Antithrombin action of phosvitin and other phosphate-containing polyanions is mediated by heparin cofactor II

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We have examined the antithrombin effects of various phosphate-containing polyanions (including linear polyphosphates, polynucleotides and the phosphoserine glycoprotein, phosvitin) on the glycosaminoglycan-binding plasma proteinase inhibitors, antithrombin III (ATIII) and heparin cofactor II (HCII). These phosphate-containing polyanions accelerate the HCII-thrombin reaction, as much as 1600-fold in the case of phosvitin. The HCII-thrombin reaction with both phosvitin and polynucleotides appears to follow the ternary complex mechanism. The HCII-thrombin complex is rapidly formed in the presence of these phosphate polyanions (each at 10 μg/ml) when ^{125}I-labeled thrombin is incubated with human plasma (ex vivo). None of these phosphate polyanions accelerate the ATIII-thrombin reaction. Our results suggest that the antithrombotic effect of these phosphate-containing polyanions is mediated by HCII activation and not by ATIII.

Heparin cofactor II; Antithrombin III; Phosphate polyanion

1. INTRODUCTION

Heparin is a glycosaminoglycan that is used therapeutically as an anticoagulant [1]. The antithrombin activity of heparin is effected through interaction with two plasma glycoproteins, antithrombin III (ATIII) and heparin cofactor II (HCII) (for review see [2–6]). ATIII inhibits all of the proteinases involved in intrinsic blood coagulation [6]. The coagulation proteinase specificity of HCII is limited to thrombin [7].

The structure of the heparin (polyanion)-binding sites in ATIII and HCII remain to be fully elucidated. However, the antithrombin action of heparin is attributed in part to its ability to bind both inhibitor (ATIII/HCII) and thrombin to form a ternary complex ([8–10] and references cited therein). Dermatan sulfate also accelerates

the HCII-thrombin reaction but it has essentially no effect on the ATIII-thrombin reaction [11–13]. We studied the interaction of various phosphate-containing polyanions with HCII and ATIII to examine further the specificity of the heparin (polyanion)-binding sites of these proteinase inhibitors.

We report here that the antithrombin action of various phosphate-containing polyanions (including linear polyphosphates, polynucleotides and phosvitin, a phosphoserine glycoprotein) is mediated through HCII and not through ATIII. The findings further suggest that these phosphate-containing polyanions are potential therapeutic antithrombotics.

2. EXPERIMENTAL

2.1. *Materials*

HCII, ATIII and thrombin were prepared from human plasma as described previously [14,15] and their purity assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Linear polyphosphates, polynucleotides, phosvitin, poly(ethyleneglycol) (M_r = 8000), and salmon protamine sulfate were obtained from Sigma (St. Louis, MO). Dansyl-Glu-Gly-

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Arg chloromethylketone (DEGR) was from Calbiochem (La Jolla, CA). Activated partial thromboplastin time (aPTT) reagents were obtained from Pacific Hemostasis (Ventura, CA). ^{125}I-labeled thrombin (with approx. 3×10^{17} dpm/mol proteinase) was prepared as detailed previously [16]. DEGR-thrombin [10] and lysine-modified HCII [17] were prepared essentially as described previously. The M_r values and extinction coefficients (ml · mg^{-1} · cm^{-1} at 280 nm) were taken as 65 600 and 0.593 for HCII, 56 600 and 0.624 for ATIII and 36 600 and 1.75 for thrombin [5].

2.2. Assays

HCII (and ATIII) activity was determined by measuring the rate of thrombin inhibition in the absence and presence of either heparin or the phosphate-containing polyanions in 50 mM triethanolamine-acetate, 100 mM NaCl, 0.1% poly(ethylene-glycol) buffer at pH 8.0 and 25°C with at least a 10-fold molar excess of proteinase inhibitor to thrombin as described previously [5,14,15]. Inhibition rate constants were calculated as detailed [16]. The anticoagulant activity of the phosphate-containing polyanions was measured using an aPTT clotting assay following the manufacturer's procedure.

