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Binding of heparin or dermatan sulfate to thrombin is essential for the sulfated polysaccharide-accelerated inhibition of thrombin by heparin cofactor II

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Heparin cofactor II (HC II) and thrombin were chemically modified with pyridoxal 5'-phosphate, and their effects on the inhibition of thrombin by HC II in the presence of heparin or dermatan sulfate were studied. The inhibition of thrombin by HC II was enhanced about 7000-fold in the presence of heparin or dermatan sulfate. However, this enhancement by heparin dwindled to 110- and 9.6-fold when the modified HC II and the modified thrombin, respectively, were substituted for native proteins. Essentially identical results were obtained from the experiments using dermatan sulfate. These results indicate that the binding of heparin or dermatan sulfate to both thrombin and HC II is required for the sulfated polysaccharide-dependent acceleration of the thrombin inhibition by HC II, and the binding to thrombin is more essential for the reaction.

Heparin; Heparin cofactor II; Thrombin; Pyridoxal 5'-phosphate; Ternary model

1. INTRODUCTION

Heparin cofactor II (HC II) is a plasma protease inhibitor which inhibits thrombin by forming a stable 1:1 molar complex [1–5]. It is well known that the thrombin inhibitory activity of HC II is dramatically accelerated by some sulfated polysaccharides such as heparin, dermatan sulfate [6], dextran sulfate [7], chondroitin polysulfates [8] and pentosan polysulfate [9]. However, the mechanism of the action of these sulfated polysaccharides to accelerate the interaction between HC II and thrombin is still controversial. Griffith [10] presented the kinetic evidence that heparin is bound to both HC II and thrombin, forming a ternary complex. On the other hand, more recently, it has been suggested from the chemical modification of HC II that the binding of heparin to HC II is essential for the reaction [11,12]. Here, we present new evidence to support the model of a ternary complex formation [10] and show that the binding of heparin or dermatan sulfate to thrombin is more essential than that to HC II for the heparin- or dermatan sulfate-accelerated thrombin inhibition by HC II.

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2. EXPERIMENTAL

Porcine mucosa heparin (168 units/mg, M_r 7000–20000) and porcine skin dermatan sulfate (M_r 20000–40000) were purchased from Sigma and Seikagaku Kogyo, respectively. Both polysac-

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charides are free from other polysaccharides [8]. HC II and thrombin were purified from human plasma as described in [7,13], and chemically modified with pyridoxal 5′-phosphate as described in [11,14], respectively. Modified proteins were applied to a heparin-Sepharose CL-6B column equilibrated with 0.1 M triethanolamine (pH 7.8)/0.1 M NaCl/0.1% polyethyleneglycol at room temperature, and the pass-through fractions were pooled and used in subsequent experiments. HC II and thrombin treated similarly but in the absence of pyridoxal 5′-phosphate were used as intact proteins. The incorporation of pyridoxal 5′-phosphate into protein was determined from absorbance at 322 nm using an extinction coefficient of 9000 $M^{-1} \cdot cm^{-1}$ [14]. The second-order rate constant (k'') of the HC II-thrombin interaction was determined using a synthetic substrate [15].

In this paper, the inhibition of intact thrombin by intact HC II and that by modified HC II are referred to as inhibitions A and B, respectively, and the inhibition of modified thrombin by intact HC II and that by modified HC II as inhibitions C and D, respectively.

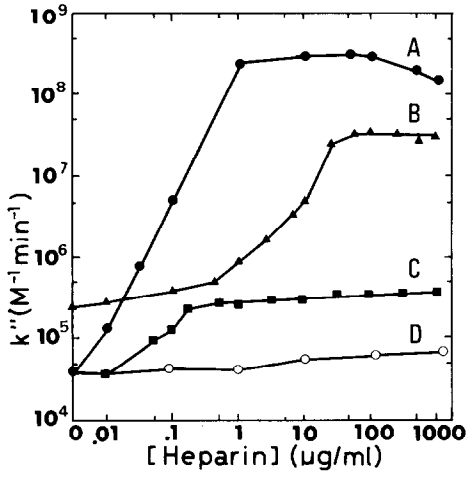

Fig.1. Kinetics of thrombin inhibition by HC II in the presence of heparin. Second-order rate constants (k'') of inhibitions A (intact thrombin and intact HC II) (●), B (intact thrombin and modified HC II) (▲), C (modified thrombin and intact HC II) (■) and D (modified thrombin and modified HC II) (○) were plotted as a function of heparin concentration. Rate constants were determined according to [15].

