Heparin-Induced Conformational Change and Activation of Mucus Proteinase Inhibitor

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Received April 2, 1992; Revised Manuscript Received June 9, 1992

ABSTRACT: Low molecular mass heparin (5.1 kDa) forms a tight complex with mucus proteinase inhibitor, the physiologic neutrophil elastase inhibitor of the upper respiratory tract. This binding strongly enhances the intrinsic fluorescence of the inhibitor and the rate of neutrophil elastase inhibitor association. One mole of this heparin fragment binds 1 mol of inhibitor with a K_d of 50 nM. From the variation of K_d with ionic strength, it is inferred that (i) 85% of the heparin–inhibitor binding energy is due to electrostatic interactions, (ii) about seven ionic interactions are involved in heparin–inhibitor binding, and (iii) about one-third of the ionized charges of heparin and inhibitor are involved in the complex. Heparin 4-fold increases the very low quantum yield of Trp30, the single tryptophan residue of the inhibitor, blue-shifts its maximum emission wavelength by 6 nm, decreases the acrylamide quenching rate constant by a factor of 4, and increases the mean intensity weighted lifetime by a factor of 2.5. These important spectroscopic changes evidence a heparin–induced conformational change of the inhibitor which buries Trp30 in a very hydrophobic environment. Heparin accelerates the inhibition of elastase in a concentration-dependent manner. When both enzyme and inhibitor are saturated by the polymer, the second-order association rate constant is 7.7 $\times 10^7$ M^{-1} s^{-1}, a value that is 27-fold higher than that measured with the free partners. This finding may have important physiologic and therapeutic bearing.

Heparin, a naturally occurring sulfated glycosaminoglycan, modulates the activity of numerous biological systems (Lane & Lindhal, 1989). Among others, it binds and activates a number of protein proteinase inhibitors that belong to the serpin superfamily, namely, antithrombin III, heparin cofactor, protease nexin, protein C, and plasminogen activator inhibitor I (Lane & Lindhal, 1989; Huber & Carrell, 1989). Although heparin does not bind α_1-proteinase inhibitor, another member of the serpin family, it strongly impairs the neutrophil elastase (NE)[1] inhibitory activity of this physiologic anti-elastase molecule. This is due to tight binding of NE to heparin: K_d = 3.3 and 89 nM for high and low molecular mass heparin, respectively (Frommherz et al., 1991). Saturating concentrations of high and low molecular mass heparin decrease the second-order rate constant (k_{ass}) for enzyme–inhibitor association by factors of 300 and 40, respectively. Thus, if heparin is given to patients with neutrophil activation, e.g., during septicemia or extracorporal circulation, α_1-proteinase inhibitor might not take up the released NE fast enough to fully prevent NE-mediated intravascular proteolysis, a feature that is commonly observed in the above clinical situations (Duswald et al., 1985). Also, from a physiologic point of view, our previous data suggest that the rate of NE inhibition in the lung is lower than expected from the in vitro determined k_{ass} value because heparin and heparin-like molecules are abundant in lung tissue (Clark et al., 1983).

MPI is the other physiologic NE inhibitor present in the lung. In contrast to α_1-proteinase inhibitor, it is a reversible inhibitor that does not belong to the serpin family. MPI is an 11.7-kDa basic protein whose structure is stabilized by eight disulfide bonds. It is formed of a single chain of 107 amino acid residues of known sequence (Seemüller et al., 1986; Thompson & Ohlsson, 1986). The inhibitor is composed of two domains of similar size and architecture (Grütter et al., 1988). Two reactive sites, one per domain, have been postulated in view of sequence similarities with other small proteinase inhibitors (Seemüller et al., 1986; Thompson & Ohlsson, 1986). One of them has been identified in the C-terminal domain (P_1 = Leu72) by diffraction studies on the MPI–chymotrypsin complex (Grütter et al., 1988). No inhibitory site could be detected as yet on the N-terminal domain, although enzymatic and physical–chemical studies indicate that 1 mol of inhibitor binds 2 mol of trypsin, chymotrypsin, and cathepsin G (Boudier & Bieth, 1991).

Recombinant MPI (Miller et al., 1989) is being tested as an anti-elastase drug for the potential treatment of destructive lung diseases (Vogelmeier et al., 1990; Lucey et al., 1990). It was thus found of interest to see whether heparin impairs its NE inhibitory capacity as it does with α_1-proteinase inhibitor.

