Heparin accelerates the inhibition of cathepsin G by mucus proteinase inhibitor: potent effect of *O*-butyrylated heparin

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Heparin tightly binds cathepsin G and so protects the enzyme from inhibition by α_1 -antichymotrypsin, α_1 -proteinase inhibitor and eglin c, three proteins which do not bind heparin [Ermolieff J., Boudier C., Laine A., Meyer B. and Bieth J. G. (1994) *J. Biol. Chem.* **269**, 29502–29508]. Here we show that heparin no longer protects cathepsin G from inhibition when the enzyme is reacted with mucus proteinase inhibitor (MPI), a heparin-binding protein. Heparin fragments of $M_r = 4500$ and 8100 and *O*-butyrylated heparin of $M_r = 8000$ form tight complexes with cathepsin G ($K_d = 0.5$ – 2.2 nM) and MPI ($K_d = 0.4$ – 0.8 μ M) and accelerate the MPI-promoted inhibition of cathepsin G by a factor of 17–26. They also accelerate the inhibition of neutrophil

elastase and pancreatic chymotrypsin. The rate acceleration is due to the binding of heparin to MPI. Butyrylation of heparin slightly decreases its affinity for cathepsin G and MPI but sharply decreases the ionic interactions between the positively charged proteins and the negatively charged polyanion. The butyrylated heparin derivative is the best rate accelerator: it increases the rate constant for the MPI-induced inhibition of cathepsin G and elastase by factors of 26 and 23, respectively. This, together with the fact that it has a good bioavailability and a very low anticoagulant activity, suggests that it might be an adjuvant of MPI-based therapy of cystic fibrosis.

INTRODUCTION

The azurophil granules of neutrophils store three serine proteinases: cathepsin G, elastase and proteinase 3, whose tertiary structures are known [1–3]. The two former enzymes are very cationic while the latter is less basic. Cathepsin G preferentially hydrolyses substrates with $P_1 = \text{Phe}$ or Leu . It cleaves type II collagen, proteoglycans, elastin, fibrinogen, coagulation factors, immunoglobulins, complement components [4] as well as angiotensinogen [5] and heparin cofactor [6]. This protein-degrading activity is normally controlled by protein proteinase inhibitors present in plasma or in lung secretions.

Mucus proteinase inhibitor (MPI) is the most abundant cathepsin G and elastase inhibitor of the upper respiratory tract where it occurs in concentrations as high as 5 μ M [7,8]. This 11.7-kDa basic polypeptide is formed of a single chain of 107 amino acid residues and consists of two domains of similar size and architecture [9]. The P_1 – P_1' residues of its reactive centre are Leu^{73} – Met^{74} [9,10].

We have recently shown that heparin accelerates the inhibition of neutrophil elastase by MPI [11]. Heparin is an anionic glycosaminoglycan that mostly consists of trisulphated disaccharide units formed of L-iduronic acid-2-sulphate linked to D-glucosamine-N,3,6-sulphate. These regular sequences are variably interrupted by irregular sequences containing either under-sulphated or oversulphated uronic or glucuronic acid and amino sugar residues. On the average, however, there are three sulphate groups per disaccharide unit [12]. It strongly binds elastase ($K_d = 3$ nM) and MPI ($K_d = 0.2$ μ M). Both unfractionated low molecular mass heparin [11] and monodisperse 4.5-kDa heparin [13] have been found to increase the rate of inhibition of elastase

by MPI. This paper examines whether a similar rate enhancement takes place with cathepsin G.

MATERIALS AND METHODS

The source and active site titration of human leucocyte elastase and cathepsin G, bovine pancreatic chymotrypsin, and MPI were the same as described before [14,15]. Heparin fragments with molecular mass of 4500 ± 500 Da (4-H) or 8100 ± 700 Da (8-H) were isolated from commercial heparin [13] while *O*-butyrylated heparin with a molecular mass of 8000 Da (but-H) was synthesized as previously described [16]. This compound contains an average of 1.3 butyryl group per disaccharide unit, ~ 3.3 mequiv/g of SO_3^- and a $\text{SO}_3^-/\text{COO}^-$ ratio of ~ 2.5 . [17]. The commercial source of the substrates succinyl-Ala₂-Pro-Phe-p-nitroanilide (Suc-Ala₂-Pro-Phe-pNA), succinyl-Ala₂-Pro-Phe-thiobenzylester (Suc-Ala₂-Pro-Phe-SBzl) and methoxysuccinyl-Ala₂-Pro-Ala-thiobenzylester (MeOSuc-Ala₂-Pro-Ala-SBzl) and of the thiol-trapping reagent 4,4'-dithiodipyridine was the same as before [13,14]. The synthetic substrates were dissolved in dimethylformamide whose final concentration in the reaction medium was 2% (v/v).

