Mapping the Heparin-Binding Site of Mucus Proteinase Inhibitor†

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ABSTRACT: Heparin accelerates the inhibition of neutrophil elastase by mucus proteinase inhibitor (MPI), the physiological antielastase of airways as a result of its binding with the inhibitor [Faller, B., Mély, Y., Gérard, D., & Bieth, J. G. (1992) *Biochemistry 31*, 8285−8290]. To explore the heparin-binding site of the inhibitor, we have modified the lysine and arginine residues of MPI and its isolated C-terminal domain by using 4-*N*,*N*-(dimethylamino)azobenzene-4′-isothiocyano-2′-sulfonic acid (S-DABITC) [Chang, J. Y. (1989) *J. Biol. Chem. 264*, 3111−3115] and (*p*-hydroxyphenyl)glyoxal (HPG) (Yamasaki, R. B., Vega, A., & Feeney, R. E. (1980) *Anal. Biochem. 109*, 32−40], respectively. The derivatizations were done in the absence and presence of a 4.5 kDa heparin fraction with a low degree of polydispersity. The effect of chemical modification of the inhibitors on their affinity for heparin was tested using two complementary procedures, one based on the ability of heparin to accelerate the inhibition of chymotrypsin by the inhibitors and the other exploiting the affinity of the inhibitors for immobilized heparin. Modification of a limited number of lysine and arginine residues in full-length MPI led to a 6-fold decrease in affinity for heparin. The presence of the polymer during the modification reactions significantly prevented this effect. Amino acid sequencing unambiguously identified the heparin-protected lysines as Lys 13 and Lys 87, located on the N-terminal and C-terminal domains of MPI, respectively. Heparin apparently protects mainly two arginine residues from modification by HPG. Reaction of S-DABITC with the C-terminal domain of MPI failed to confirm the modification of Lys 87. In addition, none of the modifiers was able to change the affinity of this inhibitor for heparin although both of them were capable of modifying the inhibitor to some extent. Comparison of the rates of chymotrypsin inhibition by MPI and its C-terminal domain in the presence and absence of heparin leads to the conclusion that the N-terminal domain of MPI is essential for both the integrity of the heparin binding site and the heparin-induced activation of the full-length inhibitor.

Heparin is a negatively-charged polysulfated glycosaminoglycan that naturally occurs in several tissues such as the lung (Clark et al., 1983), blood vessels (Cardin & Weintraub, 1989), or cartilage. Heparin in used to prevent blood clotting in many pathological situations. This effect is due mainly to the binding of heparin to the serpin proteinase inhibitor antithrombin III [for a review, see Olson and Björk (1992)]. Studies using point-mutated variants (Chang & Tran, 1986; Okajima et al., 1989) or chemical modification procedures (Blackburn & Sibley, 1980; Blackburn et al., 1984; Chang, 1989; Sun & Chang, 1989, 1990) have shown that the heparin-binding site is localized on two lysine- and arginine-rich amphypathic helices of antithrombin III.

MPI[1] is an 11.7 kDa reversible serine proteinase inhibitor (Seemüller et al., 1986; Thompson & Ohlsson, 1986; Boudier et al., 1987) found in bronchial secretions (Ohlsson et al., 1977), cervical mucus (Wallner & Fritz, 1974), seminal plasma (Schiessler et al., 1976), salivary gland secretions (Ohlsson et al., 1983), and cartilage (Böhm et al., 1991; Andrews et al., 1992). Its amino acid sequence (Seemüller et al., 1986; Thompson & Ohlsson, 1986) shows that it belongs to a new family of proteinase inhibitors together with the 6 kDa elafin, a neutrophil, and pancreatic elastase inhibitor found so far in bronchial secretions and in the skin of patients with psoriasis (Wiedow et al., 1990). MPI is made of two homologous domains, presumably issued from the duplication of a single ancestor gene. Grütter et al. (1988) unambiguously proved this two-domain organization by X-ray crystallography and showed that each domain is tightly stabilized by four disulfide bonds.

MPI inhibits a number of proteinases including neutrophil elastase, cathepsin G, pancreatic trypsin, and chymotrypsin (Thompson & Ohlsson, 1986). This protein is thought to be the physiological antielastase in airways (Ohlsson et al., 1977). We have recently shown that it forms a tight complex with heparin with resulting increase in the rate of elastase inhibition (Faller et al., 1992). This observation has now been confirmed (Ying et al., 1994). We have further shown that this rate acceleration correlates with a conformational change of the inhibitor, that ionic interactions account for most of the MPI−heparin binding energy, and that the inhibitor−glycosaminoglycan complex is held together by about seven ion−ion pairs (Faller et al., 1992). It is therefore likely that the negatively-charged heparin molecule interacts with some of the positively-charged amino acid residues found in MPI (15 lysines and 5 arginines). This paper describes the use of chemical modification procedures and binding experiments to study the importance of lysine and arginine residues in the interaction of heparin with MPI.

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[1] Abbreviations: MPI, mucus proteinase inhibitor; SLPI secretory leukoproteinase inhibitor; FPLC, fast protein liquid chromatography; RP-HPLC, reverse phase high-pressure liquid chromatography; S-DABITC, 4-(*N*,*N*-dimethylamino)azobenzene-4′-isothiocyano-2′-sulfonic acid; HPG, (*p*-hydroxyphenyl)glyoxal.