2.3. Other determinations and methods

The kinetic model and association rate equations used in this study assume that the heparin-catalyzed ATIII- or HCII-thrombin reaction is analogous to a bireactant enzyme-catalyzed reaction that follows a random order mechanism as described previously [8]. PAGE was performed in the Laemmli buffer system with 7.5% polyacrylamide gels [18]. Plasma incubation with ^{125}I-labeled thrombin was performed essentially as described previously [12,16]. Extrinsic fluorescence measurements of DEGR-thrombin in the absence and presence of various polyanions were performed as described [10].

3. RESULTS

The effect of linear polyphosphates on thrombin inhibition by HCII and ATIII was investigated. Tripolyphosphate enhanced the rate of thrombin inhibition by HCII, but not by ATIII, in a dose-dependent fashion (fig.1A). The maximal increase in activity ($\sim$60-fold) was similar when the nucleotide analog, ATP, was substituted for tri-polyphosphate in the reaction (fig.1A). Larger polyphosphate species (with average phosphate chain lengths ranging from 4 to 65) also accelerated the HCII-thrombin reaction maximally more than 800-fold but with no effect on the ATIII-thrombin reaction (fig.1B).

In order to examine the influence of the non-phosphate components of polyphosphate-containing compounds, we compared various polynucleotides in their ability to augment the rate of the HCII-thrombin reaction. The rate of thrombin inhibition by HCII in the presence of synthetic
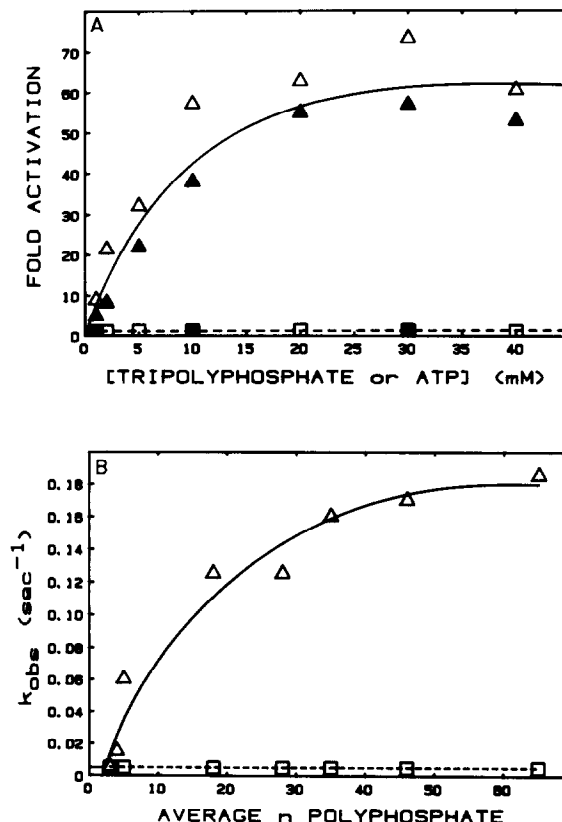

Fig.1. Thrombin inhibition by HCII (△, ▲) and ATIII (□, ■) in the presence of (A) tripolyphosphate (△, □) or ATP (▲, ■) and (B) polyphosphates with average chain lengths ranging from 4 to 65 (each at 1 mM in phosphate) (△, □). The inhibition reaction was performed as described in section 2.

Table 1

Effect of synthetic polynucleotides on the inhibition of thrombin by HCII

Polynucleotide[a]	Rate enhancement (-fold)[b]
Poly(guanylate)	400
Poly(adenylate, guanylate)	430
Poly(inosinate)	160
Poly(guanylate, uridylate)	135
Poly(uridylate)	23
Poly(adenylate)	21
Poly(cytidylate)	12

[a] With the exception of poly(G) which was 10 μg/ml, the polynucleotides were 100 μg/ml in the HCII-thrombin reaction as detailed in section 2