The K_d of the elastase-heparin and the cathepsin G-heparin complexes were determined from the tight-binding partial inhibition of the proteinases by heparin as described elsewhere [14,18]. The K_d of the MPI-heparin complexes were determined using the ability of the sulphated polysaccharides to accelerate the inhibition of chymotrypsin by MPI [15]. This procedure implies that chymotrypsin does not bind heparin as shown previously with 4-H [14,15]. Similarly, we found that chymotrypsin does not

Abbreviations used: MPI, mucus proteinase inhibitor (also called secretory leucoprotease inhibitor, SLPI); 4-H, 4.5-kDa heparin; 8-H, 8.1-kDa heparin; but-H, 8-kDa *O*-butyrylated heparin; Suc, succinyl; MeOSuc, methoxysuccinyl; pNA, p-nitroanilide; SBzl, thiobenzylester.

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bind 8-H or but-H as demonstrated by a dye displacement technique [13] and by the inability of the heparin derivatives to alter the k_{cat} and K_m values for the chymotrypsin/Suc-Ala₂-Pro-Phe-pNA system (data not shown).

Kinetics of proteinase inhibition by MPI

Proteinase ± heparin was added to a mixture of MPI + substrate and the release of product (P) was followed as a function of time. Mixing of the reagents and recording of the progress curves were done with the previously used stopped-flow apparatus [14]. The concentrations of enzyme (E), inhibitor (I), and substrate (S) were chosen so that $[I]_0 \geq 10 [E]_0$ and $[P]/[S]_0 \leq 0.05$. The inhibition of cathepsin G and chymotrypsin was studied using at least five MPI concentrations. With the former enzyme ($[E]_0 = 20$ nM) the inhibitor concentration range was 4–30 μM in the absence of heparin, 0.2–2 μM in the presence of 40 μM 4-H and 0.4–1.4 μM in the presence of 40 μM 8-H and 0.2–0.8 μM in the presence of 40 μM but-H. The cathepsin G substrate was Suc-Ala₂-Pro-Phe-SBzl ($[S]_0 = 0.14$ –0.59 mM) for these experiments. With chymotrypsin, the range of MPI concentrations was 1.6–11.2 μM in the absence of heparin, 0.1–1.6 μM and 0.2–1.6 μM in the presence of 21.5 μM 4-H and 38 μM 8-H, respectively. The chymotrypsin concentration was 0.1 $[I]_0$ in all cases, and its substrate (Suc-Ala₂-Pro-Phe-pNA) was used at concentrations of 1, 0.33 and 0.5 mM in the absence of heparin and in the presence of 4-H and 8-H, respectively. The inhibition of 80 nM chymotrypsin by 800 nM MPI in the presence of 38 μM but-H was studied using five different Suc-Ala₂-Pro-Phe-pNA concentrations ranging from 0.17 to 2 mM. The inhibition of 56 nM elastase by 0.6 μM MPI in the absence or presence of 20 μM 8-H or but-H was studied using 2 mM MeOSuc-Ala₂-Pro-Ala-SBzl as a substrate. All experiments were done in 50 mM Hepes, 100 mM NaCl, pH 7.4, 25 °C. Nonlinear regression analyses of the progress curves were done as described previously [14].

RESULTS

Protein-binding properties of heparins

We have studied the binding of MPI, cathepsin G and elastase with two fragments of natural heparin namely, 4-H ($M_r = 4500$) and 8-H ($M_r = 8100$) and with but-H, an 8000 Da butyrylated heparin derivative. These three polymers strongly interact with the three proteins (Table 1). Their affinity for cathepsin G or elastase is 260 to 2600-fold higher than that for MPI, the largest differences being observed with but-H.