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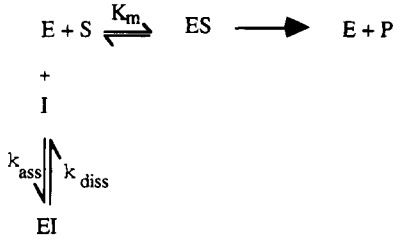
MATERIALS AND METHODS

Recombinant MPI was obtained from Synergen (Boulder, CO) through the courtesy of Dr. H. P. Schnebli (Ciba-Geigy, Basel, Switzerland). The inhibitor was titrated with an active site-titrated human neutrophil elastase solution (Boudier & Bieth, 1989). The recombinant C-terminal domain of MPI was kindly provided by Dr. R. Heinzel-Wieland (Grünenthal, Aachen, Germany) (N 55 to A 107) (Meckelein et al., 1990) and active site titrated like native MPI. The chymotrypsin substrate Suc-$(Ala)_2$-Pro-Phe-pNA was purchased from Bachem (Bubendorf, Switzerland). Heparin with a molecular mass of 4.5 kDa and a low degree of polydispersity was purified from a 5.1 kDa heparin preparation (Calbiochem, La Jolla, CA) by successive gel filtration on Sephadex G50 (Pharmacia) (M. Cadène, C. Boudier, and J. G. Bieth, to be published). S-DABITC was purchased from the Protein Institute (Broomall, PA) and HPG from Pierce. Thermolysin was from Boeringer (Mannheim, Germany).

Chymotrypsin Purification and Lack of Heparin Binding. Commercial bovine α-chymotrypsin (Biosys, France) was chromatographed on a mono-S column (Pharmacia) equilibrated with 50 mM acetate buffer, pH 5. The bound material was eluted with a linear NaCl gradient. Three minor peaks and two major peaks were separated. The fractions corresponding to the less cationic major peak were pooled and rechromatographed. The purified enzyme was not retained on heparin−Sepharose under the buffer conditions described below. In addition, heparin in large excess did not change its k_{cat} and K_m values for the hydrolysis of the substrate Suc-$(Ala)_2$-Pro-Phe-pNA.

Chymotrypsin Inhibition Kinetics. The kinetics of inhibition of chymotrypsin by MPI, its C-terminal domain, and the chemically-modified inhibitors was followed using the progress curve method (Morrison & Walsh, 1988). Chymotrypsin (13 nM) was added to a mixture of inhibitor (130 nM), substrate Suc-$(Ala)_2$-Pro-Phe-pNA (1.8 mM), and variable concentrations of heparin in 50 mM HEPES, pH 7.4, 200 mM NaCl at 37 °C. The release of product with time was followed by recording the absorbance at 410 nm.

The progress curves were analyzed assuming the following scheme:



where E is chymotrypsin, I is either the free or heparin-bound inhibitor, S is the substrate, P is the product, K_m is the Michaelis constant, k_{ass} is the second-order rate constant of EI formation, and k_{diss} is the first-order rate constant of EI dissociation. Since $[I]_0 = 10 \times [E]_0$ and since in any case the highest [P] was much lower than $[S]_0$, the resulting progress curves could be analyzed using eq 1 (Morrison & Walsh, 1988):

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

where v_z is the initial velocity, v_s is the steady-state velocity, and k is the pseudo-first-order rate constant for the approach to the steady state. The progress curves were fitted to eq 1 by nonlinear least squares analysis (ENZFITTER, Biosoft, U.K.) to obtain the best estimates of k, v_z, and v_s, which were used to calculate k_{ass}:

$$k_{ass} = k\left(1 - \frac{v_s}{v_z}\right)\frac{(1 + [S]_0/K_m)}{[I]_0} \quad (2)$$

The adequacy of the above scheme and eq 1 was checked by measuring k as a function of $[I]_0$ using MPI or its C-terminal domain saturated with heparin. k varied linearly with $[I]_0$, indicating that eq 2 is suited for the calculation of k_{ass} (Morrison & Walsh, 1988).

Equilibrium Dissociation Constant K_d of the Inhibitor−Heparin Complex. MPI (I) binds heparin (H) to form a 1:1 reversible complex (IH) which reacts much faster with neutrophil elastase than does free MPI. Elastase itself binds heparin (Faller et al., 1992). We have shown that heparin also increases the second-order rate constant, k_{ass}, for the inhibition of our chymotrypsin by MPI. Since this enzyme does not bind heparin (vide supra), the increase in k_{ass} will reflect the saturation of MPI by heparin and may thus be used to measure K_d.

If $k_{ass(0)}$ and $k_{ass(max)}$ are the rate constants of chymotrypsin inhibition by I and IH, respectively, $k_{ass(app)}$, the rate constant measured with an equilibrium mixture of I, H, and IH, will be given by:

$$k_{ass(app)} = k_{ass(0)}\frac{[I]}{[I]_0} + k_{ass(max)}\frac{[IH]}{[I]_0} \quad (3)$$

If one sets $\Delta k_{ass} = k_{ass(app)} - k_{ass(0)}$ and $\Delta k_{ass(max)} = k_{ass(max)} - k_{ass(0)}$, one gets

$$\Delta k_{ass} = \frac{[I]_0 + [H]_0 + K_d - \sqrt{([I]_0 + [H]_0 + K_d)^2 - 4[I]_0[H]_0}}{2[I]_0} \times \Delta k_{ass(max)} \quad (4)$$

K_d and $\Delta k_{ass(max)}$ were determined by measuring k_{ass} in the absence and presence of increasing concentrations of heparin. The k_{ass} were determined using the progress curve method outlined in the preceding section. The best estimates of K_d and $\Delta k_{ass(max)}$ and their confidence intervals were calculated by nonlinear regression based on eq 4.

S-DABITC Modification of the Lysine Residues of Free and Heparin-Bound MPI. The lysines were modified with S-DABITC using a slight modification of the procedure originally described by Chang (1989). Briefly, 28 μM MPI was incubated in the presence or absence of 0.48 mM heparin in 2.5 mL of 50 mM HEPES, pH 7.4, 0.2 M NaCl at 37 °C. The equilibrium dissociation constant of the MPI−heparin complex calculated with the above kinetic method predicts that the above concentration of heparin saturates MPI to the extent of 93%. S-DABITC (0.5 mg) dissolved in 0.5 mL of the same buffer was then added for a 10 min incubation. The reaction was stopped by exchanging the buffer on a PD 10 desalting column (Pharmacia) equilibrated and eluted with 0.2 M NaCl in 50 mM ammonium bicarbonate. The extent of modification was calculated assuming $\epsilon_{465nm} = 29\,000$ M^{-1} cm^{-1} for the bound reagent (Chang, 1989). The sample

