular duplex DNA (DNA Ir) in 15 min at 37°C. One unit of enzyme defines as the activity that converts 1µg of col E1 DNA I to fully relaxed form Ir under the conditions used. Col E1 DNA I was purified from *E. coli* A745 met^- thy^- (col E1) by the method of Sakakibara and Tomozawa (15). Standard reaction mixture contained in 40 µl 1µg of col E1 DNA I, 10 unit of enzyme, 0,05 M sucrose and 0,15 M sodium phosphate, pH 7.5. Reaction mixture was incubated at 37°C for15 min and the reaction was terminated by addition of equal amount of 2x strength sample buffer (40 mM tris-HCl, 0.4 mM EDTA, 20% sucrose and 2% sodium dodecyl sulfate, pH 7.5) and incubation at 45°C for 15 min. When an inhibitor was to be tested, the inhibitor and DNA was mixed first and the reaction was initiated by addition of the enzyme. We characterized the DNA product by electrophoresis on vertical 1.2% agarose slab gels using the buffer system (36 mM tris-HCl, pH 7.7, 30 mM sodium phosphate, 1 mM EDTA) described by Germond and coworkers (14). A one cm cushion of 6% acrylamide and 0.16% bisacrylamide in the same buffer was formed at the bottom of the gels prior to formation of agarose gels. After loading of sample electrophoresis was carried out at 40 volts for 15 hrs at 4°C. The gels were stained with 1 µg/ml of ethidium bromide in the same buffer for 30 min. Fluorescent DNA bands were detected by illuminating the gel with short wave ultraviolet light and photographed. Amount of DNA was determined by a densitometric scanning of photographic negatives. Fluorometric measurement of enzymatic activity was also performed by the method of Vosberg *et al.* (15). Heparin from pig intestinal mucosa was purchased from Sigma Chemical Co. Heparan sulfate from pig kindney (17), chondroitin sulfate A from whale cartilage (18), chondroitin sulfate C from shark cartilage (18), dermatan sulfate and hyaluronic acid from shark skin (19) were prepared as described previously.

RESULTS

Heparin was found to be a potent inhibitor of topoisomerase I from mouse mammary carcinoma cells. Increasing concentrations

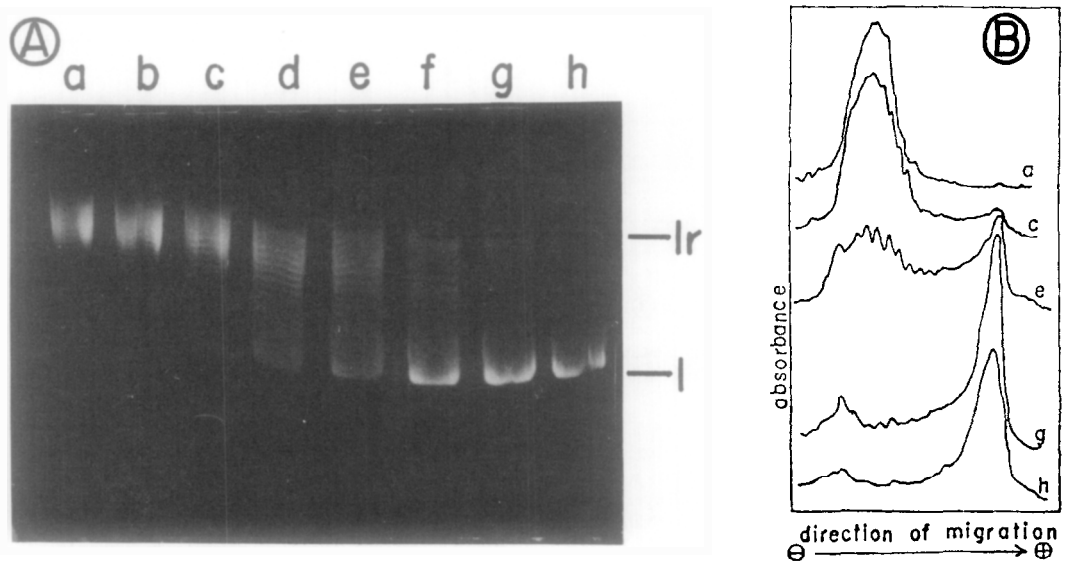

Figure 1 Inhibition of topoisomerase I by heparin. Increasing amount of heparin was added to the reaction mixture and the product DNAs were analyzed by agarose gel electrophoresis (A). Concentrations of heparin were in μg/ml, 0 (a,h), 0.03 (b), 0.08 (c), 0.13 (d), 0.18 (e), 0.25 (f), 0.38 (g). No enzyme was added to reaction tube h. Photographic negatives were scanned densitometrically (B); a, c, e, etc. in the tracing correspond to lanes a, c, e, etc. in electropherogram A. I and Ir were positions of form I and form Ir of col E1 DNA.

of heparin inhibited the enzyme in a dose-dependent manner as shown in Fig.1A and 1B. Complete inhibition at 0.50μg/ml and 50% inhibition at 0.20 μg/ml were obtained. Similar inhibition profile was obtained by fluorometric measurement, 50% inhibition being at 0.23 μg/ml (Fig. 2). To analyse the specificity of inhibition with respect to the nature of the mucopolysaccharide, chemically related mucopolysaccharides were tested. Table 1 shows the results obtained with heparin and other acid mucopolysaccharides. Dermatan sulfate was about 500 times and heparan sulfate was about 1000 times less active than heparin. No inhibition was observed with hyaluronic acid, chondroitin sulfate A and chondroitin sulfate C.