[b] The relative rate of enhancement was compared to the rate constant of thrombin inhibition by HCII in the absence of any polyanion
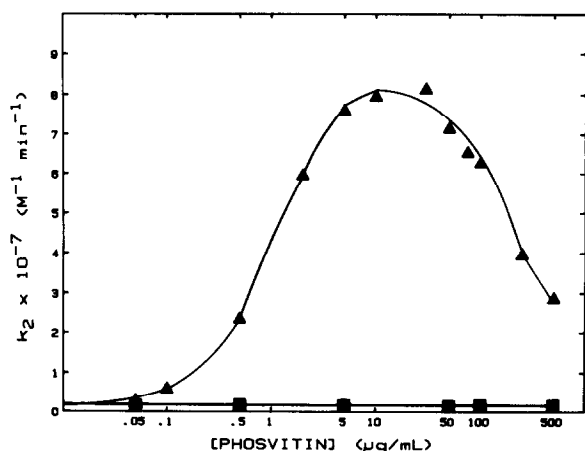
Fig.2. Apparent second order rate constants (k_2) for thrombin inhibition by HCII (▲) and ATIII (■) as a function of phosvitin concentration. Inhibition reaction conditions and rate constant determinations are detailed in section 2.

polynucleotides was significantly greater than the rate measured in the absence of any polyanion (table 1). The maximal effect on HCII-thrombin was exhibited by guanylic acid-containing species. The polynucleotides did not enhance the ATIII-thrombin inhibition rate.

The ability of phosphate groups to accelerate the rate of thrombin inhibition by HCII was further investigated with the phosphoserine-containing glycoprotein, phosvitin. Fig.2 depicts the concentration dependence for stimulation of the HCII-thrombin reaction rate by phosvitin. The rate constant for thrombin inhibition by HCII increased from 5×10^4 $M^{-1} \cdot min^{-1}$ (in the absence of phosvitin) to 8×10^7 $M^{-1} \cdot min^{-1}$ as the phosvitin concentration increased from 0.05 to 30 $\mu g/ml$ and then decreased as phosvitin was increased above 30 $\mu g/ml$. The relationship between poly-(guanylate) (poly(G)) and poly(adenylate, guanylate) concentration and the rate of HCII-thrombin inhibition was similar to that of phosvitin (not shown). As found for the other phosphate-containing polyanions, phosvitin had no effect on the ATIII-thrombin reaction (fig.2).

The kinetic mechanism of the phosvitin-catalyzed HCII-thrombin reaction was evaluated by varying the HCII and thrombin concentration at a fixed phosvitin concentration (fig.3). Saturation kinetics were observed with apparent dissociation constants for HCII-phosvitin and thrombin-phosvitin of 690 and 10 nM, respectively. A similar kinetic analysis for the poly(G)-catalyzed HCII-
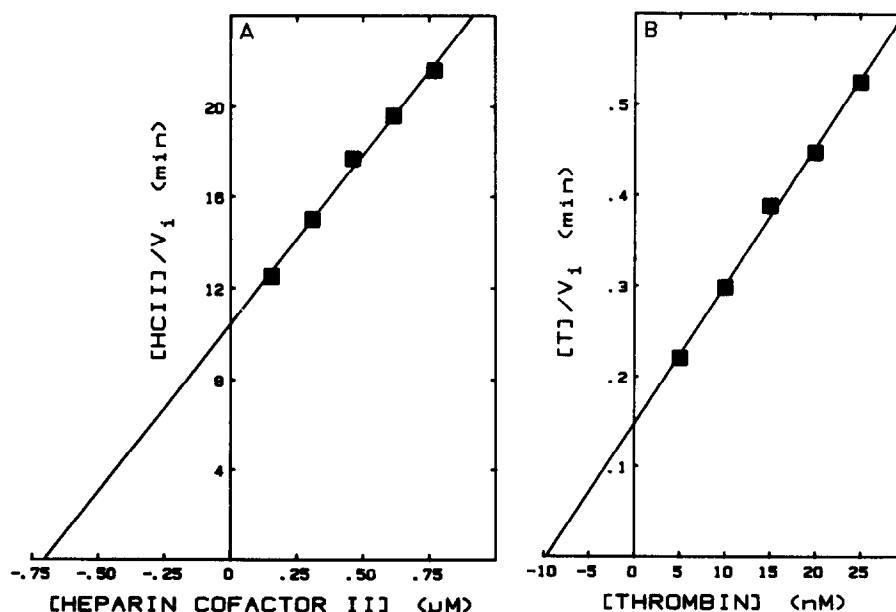

Fig.3. Kinetics of the phosvitin-HCII-thrombin reaction were analyzed by determining the initial rate (v_i) of thrombin (T) inhibition by HCII in the presence of 250 ng/ml phosvitin. (A) Initial T concentration was 5 nM. (B) Initial HCII concentration was 770 nM. Data are plotted as described in section 2.

thrombin reaction yielded apparent dissociation constants for HCII-poly(G) and thrombin-poly(G) of 520 and 15 nM, respectively.