was subsequently reduced with 0.3 M dithiothreitol in 0.5 M Tris-HCl, pH 8.5, 6 M guanidine, and 10 mM EDTA, carboxymethylated with 0.8 M iodoacetic acid, desalted on a PD 10 column, and digested overnight with 5% (w/w) thermolysin in 50 mM ammonium bicarbonate, 5 mM $CaCl_2$ at 40 °C. The resulting peptides were isolated by RP-HPLC on a Nova-Pak 3.9 × 100 mm C18 column (Waters). Buffer A was 20 mM ammonium acetate, pH 5. Eluant B was 60% acetonitrile in water. The linear gradient was run from 0 to 40% eluant B during 70 min and from 40 to 60% eluant B during 20 min. The labeled peptides were detected at 436 nm (Chang, 1989). The peptides selected for amino acid sequencing were rechromatographed on a C_8 aquapore RP 300 2.1 × 300 mm column (Brownlee) with a simultaneous detection at 436 and 225 nm. The buffer system was identical to that used for the C18 column, and the linear gradient was run from 0 to 100% buffer B during 25 min.

S-DABITC Modification of the Lysine Residues of Free and Heparin-Bound C-Terminal Domain of MPI. The chemical modification conditions were identical to those used for MPI except that (i) the heparin concentration was raised to 1.6 mM in order to saturate this inhibitor derivative to about the same extent as MPI (89%) and (ii) the S-DABITC reaction time was increased to 60 min because the reagent reacted slower with the C-terminal domain than with the intact molecule.

HPG Modification of the Arginine Residues of Free and Heparin-Bound MPI and Its C-Terminal Domain. Inhibitor and heparin concentrations, buffers, and temperature were identical with those used in the lysine modification experiments. HPG (2 mg in 0.5 mL) was added to 2.5 mL of inhibitor ± heparin, and the samples were incubated for 45 min. Excess reagent was removed as described for S-DABITC. The number of arginines modified per mole of inhibitor was calculated using $\epsilon_{340nm} = 1.83\ 10^4\ M^{-1}\ cm^{-1}$ for arginine-bound HPG (Sun & Chang, 1990).

Heparin–Sepharose Affinity Chromatography. The samples were adsorbed on a heparin Hitrap affinity column (Pharmacia) equilibrated with 50 mM HEPES buffer, pH 7.4, eluted with a linear NaCl gradient. The salt gradient was monitored with an ion detector (Pharmacia) connected next to the UV monitor, the system having a negligible dead volume. Elution of proteins was monitored at 280 nm. The flow rate was 0.5 mL/min.

Peptide Sequencing. The peptides were sequenced on a 473A pulsed liquid peptide sequencer (Applied Biosystem) on line with a PTH-amino acid analysis system.

RESULTS

Enzymatic Determination of the Inhibitor–Heparin Affinity. Measurement of the equilibrium dissociation constant, K_d, of the heparin–inhibitor complex was necessary to calculate the heparin concentration required to saturate the inhibitors before chemical modification and to evaluate the effect of chemical modification on the heparin–inhibitor binding. The procedure based on the heparin-induced enhancement of the intrinsic fluorescence of MPI (Faller et al., 1992) could not be used here because (i) some inhibitor derivatives were poorly soluble and (ii) the single tryptophan of MPI is located on the N-terminal domain so that no study was possible with the C-terminal domain. We therefore designed the enzymatic method outlined in the experimental section. Chymotrypsin was chosen because it does not bind heparin (see experimental section). Hence any effect of heparin on the rate of inhibition of chymotrypsin by MPI is accounted for by the effect of heparin binding on MPI.

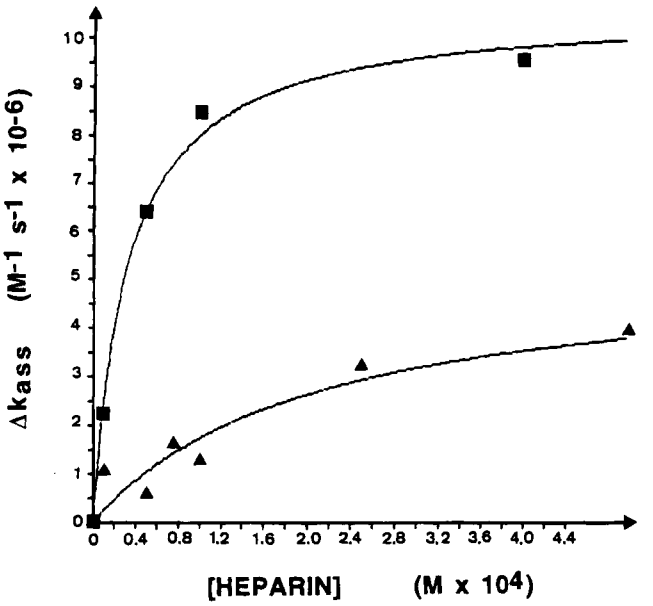


FIGURE 1: Determination of the equilibrium dissociation constant (K_d) of the heparin–MPI (■)and heparin–C-terminal domain (▲) complexes and of the rate constant for the inhibition of chymotrypsin by these complexes [$k_{ass(max)}$] at pH 7.4 and 37 °C. The k_{ass} values were determined as described in the experimental section. $\Delta k_{ass} = k_{ass(app)} - k_{ass(0)}$, the rate constants of chymotrypsin inhibition in the presence and absence of heparin, respectively. The data were fitted to eq 4 by nonlinear regression analysis. (–): theoretical curves generated using the best estimates of K_d (30 μM) and $\Delta k_{ass(max)}$ ($1.06 \times 10^7\ M^{-1}\ s^{-1}$) for MPI and K_d (190 μM) and $\Delta k_{ass(max)}$ ($4.37 \times 10^6\ M^{-1}\ s^{-1}$) for the C-terminal domain.