Cathepsin G and MPI are basic proteins whose binding with natural heparin is governed by electrostatic interactions [14,15]. We wanted to see whether ionic interactions are also involved in their binding with but-H. We therefore investigated the ionic strength dependence of the equilibrium dissociation constants K_d of the complexes formed from cathepsin G or MPI with but-H or

Table 1 Equilibrium dissociation constants K_d (in M) of heparin-protein complexes at pH 7.4, 100 mM NaCl, and 25 °C

Proteins	Heparins		
	4-H	8-H	but-H
MPI	$(8.0 \pm 1.6) \times 10^{-7}$	$(4.0 \pm 0.9) \times 10^{-7}$	$(8.0 \pm 0.3) \times 10^{-7}$
Cathepsin G	$(2.2 \pm 0.3) \times 10^{-9}$	$(0.5 \pm 0.15) \times 10^{-9}$	$(0.8 \pm 0.12) \times 10^{-9}$
Elastase	$(3.0 \pm 0.4) \times 10^{-9}$	$(1.9 \pm 0.5) \times 10^{-9}$	$(0.3 \pm 0.18) \times 10^{-9}$

Figure 1 Effect of ionic strength on the equilibrium dissociation constant K_d of cathepsin G-but-H (○), cathepsin G-8-H (●), MPI-but-H (□) and MPI-8-H (■) complexes at pH 7.4 and 25 °C

Plots in accordance with eqn. (1).

Table 2 Effect of ionic strength on the binding of heparin derivatives to cathepsin G and MPI at pH 7.4 and 25 °C

The equilibrium dissociation constant, K_d , was measured between 0.05 and 0.25 M NaCl. Z , the number of ionic interactions involved in heparin-protein binding and $K_{d(1\text{ M NaCl})}$ have been calculated from a least square fit of the data of Figure 1 to eqn. 1.

Proteins	Heparins	Z	$K_{d(1\text{ M NaCl})}$ (M)
Cathepsin G	8-H	2	5.8×10^{-8}
Cathepsin G	but-H	0	1.5×10^{-11}
MPI	8-H	4	2.3×10^{-3}
MPI	but-H	2	2.1×10^{-5}

8-H, two compounds of similar size. We analysed the data according to the theory of protein-polyelectrolyte interactions [11,19–22], which predicts that:

$$\log K_d = \log K_{d(1\text{ M NaCl})} + Z\Psi \log [\text{NaCl}] \quad (1)$$

where K_d is the experimental equilibrium dissociation constant measured at variable $[\text{NaCl}]$, $K_{d(1\text{ M NaCl})}$ is the extrapolated dissociation constant at $[\text{NaCl}] = 1$ M, Z is the number of purely ionic interactions formed between the protein and the polyelectrolyte and Ψ represents the fraction of Na^+ cations bound to the polyelectrolyte. In our calculations we used $\Psi = 0.8$ [11,21,22]. Plots of $\log K_d$ vs. $\log [\text{NaCl}]$ (Figure 1) are linear for the four protein-heparin complexes but only those corresponding to the cathepsin G-8-H, the MPI-8-H and the MPI-but-H complexes have positive slopes as predicted by eqn. (1). The data computed from the curves of Figure 1 are compiled in Table 2. Assuming that at $[\text{NaCl}] = 1$ M all ionic interactions are neutralized, $K_{d(1\text{ M NaCl})}$ may be considered as the dissociation constant for nonionic protein-heparin interactions so that the contribution of ionic interactions to the binding energy at 0.1 M NaCl may be calculated [11,21,22]. This shows that ionic interactions are responsible for 22, 59 and 23 % of the binding

Figure 2 Effect of MPI concentration on k , the pseudo-first-order rate constant of cathepsin G inhibition at pH 7.4 and 25 °C in the absence (A) or presence of a saturating concentration of 8-H (B)

Each point represents the average data collected from at least four stopped-flow traces.