Lysine-modified HCII (phosphopyridoxylated to an extent of 4 mol of reagent incorporated/mol protein) and DEGR-thrombin were used to assess the importance of phosphate-containing polyanion binding to both inhibitor and proteinase during thrombin inhibition. Modified HCII lost >80% of the heparin (and dermatan sulfate) cofactor activity compared to the unmodified proteinase inhibitor. Enhanced thrombin inhibition in the presence of phosphate-containing polyanions [for instance, poly(G), phosvitin and polyphosphate (average chain length of 65)] was greatly reduced (an average of 84%) with lysine-modified HCII. Poly(G) and polyphosphate (average chain length of 65) produced an extrinsic fluorescence signal enhancement (~3-fold) in DEGR-thrombin, indicating that their binding altered the environment of the dansyl moiety in the active site of thrombin.

Calcium and protamine were added to phosvitin and poly(G) to investigate the importance of the polyanion charge on the HCII-thrombin reaction. The phosvitin-catalyzed HCII-thrombin reaction was inhibited more than 97% with rate constants of 6.6×10^6 and $<1.6 \times 10^5\ M^{-1} \cdot min^{-1}$ in the absence and presence of calcium (10 mM), respectively. Protamine (at a 100-fold excess by weight) eliminated >98% of the poly(G) effect on the HCII-thrombin reaction.

The anticoagulant activity of phosvitin, poly(G) and polyphosphate (average chain lengths of 5 and 65) was assessed in plasma. In an aPTT clotting assay, heparin (by weight) is about 100 times more potent as an anticoagulant in plasma than these phosphate-containing polyanions.

The ability of various phosphate-containing polyanions to activate HCII was further investigated in a plasma system. This ex vivo system consisted of incubating ^{125}I-labeled thrombin with plasma and then analyzing the reaction products by SDS-PAGE and autoradiography. As shown in fig.4, incubation of ^{125}I-labeled thrombin with plasma either in the presence of phosvitin, poly(G) or polyphosphate with a chain length of 65 (each at 10 μg/ml) was correlated with incorporation into a complex with HCII. There was no increase in the amount of ^{125}I-labeled thrombin incorporated into a complex with ATIII (fig.4).
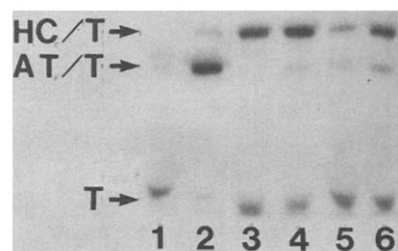

Fig.4. Activation of HCII in human plasma was assessed by incubating ^{125}I-labeled thrombin (T) (5 nM), for 10 min at 25°C, in citrated plasma (diluted 1:50) in the presence of phosphate-containing polyanions (each at 10 μg/ml), followed by SDS-PAGE and autoradiography as detailed in section 2. T, purified ATIII-heparin and HCII-dermatan sulfate complexes with T are shown in lanes 1–3, respectively; the plasma system with phosvitin, poly(G) and polyphosphate (average chain length of 65) are in lanes 4–6, respectively.

4. DISCUSSION

In the present study we have shown that various phosphate-containing polyanions greatly enhance the rate of the HCII-catalyzed thrombin inhibition reaction in vitro. Furthermore, the HCII-thrombin complex is rapidly formed when ^{125}I-labeled thrombin is incubated with human plasma (ex vivo) in the presence of either phosvitin, poly(G) or polyphosphate. In all cases, these phosphate-containing polyanions have no effect on the ATIII-catalyzed thrombin reaction in vitro or ex vivo.