Table 1: K_d Values for Heparin–Inhibitor Complexes as Determined Using the Enzymatic Method Illustrated in Figure 1

chemical modification conditions	inhibitors: MPI	inhibitors: C-terminal domain
native inhibitors	$(3.2 \pm 0.4) \times 10^{-5}$	$(2 \pm 0.6) \times 10^{-4}$
S-DABITC in the absence of heparin	$(2.4 \pm 0.7) \times 10^{-4}$	$(2 \pm 0.8) \times 10^{-4}$
S-DABITC in the presence of heparin	$(3.8 \pm 0.6) \times 10^{-5}$	$(4.9 \pm 1) \times 10^{-4}$
HPG in the absence of heparin	$(2 \pm 0.4) \times 10^{-4}$	$(1.6 \pm 1) \times 10^{-4}$
HPG in the presence of heparin	$(8.7 \pm 1.8) \times 10^{-5}$	$(2.9 \pm 1.3) \times 10^{-4}$

Figure 1 shows that the k_{ass} for the inhibition of chymotrypsin by MPI and its C-terminal domain increases hyperbolically with the heparin concentration. There is a good fit between the experimental points and the theoretical curve calculated using eq 4 and the best estimates of K_d and $\Delta k_{ass(max)}$. MPI has a 6-fold better affinity for heparin than its C-terminal domain (see Table 1). This difference in affinity is also diagnosed by heparin–Sepharose affinity chromatography (see Figures 2a and 3a). On the other hand, the two inhibitors have identical association rate constants in the absence of heparin [$k_{ass(0)} = (3.4 \pm 0.03) \times 10^6$ and $(3.6 \pm 0.06) \times 10^6\ M^{-1}\ s^{-1}$ for MPI and the C-terminal domain, respectively] but significantly different rate constants following saturation by heparin [$k_{ass(max)} = (1.4 \pm 0.05) \times 10^7$ and $(9 \pm 0.8) \times 10^6\ M^{-1}\ s^{-1}$ for MPI and the C-terminal domain, respectively].

Lysines 13 and 87 Are Shielded by Heparin during S-DABITC Modification of MPI. In the original procedure

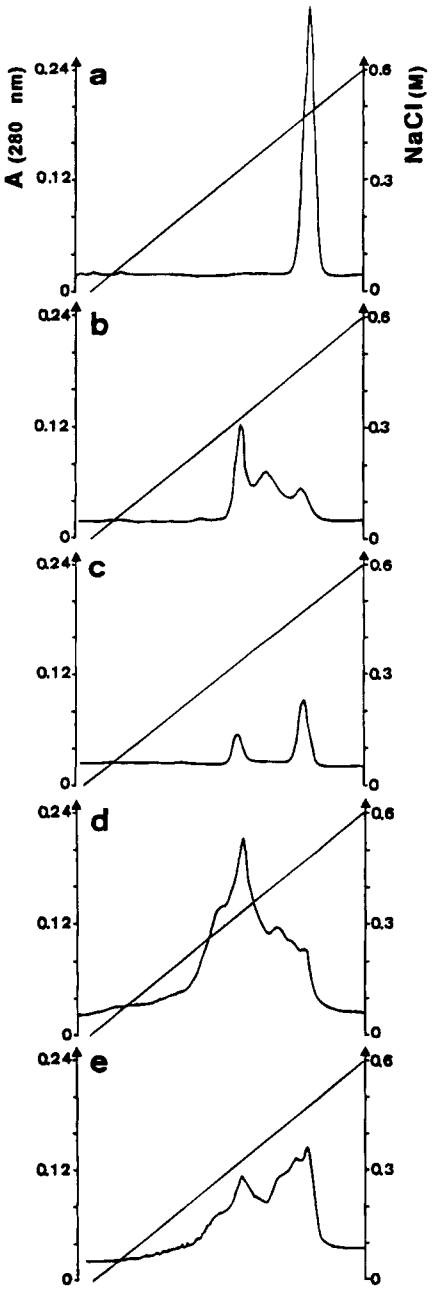


FIGURE 2: Heparin–Sepharose affinity chromatography of MPI and its derivatives; (a) native inhibitor, (b) modified with S-DABITC, (c) modified with S-DABITC in the presence of heparin, (d) modified with HPG, and (e) modified with HPG in the presence of heparin.

described by Chang (1989), the lysine modification was done in 50 mM sodium bicarbonate buffer, pH 8.3, at 25 °C. We used pH 7.4 in order to get a significantly strong interaction of MPI with heparin. In addition, we had to use a higher ionic strength (0.2 M NaCl) and temperature (37 °C) to avoid precipitation of the heparin–MPI complex. Using these buffer and temperature conditions, we varied the time of S-DABITC incubation for the determination on an optimal labeling time. The MPI–heparin complex was found to precipitate slowly after 15 min, whereas free MPI precipitated after 20 min. Thus, an incubation time of 10 min was chosen in order to avoid precipitation. In these conditions, assuming a molar extinction coefficient of 29 000 M^{-1} cm^{-1} at 465 nm for lysine-bound S-DABTC (Chang, 1989), an average extent of labeling of 1.3 lysines per MPI molecule was achieved in the absence of heparin. In the presence of

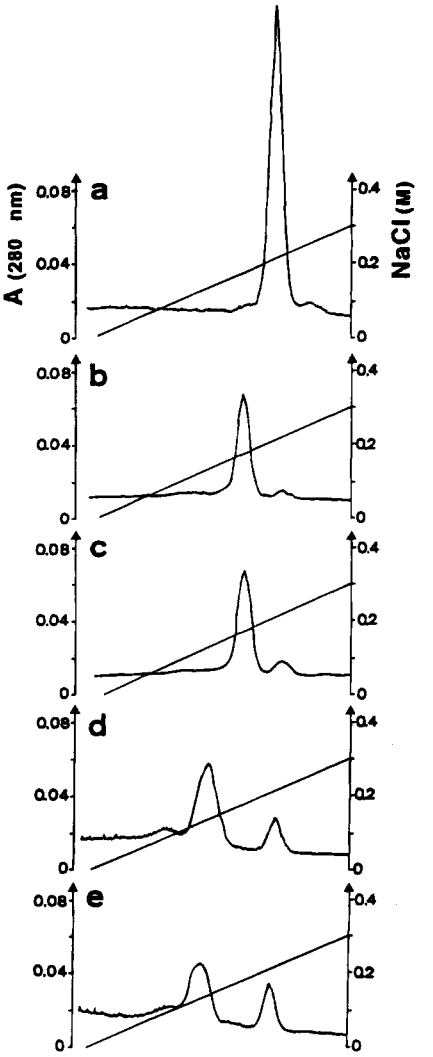


FIGURE 3: Heparin–Sepharose affinity chromatography of the C-terminal domain of MPI and its derivatives; (a) native inhibitor, (b) modified with S-DABITC, (c) modified with S-DABITC in the presence of heparin, (d) modified with HPG, and (e) modified with HPG in the presence of heparin.

heparin, an average of only 0.25 lysine per MPI molecule was labeled, indicating that a strong protection of some reacting lysines took place.