3. RESULTS

To abolish the heparin-binding abilities of HC II and thrombin, these two proteins were phosphopyridoxylated according to [11,14]. When the modified proteins were applied to a heparin-Sepharose column as described in section 2, about 95 or 60% of HC II or thrombin, respectively, was passed through the column. The pass-through fractions were pooled and used in subsequent experiments so that proteins retaining the heparin-binding ability were completely avoided.

HC II and thrombin incorporated 4.1 and 2.7 mol per mol, respectively, of pyridoxal 5′-phosphate, which resulted in the loss of their heparin-binding abilities without significant loss of their inhibitory and amidolytic activities, respectively. These results are consistent with [11,14].

Heparin-independent k'' of the inhibition of modified thrombin by intact HC II (inhibition C) and that by modified HC II (inhibition D) were almost identical to that of the inhibition of intact thrombin by intact HC II (inhibition A), giving a value of about 5×10^4 $M^{-1} \cdot min^{-1}$. This value of heparin-independent k'' of inhibition A is about 10-fold lower than that reported by Tollefsen et al. [3] but is equal to that reported by Griffith et al. [5,11]. However, heparin-independent k'' of the

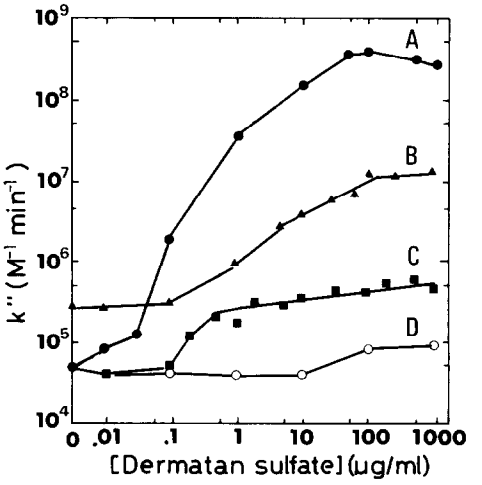

Fig.2. Kinetics of thrombin inhibition by HC II in the presence of dermatan sulfate. Experiments were conducted as described in fig.1 except that dermatan sulfate was used instead of heparin.

inhibition of intact thrombin by modified HC II (inhibition B) increased to 3.3×10^5 $M^{-1} \cdot min^{-1}$ for unknown reasons. This phenomenon has also been described in [11].

Effects of phosphopyridoxylations of HC II and/or thrombin on the heparin-dependent inhibition of thrombin by HC II are shown in fig.1. A modification of either protein resulted in a remarkable decrease of the maximum k'' of the reaction between HC II and thrombin. The decrease in k'' of inhibition C was much more striking than that of inhibition B, giving the maximum k'' of 3.2×10^8, 3.6×10^7, 4.7×10^5 and 5.7×10^4 $M^{-1} \cdot min^{-1}$ for inhibitions A, B, C and D, respectively.

Unexpectedly, no decrease in k'' of inhibition B and C was observed even in the presence of excess amounts of heparin, while k'' of inhibition A decreased at heparin concentrations above 50 μg/ml. It should also be pointed out that k'' of inhibition B was saturated at a heparin concentration of 100 μg/ml, while inhibition C was saturated at as low as 1 μg/ml heparin.