We investigated the nature of heparin inhibition of the enzyme by increasing the concentration of enzyme in the assay. As shown in Fig. 3 two fold excesses of enzyme protein overcame inhibition

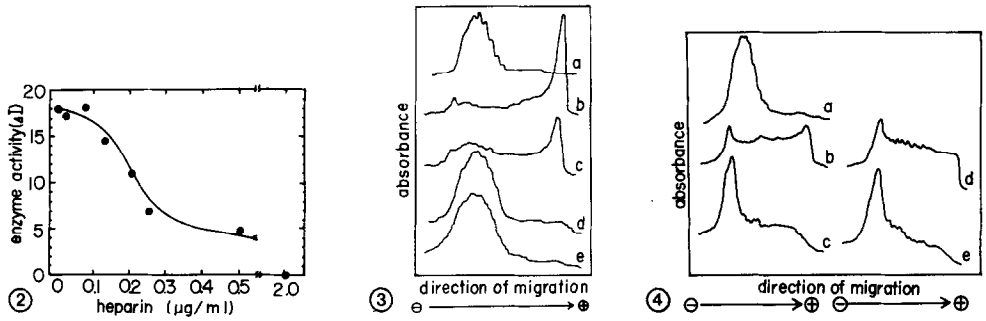

Figure 2 Fluorometric measurement of inhibition of topoisomerase I by heparin. Increasing amount of heparin was added to the reaction mixture containing 3 μg of DNA I. Enzymatic activity was measured fluorometrically (16).

Figure 3 Reversion of heparin inhibition by increasing the amount of enzyme. The reaction was initiated by the addition of 10 units (a,b), 14 units (c), 20 units (d) and 24 units (e) of enzyme to the reaction mixture containing 0.38 μg/ml of heparin. No heparin was added to reaction tube a. Reaction products were analyzed as described in figure 1.

Figure 4 Time course of inhibition of topoisomerase I by heparin. Tubes containing standard reaction mixture were preincubated in ice bach at 0°C for 20 min (b,d) or 40 min (a,c,e). Sodium dodecyl sulfate was added to reaction tubes d and e to terminate the reaction and heparin was added to tubes b and c at 0.75 μg/ml. Tubes a, b and c were further incubated at 37°C for 15 min. Products were analyzed as described in figure 1.

of heparin (cf. Fig. 3b and 3d). Addition of extra substrate DNA partially overcame heparin inhibition. At three fold or more concentrations of DNA partial revertion of inhibition was obtained (data not shown). Furthermore, heparin proved to immediatly inhibit an ongoing reaction when added to the reaction mixture. As shown in Fig. 4, when added at 20 or 40 min after the initiation of the reaction at 0°C when the relaxation of DNA was in progress heparin inhibited the reaction instantaneously.

DISCUSSION

Topoisomerase I has been found in a wide variety of prokaryotic and eukaryotic cells (1,2). The enzyme has been purified from bacteria (20-21), mammalian cells (23,24) and from vaccinia virion (25). The types of reactions catalyzed by this enzyme were elucidated, i.e. removal of superhelical turns, interconversion between single-stranded DNA rings with and without topological knots, combination of two single-stranded DNA rings of complementary

base sequences to give a covalently closed double-stranded ring and catenation of double-stranded DNA rings. In spite of these extensive enzymological studies no evidence has yet been obtained suggesting the physiological role of this enzyme. The ways to approach this problem may be two fold. First, if one could isolate mutant strains which possess some defect in this enzyme, one could speculate the physiolosical role of the enzyme from the behavior of the mutant. E. coli mutant deficient in this enzyme was isolated (26). Second, if one could obtain a specific inhibitor of this enzyme, one could also do the same from the response of the cells to the inhibitor. In the present paper we described for the first time an inhibitor of topoisomerase I. However, heparin might not be a specific inhibitor since it was shown to alter the activity of a number of enzymes including DNA-dependent RNA polymerase (7,8), various DNA polymerases (9), initiation and elongation factors (10,11), ribonuclease (27) and casein kinase II (28). Due to the lack of specificity of this inhibitor it might be of great interest to explore mechanism of the non-specific inhibition by this mucopolysaccharide. In this communication we have also shown that heparin inhibition was reversed by doubling the amount of enzyme relatative to the substrate and further that heparin inhibits tha reaction in progress, suggesting that heparin binds to enzyme and/or enzyme-substrate complex and whereby inhibits the reaction. The same was also reported with DNA polymerase (9) and DNA-dependent RNA polymerase working on single-stranded DNA template (7). It was found quite difficult to perform an accurate kinetic study due to the instability of the enzyme. Further investigation is under way to dissolve this problem.