Comparative RP-HPLC analysis of the thermolysin digests of free or heparin-protected MPI (Figure 4) shows that heparin reduces the intensity of 12 peaks to the extent of 80–90%. Aliquots from peak 1–12 of Figure 4A were rechromatographed on a less polar C_8 aquapore column before amino acid sequencing. Their elution was monitored at both 436 and 225 nm in order to assess their homogeneity. Typical elution profiles are shown in Figure 5. All fractions from Figure 4A except numbers 2 and 10 eluted from the C_8 column as a single peak whose retention time was the same at both detection wavelengths. The remarkable purity of these peptides can be partly attributed to the shallow gradient used and confirms that the pH 5.0 ammonium acetate/acetonitrile elution system is perfectly well suited for the efficient separation of S-DABITC-labeled peptides from unlabeled ones as previously demonstrated by Chang and co-workers (Chang, 1991; Chang et al., 1992). Despite their heterogeneity, peaks 2 and 10 were sequenced as a mixture of peptides due to paucity of material.

The partial amino acid sequences of the peptides isolated by RP-HPLC are reported in Table 2 and were compared to

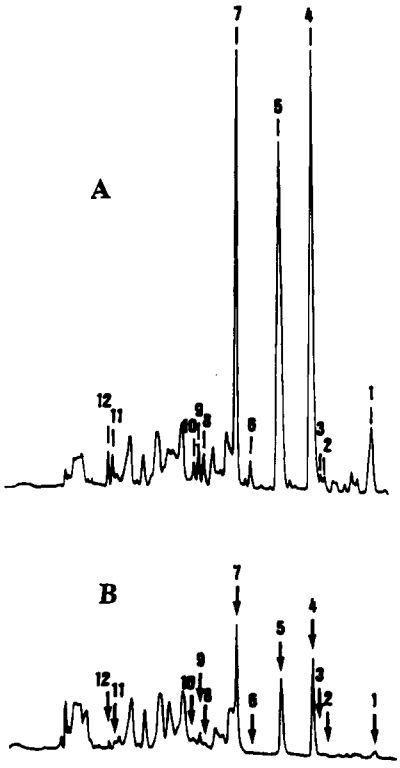


FIGURE 4: RP-HPLC analysis of the thermolysin digests of S-DABITC-modified MPI. The samples were reduced, carboxymethylated, digested with thermolysin, and analysed on a C18 column as described in the experimental section. The peptides were detected at 436 nm. A: Chemical modification done in the absence of heparin. B: Chemical modification done in the presence of a saturating concentration of heparin. The arrows indicate the depleted peaks. The numbers refer to the sequences shown in Table 2.

Table 2: Partial Amino Acid Sequences of the Peptides Whose RP-HPLC Elution Profile Is Shown in Figure 4[a]

cycle no.	peptide no.									
	1	3	4	5	6	8	9	10	11	12
1	V_9	F_{79}	M_{82}	F_{79}	G	M_{82}	V_9	$L_{48}Y_{21}F_{79}$	F_{79}	M_{82}
2	C_{10}	C_{80}	D_{83}	C_{80}	K	D_{83}	C_{10}	$D_{49}K_{22}C_{80}$	C_{80}	D_{83}
3	P_{11}	E_{81}	G_{84}	E_{81}	S	G_{84}	P_{11}	$P_{50}K^*_{23}E_{81}$	E_{81}	G_{84}
4	P_{12}	M_{82}	Q_{85}	M_{82}	ls	Q_{85}	P_{12}	$V_{51}P_{24}M_{82}$	M_{82}	Q_{85}
5	K^*_{13}	D_{83}	C_{86}	D_{83}		C_{86}	K^*_{13}	$D_{52}E_{25}D_{83}$	D_{83}	C_{86}
6	K_{14}	G_{84}	K^*_{87}	G_{84}		K^*_{87}		$T_{53}C_{26}G_{84}$	G_{84}	K^*_{87}
7	S_{15}	Q_{85}	R_{88}	Q_{85}		R_{88}		$P_{54}Q_{27}Q_{85}$	ls	R_{88}
8		C_{86}	D_{89}	C_{86}				$N_{55}S_{28}C_{86}$		D_{89}
9		K^*_{87}		K^*_{87}				/ / K^*_{87}		
10		ls		R_{88}				T_{57}/ R_{88}		
11				D_{89}						

[a] The peptide corresponding to peak 2 in Figure 4 gave unreadable multiple sequences. Peak 7 corresponds to N-terminally-blocked undigested MPI. K*: S-DABITC-modified lysine. /: gap in the sequence. ls: low signal.

the sequence of native MPI (Thompson & Ohlsson, 1986). During sequencing, αN-PTH-εNS-DABTC-lysines were identified as a gap in the place of an expected lysine or as an additional peak whose retention time was slightly longer than that of PTH-valine. Peptide 1 contains Lys 13 located in the N-terminal domain. Peptides 4 and 5 both contain Lys 87 from the C-terminal domain, indicating that thermolysin cleaves at Phe 79 or Met 82 with equal efficiency in our conditions. Sequence given by peptide 6 is too short for unambigous identification. Peptide 7 gave no sequence. Amino acid analysis (not shown) suggested that it consisted of undigested MPI that was probably N-terminally blocked