Essentially identical results were obtained from experiments using dermatan sulfate as sulfated polysaccharide (fig.2), although the difference of k'' between inhibition B and C was slightly smaller than that in the case of heparin, giving a maximum k'' of 3.8×10^8, 1.6×10^7, 5.4×10^5 and 9.6×10^4 $M^{-1} \cdot min^{-1}$ for inhibitions A, B, C and D, respectively.

4. DISCUSSION

The results presented here indicate that the binding of heparin to thrombin is essential for the heparin-accelerated interaction between HC II and thrombin. The rate of the heparin-dependent thrombin inhibition by HC II was remarkably decreased by a modification of either thrombin or HC II, and when both modified thrombin and modified HC II were mixed in the presence of heparin (inhibition D), essentially no acceleration by heparin could be observed, indicating that the binding of heparin to both thrombin and HC II is required for the acceleration of thrombin-HC II interaction by heparin. These results support the ternary complex model proposed by Griffith [10]. However, it should be pointed out that the effect of the modification of thrombin was much more striking than that of HC II, indicating that the binding of heparin to thrombin is more essential for the acceleration by heparin.

Essentially identical results were obtained from the experiments using dermatan sulfate. In both cases, the loss of heparin-binding or dermatan sulfate-binding ability of thrombin resulted in a more drastic decrease of the sulfated polysaccharide-dependent acceleration of the thrombin inhibition by HC II. These results also indicate that lysine residues of both HC II and thrombin are essential for their dermatan sulfate-binding abilities.

Results analogous to ours have been reported for the inhibition of thrombin by antithrombin III in the presence of heparin. Pomerantz and Owen [16] and Griffith [14] demonstrated that heparin could not enhance the thrombin inhibition by antithrombin III when the heparin-binding ability of thrombin was abolished by a modification of arginyl or lysyl residues. Based on these and subsequent studies [17], a ternary complex (or a template) model in which heparin is simultaneously bound to both thrombin and antithrombin III has generally been accepted.

REFERENCES

[1] Briginshaw, G.F. and Shanberge, J.N. (1974) Arch. Biochem. Biophys. 161, 683–690.
[2] Tollefsen, D.M. and Blank, M.K. (1981) J. Clin. Invest. 68, 589–596.
[3] Tollefsen, D.M., Majerus, D.W. and Blank, M.K. (1982) J. Biol. Chem. 257, 2162–2169.
[4] Wunderwald, P., Schrenk, W.J. and Port, H. (1982) Thromb. Res. 25, 177–192.
[5] Griffith, M.J., Noyes, C.M. and Church, F.C. (1985) J. Biol. Chem. 260, 2218–2225.
[6] Tollefsen, D.M., Pestka, C.A. and Monafo, W.J. (1983) J. Biol. Chem. 258, 6713–6716.
[7] Yamagishi, R., Niwa, M. and Sakuragawa, N. (1986) Thromb. Res. 44, 347–354.
[8] Yamagishi, R., Niwa, M., Kondo, S., Sakuragawa, N. and Koide, T. (1984) Thromb. Res. 36, 633–642.
[9] Scully, M.F., Ellis, V. and Kakkar, V.V. (1986) Thromb. Res. 41, 489–499.

[10] Griffith, M.J. (1983) Proc. Natl. Acad. Sci. USA 80, 5460–5464.
[11] Church, F.C. and Griffith, M.J. (1984) Biochem. Biophys. Res. Commun. 124, 745–751.
[12] Church, F.C., Villanueva, G.B. and Griffith, M.J. (1986) Arch. Biochem. Biophys. 246, 175–184.
[13] Lundblad, R.L., Kingdon, H.S. and Mann, K.G. (1976) Methods Enzymol. 45, 156–176.
[14] Griffith, M.J. (1979) J. Biol. Chem. 254, 3401–3406.
[15] Griffith, M.J. (1982) Thromb. Res. 25, 245–253.
[16] Pomerantz, M.W. and Owen, W.G. (1978) Biochim. Biophys. Acta 535, 66–77.
[17] Björk, I. and Lindahal, U. (1983) Mol. Cell. Biochem. 48, 161–182.