We also analyzed the specificity of heparin inhibition of the enzyme with respect to the chemical nature of the mucopolysaccharide. Heparin proved to be the most potent inhibitor among

Table 1 Effect of acid mucopolysaccharides on the activity of topoisomerase I.

Compound	IC_{50} (μg/ml)[a]
Heparin	0.20
Heparan sulfate	200
Dermatan sulfate	100
Hyaluronic acid	NI[b]
Chondroitin sulfate A	NI
Chondroitin sulfate C	NI

[a]Concentration giving 50% inhibition.
[b]NI, no inhibition observed.

various chemically related mucopolysaccharides (Table 1). Preliminary experiment indicated that N-desulfation of heparin by the method of Foster et al. (29) resulted in loss of the inhibitory activity of the enzyme. These results suggest that the total sulfate content and/or glucosamine N-,6-disulfate might play a crucial role in inhibition, since most related mucopolysaccharide heparan sulfate which possesses only glucosamine N-sulfate (17) had little activity. Polymer nature of heparin might be of some importance. It is relevant to note that Lindahl et al. (30) obtained minimum fragment consisting of 12-16 suger residues from heparin which possesses inhibitory activity on antithrombin III. We are currently under way to investigate these problem.

ACKNOWLEDGEMENTS

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

REFERENCES

1. Wang, J. C. and Liu, L. F. (1979) in Molecular Genetics, Taylor, J. H. Ed. Part 3, pp 65-88, Academic Press, New York.
2. Champoux, J. J. (1978) Ann. Rev. Biochem. 47, 449-479.
3. Yoshida, S., Ungers, G. and Rosenberg, B. H. (1977) Nucl. Acids Res. 4, 223-228.
4. Poccis, D. L., Levine, D. and Wang, J. C. (1978) Dev. Biol. 64, 273-283.
5. Tse, Y-C. and Wang, J. C. (1980) Cell 22, 269-276.
6. Rosenberg, R. D. (1977) Fed. Proc. 36, 10-18.
7. Walter, G., Zillig, W., Palm, P. and Fuchs, E. (1967) Eur. J. Biochem. 3, 194-201.
8. Pfeffer, S. R., Stahl, S. J. and Chamberlin, M. J. (1977) J. Biol. Chem. 252, 5403-5407.
9. DiCioccio, R. A. and Srivastava, B. I. S. (1978) Cancer Res. 38, 2401-2407.

10. Waldman, A. A. and Goldstein, J. (1973) Biochem. 12, 2706-2711.
11. Slobin, L. I. (1976) Biochem. Biophys. Res. Commun. 73, 539-547.
12. Jaques, L. B. (1979) Science 206, 528-533.
13. Nakano, M. (1966) Tohoku J. Exp. Med. 88, 69-84.
14. Germond, J. E., Hirt, B., Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) Proc. Nat. Acad. Sci. USA 72, 1843-1847.
15. Sakakibara, Y. and Tomizawa, J-I. (1974) Proc. Nat. Acad. Sci. USA 71, 802-806.
16. Vosberg, H-P., Grossman, L. I. and Vinograd, J. (1975) Eur. J. Biochem. 55, 79-93.
17. Akiyama, F. and Seno, N. (1978) Nat. Sci. Rep. Ochamomizu Univ. 29, 147-153.
18. Seno, N., Anno, K., Yaegashi, Y. and Okuyama, T. (1975) Connective Tiss. Res. 3, 87-96.
19. Seno, N. and Meyer, K. (1963) Biochem. Biophys. Acta 78, 258-264
20. Burrington, M. G. and Morgan, A. R. (1976) Can. J. Biochem. 54, 301-306.
21. Kund, V. and Wang, J. C. (1977) J. Biol. Chem. 252, 5398-5402.
22. Depew, R. E., Liu, L. F. and Wang, J. C. (1978) J. Biol. Chem. 253, 511-518.
23. Champoux, J. J. and McConaughy, B. L. (1976) Biochem. 15, 4638-4643.
24. Keller, W. (1975) Proc. Nat. Acad, Sci. USA 72, 2550-2554.
25. Bauer, R. W., Ressner, E. C., Kates, J. and Patzke, J. V. (1977) Proc. Nat. Acad. Sci. USA 74, 1841-1845.
26. Strenglanz, R., DiNardo, S., Voelkel, K. A., Nishimura, Y., Hirota, Y., Becherer, K., Zumstein, L. and Wang, J. C. (1981) Proc. Nat. Acad. Sci. UAS 78, 2747-2751.
27. Flanagan, J. F. (1967) J. Cell. Physiol. 69, 117-124.
28. Hathaway, G. M., Lubben, T. H. and Traugh, J. A. (1980) J. Biol. Chem. 255, 8038-8041.
29. Foster, A. B., Harrisson, R., Inch, T. D., Stacey, M. and Wegger J. M. (1963) J. Chem. Soc., 2279-2287.
30. Lindahl, U., Backstrom, G., Höök, M. and Thunberg, L. (1979) Proc. Nat. Acad. Sci. USA 76, 3198-3202.