energy of the cathepsin G-8-H, MPI-8-H and MPI-but-H complexes, respectively. While butyrylation of heparin has little effect on the cathepsin G-heparin affinity at 0.1 M NaCl (Table 1) it fully abolishes the ionic interactions between the two molecules as evidenced by the negative slope of the curve corresponding to the cathepsin G-but-H complex in Figure 1. It is noteworthy that

at 1 M NaCl cathepsin G has a 4000-fold higher affinity for but-H than for 8-H. Butyrylation affects the MPI-heparin interaction in a way similar to that observed with the cathepsin G-heparin binding although the effects are less pronounced. Altogether these data show that but-H has a very high binding energy for elastase ($\Delta G^\circ = -13.3 \text{ kcal} \cdot \text{mol}^{-1}$) and cathepsin G ($\Delta G^\circ = -12.7 \text{ kcal} \cdot \text{mol}^{-1}$) and that its binding with cathepsin G and MPI is mainly governed by nonionic interactions.

Effect of heparin on the rate of inhibition of cathepsin G by MPI

Rapid mixing of cathepsin G $\pm$ heparin with MPI + substrate and recording the product (P) as a function of time (t) gave stopped-flow traces characteristic of reversible inhibition [11] (data not shown). Since $[I]_0 \geq 10 [E]_0$ and $[P]/[S]_0 \leq 0.5$, these progress curves could be fitted to the following integrated rate equation [23]:

$$[P] = v_s t + \frac{v_z - v_s}{k} (1 - e^{-kt}) \quad (2)$$

where v_z is the velocity at $t = 0$, v_s is the steady-state velocity, and k is the apparent first-order rate constant for the approach to the steady-state. Nonlinear regression analyses showed that the fits were satisfactory in all cases and was used to compute the best estimates of k . Under our concentration conditions, v_s was small as compared with v_z .

The rate of inhibition was measured using variable MPI concentrations. In the absence of heparin, the highest inhibitor concentration compatible with a reliable measurement of k was 30 μM . In the presence of heparin the highest MPI concentration that could be used was 1–2 μM because with higher concentrations the reaction medium became cloudy. Figure 2 shows the data obtained in the absence of heparin and in the presence of a saturating concentration of 8-H. In both cases, the rate constant k increases linearly with the MPI concentration indicating that the inhibition reaction conforms to a simple bimolecular reaction

$(E + I \xrightleftharpoons[k_{\text{diss}}]{k_{\text{ass}}} EI)$. For such a mechanism, k is related to k_{ass} and k_{diss}

through the following relation [23]:

$$k = \frac{k_{\text{ass}} [I]_0}{1 + [S]_0/K_m} + k_{\text{diss}} \quad (3)$$

The curves shown in Figure 2 as well as those obtained with 4-H and but-H cross the y axis at positions barely distinguishable

Table 3 Second-order rate constants k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$) for the inhibition of cathepsin G, chymotrypsin and elastase by MPI in the absence and presence of heparin at pH 7.4 and 25 °C

The numbers in square brackets are relative values, relative to the column 'None'. The data for free elastase and the elastase-4-H complex are from Cadène et al. [13].

Proteinase	Heparin			
	None	4-H	8-H	but-H
Cathepsin G*	$(1.7 \pm 0.05) \times 10^5$ [1]	$(4.0 \pm 0.12) \times 10^6$ [24]	$(2.9 \pm 0.04) \times 10^6$ [17]	$(4.4 \pm 0.2) \times 10^6$ [26]
Chymotrypsin†	$(6.5 \pm 0.2) \times 10^5$ [1]	$(6.4 \pm 0.2) \times 10^6$ [10]	$(2.3 \pm 0.8) \times 10^7$ [35]	$(3.7 \pm 0.1) \times 10^7$ [57]
Elastase‡	$(3.1 \pm 0.5) \times 10^6$ [1]	$(2.6 \pm 0.6) \times 10^7$ [8]	$(2.3 \pm 0.7) \times 10^7$ [7]	$(7.2 \pm 1.8) \times 10^7$ [23]

* [heparin] = 40 μM throughout.

† [heparin] = 21.5 μM for 4-H, 38 μM for 8-H and but-H.

‡ [heparin] = 40 μM for 4-H, 20 μM for 8-H or but-H.