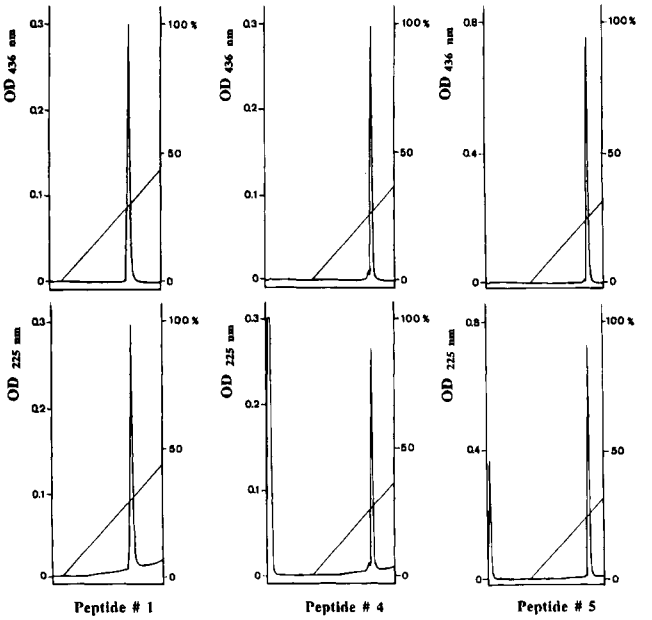


FIGURE 5: Rechromatography of fractions 1, 4, and 5 from Figure 4A on a C_8 column developed as described in the experimental section. The absorbance (OD) was monitored at 225 and 436 nm. The right hand ordinates represent the percentage of solvent B. The 225 nm peaks appearing before the start of the gradient are artifacts due to the injection of the samples.

by the lysine reagent. Table 2 shows that the minor shielded peaks (except peaks 2 and 10) result from secondary enzymatic cleavages of peptides bearing Lys 13 or 87. Peak 2 contained unreadable multiple sequences, while peak 10 gave three sequences: an unlabeled one (Leu 48-Thr 57) and two peptides containing either Lys 87 or Lys 23. The latter residue can, however, not be claimed as being an additional protected lysine because Lys 87 may interfere with the decrease of peak 10.

Table 1 shows that MPI modified by S-DABITC in the absence of heparin has a K_d for heparin that is 6-fold higher than that of the native MPI, while the inhibitor modified in the presence of heparin has about the same K_d as the native inhibitor.

Heparin−Sepharose chromatography of MPI modified by S-DABITC in the absence of heparin (Figure 2b) yields one minor peak eluting at about the same ionic strength as native MPI and two major peaks eluting at 340 and 400 mM NaCl. In contrast, the heparin-protected sample elutes as a minor peak at 340 mM NaCl and a major peak at 470 mM NaCl (Figure 2c), close to the elution position of native MPI. These data confirm that S-DABITC modification lowers the heparin−MPI affinity and indicate that the chemical modification reaction leads to a a heterogeneous population of modified molecules. Such a heterogeneity may be expected for any chemical modification of protein and suggests that the K_d must be considered as an apparent constant. Altogether these results demonstrate that lysines 13 and 87, which are modified by S-DABITC and whose modification is prevented by heparin, are essential for heparin binding.

Role of Arginine Residues in the MPI−Heparin Interaction. The three-dimensional representation of MPI reveals that the ϵ-NH_2 of lysine 87 is distant by 4.2 and 3.6 Å from the guanidinium groups of arginines 37 and 88 (Figure 6). We have therefore modified the arginine residues of MPI in order to delineate their role in heparin binding. An average of 1.5 arginines per MPI molecule was modified in the

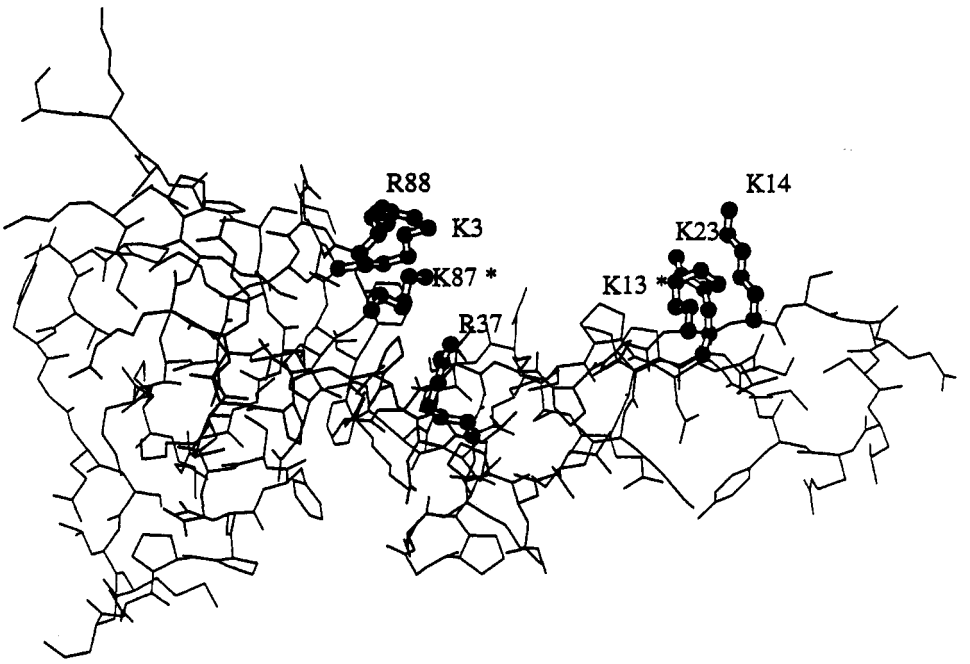


FIGURE 6: Stick drawing of MPI. The plot was generated using Molscript (Kraulis, 1991). *: lysine residues (Lys 13 and 87) protected by heparin from chemical modification by S-DABITC. ●═●: lysine or arginine residues that are potentially involved in heparin binding.

absence of heparin, whereas one residue was modified in the presence of heparin. Table 1 shows that the K_d of MPI modified by HPG in the absence of heparin is 6-fold higher than that of the control MPI sample, indicating that arginine residues are indeed involved in heparin−inhibitor binding. Modification in the presence of heparin leads to a derivative with an improved but not fully restored affinity for heparin.