HCII is apparently activated by the multiple negative charges of these phosphate polyanions, since the polyanion effect can be negated by complexing the phosphate with calcium or protamine. The effective phosphate polyanions must also possess a specific structure for maximal acceleration of the HCII-thrombin reaction, as shown by the range of rate constants for thrombin inhibition by HCII in the presence of various polynucleotides (also see [19]).

The characteristics of the phosvitin- and polynucleotide-catalyzed HCII-thrombin reaction are similar to those found for HCII and thrombin interactions with heparin or dermatan sulfate [8–10,12,17]. The results with chemically modified HCII illustrate the importance of phosphate polyanion binding to HCII for the catalytic effect during thrombin inhibition. The results with DEGR-thrombin suggest that phosphate polyanion binding to the proteinase is important; this effect is

similar to that reported for heparin-DEGR-thrombin [10]. The rate constant for phosvitin-accelerated thrombin inhibition by HCII increases in a concentration-dependent manner, reaches a maximum, and then decreases as phosvitin concentration is further increased. The shape of the curve implies that phosvitin (and polynucleotides) forms a ternary complex ('template') with binding to both HCII and thrombin. The binding sites for HCII and thrombin probably reside in the phosphoserine-rich core region of phosvitin [20]. This is the first demonstration that a protein or a polynucleotide, not a glycosaminoglycan like heparin or dermatan sulfate, can serve as a surface (or template) for thrombin inhibition by a proteinase inhibitor.

Although an in vivo role can be proposed for thrombin inhibition by HCII, the physiological function of this inhibitor remains to be fully understood. Nevertheless, our results support the concept of a new class of antithrombotics that are mediated through HCII and not through ATIII. The common feature of these compounds is a high charge density of phosphate polyanions. Further investigation with these antithrombotics may permit us to evaluate the biological functions of HCII.

REFERENCES

[1] Jacques, L.B. (1979) Science 206, 528–533.
[2] Rosenberg, R.D. (1979) Fed. Proc. 36, 10–18.
[3] Bjork, I. and Lindahl, U. (1982) Mol. Cell. Biochem. 48, 161–182.
[4] Blinder, M.A., Marasa, J.C., Reynolds, C.H., Deaven, L.L. and Tollefsen, D.M. (1988) Biochemistry 27, 752–759.
[5] Church, F.C., Meade, J.M. and Pratt, C.W. (1987) Arch. Biochem. Biophys. 259, 331–340.
[6] Travis, J. and Salvesen, G.S. (1983) Annu. Rev. Biochem. 52, 655–709.
[7] Parker, K.A. and Tollefsen, D.M. (1985) J. Biol. Chem. 260, 3501–3505.
[8] Griffith, M.J. (1983) Proc. Natl. Acad. Sci. USA 80, 5460–5464.
[9] Danielsson, A., Raub, E., Lindahl, U. and Bjork, I. (1986) J. Biol. Chem. 261, 15467–15473.
[10] Nesheim, M., Blackburn, M.N., Lawler, C.M. and Mann, K.G. (1986) J. Biol. Chem. 260, 3214–3221.
[11] Teien, A.N., Abildgaard, U. and Hook, M. (1976) Thromb. Res. 8, 859–867.
[12] Tollefsen, D.M., Pestka, C.A. and Monafo, W.F. (1983) J. Biol. Chem. 258, 6713–6716.
[13] Griffith, M.J. and Marbet, G.B. (1983) Biochem. Biophys. Res. Commun. 112, 663–670.
[14] Griffith, M.J., Church, F.C. and Noyes, C.M. (1985) J. Biol. Chem. 260, 2218–2225.
[15] Church, F.C. and Whinna, H.C. (1986) Anal. Biochem. 157, 77–83.
[16] Church, F.C., Treanor, R.E., Sherrill, G.B. and Whinna, H.C. (1987) Biochem. Biophys. Res. Commun. 148, 362–368.
[17] Church, F.C. and Griffith, M.J. (1984) Biochem. Biophys. Res. Commun. 124, 745–751.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Saenger, W. (1984) Principles of Nucleic Acid Structure, pp. 298–320, Springer-Verlag, New York.
[20] Marion Byrne, B., Van het Schip, A.D., Van de Klundert, J.A.M., Arnberg, A.C., Gruber, M. and Ab, G. (1984) Biochemistry 23, 4275–4279.