Figure 3 Effect of but-H on k_{ass} , the second-order rate constant of inhibition of cathepsin G by MPI at pH 7.4 and 25 °C

Cathepsin G (20 nM) $\pm$ but-H was rapidly mixed with 250 nM MPI + 3 mM Suc-Ala₂-Pro-Phe-pNA and the release of pNA was recorded as a function of time using a stopped-flow apparatus. The pseudo-first-order rate constants, k , were calculated from the progress curves using eqn. (2) (see text). The rate constants k_{ass} were calculated using $k_{ass} = k(1 - v_s/v_2)/(1 + [S]_0/K_m)[I]_0$ [21]. The degree of saturation of cathepsin G and MPI by but-H was calculated using the K_d values from Table 1 and a published program for computing distribution diagrams for multiple equilibria [39].

from zero, precluding a reliable estimation of k_{diss} . This is due to the high MPI concentrations used in the stopped-flow experiments, which render k_{diss} negligibly small with respect to k_{ass} $[I]_0/(1 + [S]_0/K_m)$. The k_{ass} values were derived from the slopes of the k versus [MPI] plots using the known values of $[S]_0$ and the experimentally determined values of K_m , i.e. 35, 58, 35 and 52 μ M in the absence of heparin and the presence 4-H, 8-H and but-H, respectively. Table 3 shows that heparin significantly increases the k_{ass} for the inhibition of cathepsin G by MPI, the three polyanions being about equally effective.

Heparin saturates both MPI and cathepsin G in the preceding experiments, described above. We wanted to know whether saturation of both reaction partners is required for observing an increase in k_{ass} . To this end we chose but-H as a ligand because it has a 1000-fold greater affinity for cathepsin G than for MPI (see Table 1). We also chose a relatively low MPI concentration to be able to saturate cathepsin G with but-H without significantly saturating MPI. We then measured k_{ass} using constant cathepsin G and MPI concentrations and variable but-H concentrations. The results reported in Figure 3 show that within experimental errors, heparin does not significantly alter k_{ass} as long as it binds only to cathepsin G. However, with an 11% MPI saturation, a 3-fold increase in k_{ass} is observed. We conclude that free MPI inhibits the cathepsin G-heparin complex as fast as it inhibits free cathepsin G and that binding of heparin to MPI is required to get an acceleration of the rate of inhibition.

Effect of heparin on the rate of inhibition of chymotrypsin by MPI

We wanted to confirm the above results by using a proteinase that does not bind heparin. Bovine pancreatic α -chymotrypsin, further purified by ion exchange chromatography, was previously shown to be unable to bind 5-kDa heparin [14]. Here, we

demonstrated that it does not react with 4-H, 8-H and but-H (see Materials and Methods). The k_{ass} for the chymotrypsin-MPI association in the absence and presence of 4-H and 8-H was determined as described above for cathepsin G. The pseudo-first order rate constant of inhibition, k , was again linearly related to the MPI concentration (data not shown) so that eqn. (3) was again used to calculate k_{ass} . As for cathepsin G, k_{diss} could not be measured. The inhibition of chymotrypsin by MPI in the presence of but-H was studied using constant enzyme, inhibitor and heparin concentrations and variable substrate concentrations. A plot of k versus $K_m/(K_m + [S]_0)$ gave a straight line passing through the origin which indicates that eqn. (3) adequately describes the data and that k_{diss} cannot be measured. Table 3 shows that heparin significantly accelerates the inhibition of chymotrypsin by MPI, 8-H and but-H being twice more efficient on chymotrypsin inhibition than on cathepsin G inhibition. This experiment confirms that the heparin-promoted acceleration of proteinase inhibition results from the binding of heparin with MPI.

Effect of heparin on the rate of inhibition of elastase by MPI

4-H has already been shown to accelerate the inhibition of elastase by MPI [13]. For pharmacological reasons we wanted to compare this effect with that brought about by 8-H and but-H. MPI was used at a single concentration of 0.6 μ M. Table 3 shows that but-H is a significantly better rate accelerator than 4-H and 8-H.