Heparin affinity chromatography separates the arginine-modified inhibitor into at least four derivatives: two major ones eluting at 290 and 330 mM NaCl and two minor ones (see Figure 2d). Comparison of this profile with that of the lysine-modified inhibitor suggests that HPG has a broader reactivity than S-DABITC. Arginine modification in the presence of heparin shifts the elution profile toward higher ionic strengths, the major peak being now eluted at the position of the control MPI (Figure 2e). Thus, the protecting effect of heparin is more visible by affinity chromatography than by the kinetic measurement of K_d. The presence of two major low-affinity inhibitor derivatives in the HPG-modified sample and the almost complete disappearance of these derivatives following protection by heparin strongly suggest that at least two arginine residues participate in the binding of heparin to MPI.

Lysine and Arginine Modification in the C-Terminal Domain of MPI. The C-terminal domain of MPI was reacted with S-DABITC under the same conditions as the intact inhibitor except that the incubation time was increased from 10 to 60 min in order to obtain an extent of labeling comparable to that of intact MPI. The relative extent of labeling, i.e., labeling of inhibitor−heparin complex/labeling of free inhibitor, was the same for both incubation times. This excludes the possibility of a significant displacement of heparin by S-DABITC. An average of 0.61 and 0.53 lysine per inhibitor molecule were modified in the absence and presence of heparin, respectively.

In contrast to the clear-cut effect of heparin observed with intact MPI, no important difference was noticed between the control C-terminal domain-labeled peptide map and the heparin-protected sample (Figure 7). In addition, the pattern

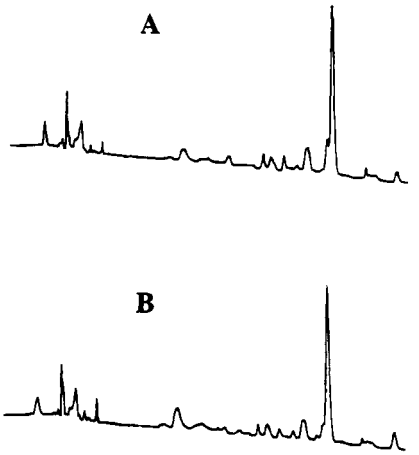


FIGURE 7: RP-HPLC analysis of the thermolysin digests of the S-DABITC-modified C-terminal domain of MPI in the absence (A) and presence (B) of a saturating concentration of heparin. For conditions, see legend to Figure 2 and text.

of labeled peptides did not match at all that of MPI. The most remarkable difference between the two maps is that the modified C-terminal domain generates none of the lysine 87-containing peptides yielded by intact MPI (compare figures 4 and 7). No attempt was made to identify the unprotected labeled peptides.

Table 1 shows that the S-DABITC modification does not strongly affect the heparin−inhibitor affinity. There is, however, a slight decrease in the affinity of the modified inhibitor for immobilized heparin since this derivative elutes at 175 mM NaCl instead of eluting at 210 mM NaCl as does the intact inhibitor (see Figure 3a−c). Protection by heparin does not prevent this slight decrease in affinity.

HPG modifies an average of 0.3 arginine residue per molecule of free C-terminal domain and 0.23 residue per molecule of heparin−inhibitor complex. The affinities of the two derivatives for heparin are close to each other and to that of the native C-terminal domain (see Table 1). Surprisingly, however, the two derivatives are eluted from heparin−Sepharose at NaCl concentrations (135 and 140

mM) lower than those of the native C-terminal domain (210 mM NaCl) of the S-DABITC-modified protein (see Figure 3d,e). The discrepancy between the K_d and the affinity chromatography data is not clearly understood. The chromatographic results nevertheless suggest that arginine residues might participate in the binding of heparin to the C-terminal domain of MPI.

DISCUSSION

Literature is well documented on heparin−protein interactions. For example, growth factors (Sudhalter et al., 1989), interleukines (Lortat-Jacob, 1992), proteinases, and proteinase inhibitors [for a review, see Bourin and Lindahl (1993)] are able to form complexes with heparin or heparin-like molecules. Although the three-dimensional structure of a heparin−protein complex has not yet been reported, a few heparin-binding sites have been partially identified. The structural requirements for heparin binding are however not well understood, probably because each protein has a unique binding site. This paper describes the initial approach to solving the structural basis of the MPI−heparin binding. Since the inhibitor−polymer complex is held together by salt bridges (Faller et al., 1992), lysine and/or arginine residues of the inhibitor are likely candidates for the interaction with negatively-charged groups of heparin. We have derivatized both of these amino acid residues using S-DABITC and HPG, respectively. The former reagent was used because the peptides generated by proteolytic cleavage of the modified protein may contain dye-labeled lysines and can thus be selectively detected with great sensitivity (Chang, 1989). On the other hand, the arginines were modified using HPG, a reagent that is specific and efficient under mild pH conditions (Yamasaki et al., 1980). Both modifications were done at pH 7.4 where heparin exhibits a good affinity for MPI and its C-terminal domain. This affinity was assessed using two procedures, one based on the abiilty of soluble heparin to accelerate the inhibition of chymotrypsin by the inhibitors and the other exploiting the affinity of the inhibitors for immobilized heparin. These two methods yield complementary information: the enzymatic method diagnoses only that or those heparin−inhibitor binding mode(s) that lead(s) to an activation of the inhibitor, while the affinity chromatography recognizes all possible complexes. The enzymatic method enabled us to measure K_d values with all inhibitor samples, indicating that heparin was able to activate all forms of the inhibitor, i.e., native or chemically-modified full-length MPI and C-terminal domain. Sepharose−heparin affinity chromatography gave additional information: (i) when chemical modification increased K_d, it showed that the modification medium may contain more than one form of the modified inhibitor and (ii) in cases where chemical modification did not change K_d, it indicated nevertheless, that some derivatization had occurred (compare Table 1 and Figure 3). In the latter case, the modified inhibitor binds heparin, but this binding does not lead to inhibitor activation.