DISCUSSION

Heparin is able to regulate proteolysis by reacting with proteinases or proteinase inhibitors. For example, it promotes the binding of thrombin to fibrin [24], stimulates the activity of plasminogen activator [30] and partially inhibits the activity of neutrophil elastase and cathepsin G [14,18]. It also binds a number of serine proteinase inhibitors that belong to the serpin superfamily, namely antithrombin, heparin cofactor, protein C inhibitor, protease nexin and plasminogen activator inhibitor I [25]. As a rule, this binding increases the rate of proteinase inhibition. Acceleration of proteinase inhibition also occurs when heparin binds MPI, a non-serpin inhibitor. Thus, the sulphated glycosaminoglycan has been shown to significantly increase the rate constant for the inhibition of neutrophil elastase [13] and mast cell chymase [26] by MPI. Here we show that it also accelerates the inhibition of neutrophil cathepsin G.

The aforementioned rate-enhancing effect of heparin sharply contrasts with the previous finding that cathepsin G in complex with 5-kDa heparin reacts extremely slowly with α_1 -antichymotrypsin, α_1 -proteinase inhibitor and eglin c, and 8-kDa peptide [14]. Thus, MPI and eglin c, two inhibitors of comparable size, behave quite differently with cathepsin G bound to 4.5-kDa or 5-kDa heparin: MPI inhibits heparin-bound cathepsin G 24-fold faster than free cathepsin G (Table 3) while eglin c inhibits cathepsin G-heparin 10000-fold slower than free cathepsin G [14]. As discussed earlier [14], the almost complete inability of α_1 -antichymotrypsin, α_1 -proteinase inhibitor and eglin c to inhibit heparin-bound cathepsin G is probably due to the fact that the polymer causes steric hindrance to the access of the inhibitors to the enzyme's active centre. Since these three inhibitors do not bind heparin [14,18] while MPI does, one may suggest that heparin activates the inhibitor and thus renders it able to overcome the above steric hindrance. Similar observations have previously been made with neutrophil elastase: heparin decelerates the inhibition of elastase by α_1 -proteinase inhibitor and eglin c [18] but accelerates the inhibition of this enzyme by

MPI [11,13]. The effects are, however, less pronounced than with cathepsin G. For instance, 5-kDa heparin decreases the k_{ass} for the inhibition of elastase by α_1 -proteinase inhibitor by a factor of 5.5 only [14] and 4.5-kDa heparin increases the k_{ass} for the elastase + MPI binding by a factor of 8 (Table 3). In summary, the enzymatic activity of heparin-bound cathepsin G or elastase is more or less shielded from inhibitors that do not bind heparin but is rapidly inactivated by the MPI-heparin complex.

Using the progress curve method [23] and a stopped-flow apparatus, we have measured k , the pseudo-first order rate constant of inhibition as a function of MPI concentration in order to decide whether the inhibition of cathepsin G or chymotrypsin by MPI occurs in one step,

or in two steps,

and to see whether heparin influences the inhibition mechanism. The rate constant k was found to vary linearly with the inhibitor concentration whatever the proteinase and the heparin used. This indicates either that the inhibition indeed occurs in one step or that it takes place in two steps but that EI^* is not seen kinetically, i.e. that the highest inhibitor concentration used in the stopped-flow experiments is lower than $K_1^* (1 + [S]_0/K_m)$ (see for example ref. [13]). If this assumption is correct, one may conclude that $K_1^* > 3 \mu\text{M}$ for the cathepsin G/MPI system in the absence of heparin. A similar limit was previously found for the elastase/MPI pair in the absence of heparin ($K_1^* > 2 \mu\text{M}$; ref. [13]). On the other hand, K_1^* should be greater than $0.2 \mu\text{M}$ for the cathepsin G/MPI/4-H system. This contrasts with the finding that the elastase/MPI/4-H system shows a reaction intermediate EI^* ($K_1^* = 80 \text{ nM}$; ref. [13]).

It has been suggested previously that the binding of both thrombin and antithrombin to the same heparin chain is essential for heparin acceleration of the thrombin-antithrombin reaction. In this template mechanism, heparin acts as a surface to approximate the proteinase and the inhibitor [27]. This mechanism does probably not hold for the heparin-promoted acceleration of proteinase inhibition by MPI since it occurs with chymotrypsin, an enzyme that does not bind heparin. Interestingly, the acceleration of inhibition by 8-H and but-H is even more pronounced with chymotrypsin than with the two heparin-binding proteinases cathepsin G and elastase (Table 3). On the other hand, no rate acceleration occurs when the cathepsin G-but-H complex is reacted with free MPI (Figure 3). Saturation of the inhibitor with heparin thus appears to be a necessary and sufficient condition for increasing its rate of proteinase inhibition. Perhaps the heparin-induced conformational change [11] activates the inhibitor by increasing the accessibility of its active site to the enzymes.

Heparin-protein binding is sensitive to ionic strength and is commonly thought to be mainly governed by electrostatic interactions. Analysis of the ionic strength dependence of K_d according to the theory of protein-polyelectrolyte interactions [20], however, shows that the binding is not necessarily dominated by ionic interactions. For example, while such interactions are responsible for 80 % of the thrombin-heparin binding energy [28] they account only for 30 % of the binding of heparin to basic fibroblast growth factor [22]. The present study shows that heparin forms more ionic interactions with MPI (59 %) than

with cathepsin G (22 %). We therefore confirm here that the contribution of ionic interactions to the heparin-protein binding energy depends upon the nature of the protein and may be exceedingly small. On the other hand, it is worthwhile noticing that heparin has a dramatically higher affinity for cathepsin G than for MPI but forms less ionic interactions with the former than with the latter (Table 2). This suggests that nonionic interactions are much more energetic than ionic ones. Although butyrylation does not significantly alter the sulphate content of heparin [17], it strongly decreases the ionic interactions within the heparin-protein complexes. The most dramatic effect is observed with cathepsin G which, despite its 34 Arg and 4 Lys residues, no longer binds electrostatically with but-H (Table 2). A 10-fold increase in ionic strength leads to a 53-fold decrease in the K_d of the but-H-cathepsin-G complex (compare tables 1 and 2). This corresponds to an increase in free energy of binding of $2.4 \text{ kcal} \cdot \text{mol}^{-1}$ and suggests that hydrophobic interactions participate in binding. One may speculate that in the but-H-cathepsin G complex ionic interactions serve as guides for the formation of a loose initial complex which then isomerizes into a stable complex in which the partners are held together by nonionic interactions.

Apart from its well-known anticoagulant activity, heparin has numerous pharmacological effects including inhibition of HIV replication [29]. Butyrylated heparin is a potential drug for the treatment of HIV infections because it has a potent activity against HIV-induced cytopathicity, a very low anticoagulant activity, a fair bioavailability and a very low toxicity [17]. The present data suggest that but-H may also be a potential adjuvant of MPI-based therapy of cystic fibrosis, a frequent and fatal disease. Cystic fibrosis lung secretions contain very high levels of active elastase and cathepsin G: one study reports elastase concentrations ranging from 1.2 to $85.7 \mu\text{M}$ [30]. Aerosol-delivered recombinant MPI was shown to depress this elastase activity significantly. Considerable amounts of MPI (200 mg/day) were, however, required [31]. This may be due to several reasons. Firstly part of MPI, a DNA-binding protein [16,35], may be sequestered by the large quantities of DNA (4 mg/ml) present in cystic fibrosis secretions; part of MPI may also bind to the mucins from bronchial secretions [34]. Secondly, DNA binds elastase [32] and cathepsin G (unpublished data) and considerably hinders their inhibition by MPI [32]. Thirdly, elastase and cathepsin G bind to the surface of activated neutrophils or to neutrophil membranes [35,36]; while this binding preserves their enzymatic activity, i.e. their tissue destructive function, it hinders their inhibition by natural proteinase inhibitors, including MPI [36]. Heparin has already been administered via an aerosol [37]. Treatment of cystic fibrosis patients with an aerosol formed of MPI and an excess of but-H should have the following advantages: (i) reacting with MPI, but-H should (at least partially) prevent MPI from binding with DNA and mucins, (ii) due to its very high affinity for elastase and cathepsin G (Table 1), but-H should be able to displace the two enzymes from their binding with DNA and neutrophil surface so that the bulk of them would occur as but-H-proteinase complexes, (iii) but-H-MPI inhibits the but-H-proteinase complexes at a rate more than 20-fold higher than that measured in the absence of but-H (Table 3); this rate enhancement has a therapeutic advantage since faster inhibition *in vitro* corresponds to more efficient inhibition *in vivo* [38]. Because MPI is likely to be much more efficient when administered as a cocktail with but-H, it should be efficient at a much less expensive therapeutic dose.

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