The most straightforward chemical modification data were obtained with MPI. The derivatization of lysines and arginines led to a 6-fold increase in K_d. Heparin significantly prevented these effects. Amino acid sequencing unambiguously identified the heparin-protected lysines as Lys 13 and 87 located on the N- and C-terminal domains, respectively. Interestingly, those heparin-protected ϵ-NH_2 groups which are the most reactive with S-DABITC at pH 7.4 (see Figure 4 and Table 2) are also the most important for heparin binding and inhibitor activation. Sepharose−heparin affinity chromatography suggests the involvement of two arginine residues mainly in polymer binding and inhibitor activation, presumably Arg 37 and 88 distant by 4.2 and 3.6 Å from Lys 87. Thus, each domain of MPI bears at least two positively-charged residues that interact with heparin: Lys 13 and Arg 37 for the N-terminal domain and Lys 87 and Arg 88 for the C-terminal one. Since the residues around Lys 87 are separated from each other by short distances, one may speculate that heparin strengthens the interdomain interactions that already take place in the absence of the polymer (Grütter et al., 1988).

S-DABITC modification and sequencing of the fragmented C-terminal domain of MPI failed to confirm the participation of Lys 87 in heparin binding. In addition, the derivatization did not lead to a change in K_d, although 0.6 lysine per molecule were modified. HPG also gave essentially negative results. Following each modification, however, affinity chromatography diagnosed a species with a reduced affinity for immobilized heparin (Figure 3). Soluble heparin was unable to prevent the occurrence of these low affinity derivatives as it did with the full-length inhibitor. These results, together with the observed low affinity of the C-terminal domain for soluble and insoluble heparin, suggest that the polymer binds the truncated inhibitor via a number of binding modes, none of which is predominant. This lack of preferential binding might result from a change in the environment of Lys 87. Such an alteration is expected since in full-length MPI Lys 87 is close to several charged amino acid residues located on the N-terminal domain (figure 6). Alternatively, the disconnection of the C-terminal domain from the N-terminal part of the inhibitor could cause a conformational change in the former domain. The fact that four disulfide bonds tightly lock the spatial arrangement of each domain (Grütter et al., 1988) gives, however, little credence to this hypothesis.

The three-dimensional representation of MPI shows that both Lys 13 and Lys 87 clearly rise above the average surface of the molecule (Figure 6). This topology might explain their favorable reaction with S-DABITC. Each of these residues is located on one side of the concave surface of the boomerang-like-shaped molecule, and they are distant from each other by 16 Å. Moreover, each of them is surrounded by other cationic residues potentially able to bind heparin. For instance, Lys 87 is distant from Lys 3, Arg 88, and Arg 37 by only 6.3, 3.6, and 4.2 Å, respectively. The above four residues, therefore, form a compact cluster of positive charges that might serve as a major anchorage point for heparin. On the other hand, Lys 13 is surrounded by Lys 14 and 23 located at 5.9 and 12.8 Å from it. These three residues form a less compact cluster of charges that might serve as a secondary binding site for heparin. The overall organization and orientation of lysine 13 and 87 and of the other potentially implicated amino acid residues (Figure 6) suggest that heparin could act as a link nested in the concave surface delimited by the two domains of the inhibitor. This may strengthen interdomain interactions and hence freeze the conformation of the protein. This view is supported by our previous observation of a heparin-induced conformational change of the inhibitor (Faller et al., 1992). In addition, one may suggest that the two domains play a cooperative function in heparin binding, the occupancy of one domain favoring the productive binding of heparin with the other domain.

Lysine 3 could not be identified as a heparin-shielded group although it is distant by only 6.3 Å from lysine 87. Perhaps the pK_a of this residue is higher than that of lysine 13 or 87 which would slow down the reaction with S-DABITC. Alternatively, one may hypothesize that the ionic interaction between lysine 3 and heparin is so weak that it may be readily displaced by S-DABITC. We do not know whether lysine 23 is protected by heparin since this residue was found in an unresolved HPLC peak. Further investigations are therefore required to decide whether all of the afore-mentioned basic residues participate in heparin binding.

The enzymatic method we have designed for measuring the K_d of the heparin−inhibitor complex also yields $k_{ass(0)}$ and $k_{ass(max)}$, the rate constants of chymotrypsin inhibition in the absence of heparin and in the presence of a saturating concentration of the polymer, respectively. These measurements show that MPI and its C-terminal domain have identical $k_{ass(0)}$ values but significantly differ in $k_{ass(max)}$, the highest rate constant being observed with the full-length inhibitor. This, together with the observation that the heparin−inhibitor affinity is higher with MPI than with its C-terminal domain, indicates that the N-terminal domain favors both heparin binding and heparin-induced activation of the full-length inhibitor.

MPI and α_1-proteinase inhibitor are the physiological antielastases that normally protect the lung against the connective tissue protein-degrading activity of neutrophil elastase (Bieth, 1986). Chronic pulmonary infection, commonly associated with cystic fibrosis, leads to an important recruitment of neutrophils in airways with a resultant enormous load of neutrophil elastase which frequently overcomes the local antielastase potential (Mc Elvaney et al., 1993) and leads to lung tissue destruction, the major cause of mortality in cystic fibrosis. This prompted research aimed at testing MPI as a potential drug in cystic fibrosis (Vogelmeier et al., 1990; Mc Elvaney et al., 1993). In preliminary clinical trials, very large amount of MPI had to be used to get significant effects on the elastase burden (Mc Elvaney et al., 1993). We believe that the concomitant use of heparin would significantly decrease the therapeutic doses of MPI. In this context, investigation of the heparin-binding site of MPI may help to model the MPI−heparin complex and design more efficient glycosaminglycans or a covalently-linked inhibitor−heparin derivative useful in therapy.

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