EXPERIMENTAL PROCEDURES

Materials

Human NE was isolated from purulent sputum (Martodam et al., 1979) and active-site-titrated with acetyl-Ala_2-AzaAla *p*-nitrophenyl ester (Powers et al., 1984) from Enzyme System Products, Livermore, CA. Recombinant MPI, prepared by Synergen, Boulder, CO, was kindly provided by Dr. H. P. Schnebli from Ciba-Geigy, Basel, Switzerland, and was active-site-titrated with NE (Bruch & Bieth, 1986). MeOSuc-Ala_2-Pro-Val-SBzl was purchased from Bachem, Bubendorf,

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[1] Abbreviations: MPI, mucus proteinase inhibitor, secretory leukoprotease inhibitor; NE, neutrophil elastase; MeOSuc-Ala_2-Pro-Val-SBzl, methoxysuccinyl-Ala_2-Pro-Val thiobenzyl ester.

0006-2960/92/0431-8285$03.00/0

Switzerland. Heparin was a 5.1-kDa fragment purchased from Calbiochem, La Jolla, CA. Unless otherwise stated, all experiments were done in 50 mM Hepes/100 mM NaCl, pH 7.4, a solution that will be referred to as the "buffer" throughout the text.

Spectroscopic Methods

Fluorescence spectroscopy was done with 2 μM MPI ± 4 μM heparin dissolved in the buffer. This heparin concentration saturates 2 μM MPI to the extent of 97% as calculated using eq 3 and K_d = 50 nM (see below).

Absorption Spectra. Absorption spectra were recorded with a CARY-1 spectrophotometer and 10 μM MPI dissolved in the buffer. The value of 8770 M^{-1} cm^{-1} was taken as the molar extinction coefficient at 280 nm (Smith & Johnson, 1985).

Steady-State Fluorescence. Fluorescence spectra were recorded with an SLM 48000 spectrofluorometer. The excitation wavelength was set either at 295 nm for the selective excitation of tryptophan or at 280 nm for the excitation of both tyrosine and tryptophan residues. The solution absorbance was always less than 0.1 at the excitation wavelength. Quantum yields of free or heparin-bound MPI were determined using L-tryptophan in water as a reference (ϕ = 0.14 at 20 °C) (Eisinger & Navon, 1969) and were corrected for the screening effect of scattered light (Hélène et al., 1971).

Fluorescence Lifetimes. Lifetime measurements were made with a single photon counting technique using a frequency-doubled rhodamine 6G laser, dumped and synchronously pumped by a mode-locked argon laser (Spectra Physics). Temperature was maintained at 20 °C. The excitation and emission wavelengths were set at 295 and 350 nm, respectively. The emission was detected with a Phillips XP 2020 photomultiplier at right angle to the excitation beam and through a polarizer set at the magic angle (54.7°) to the direction of the excitation polarization. The instrumental response function was approximated by the observed decay of a strongly quenched fluorophore of negligible lifetime (<20 ps), i.e., *p*-terphenyl dissolved in a mixture of 2 volumes of cyclohexane and 1 volume of CCl_4 (Kolber & Barkley, 1986). The fluorescence intensity decays, $I(t)$, were analyzed assuming a sum of exponentials as shown in eq 1 where I_0 is the intensity

$$I(t) = I_0 \sum_i \alpha_i e^{-t/\tau_i} \quad (1)$$

at $t = 0$, α_i stands for the normalized preexponential terms obeying the relationship $\sum_i \alpha_i = 1$, and τ_i are the fluorescence lifetimes. The fit between the experimental and theoretical curves was estimated with a reduced χ^2 test using an iterative nonlinear least-squares convolution procedure based on the Marquardt algorithm. The fractional intensities, f_i, associated with each lifetime were calculated using $f_i = \alpha_i \tau_i / \sum_i \alpha_i \tau_i$. The mean intensity-weighted lifetime, τ_m, was calculated using $\tau_m = \sum_i f_i \tau_i$.

Acrylamide Fluorescence Quenching. Aliquots of a concentrated stock solution of acrylamide were added to MPI in the absence or presence of a saturating heparin concentration. Changes in the fluorescence intensity at 350 nm were recorded. The data were corrected for the dilution of the sample and the screening effect due to the absorption of acrylamide at 295 nm (Hélène et al., 1971) and were analyzed using the modified Stern–Volmer relationship (Eftink & Gihron, 1976):

$$I_0/I = (1 + K_{sv}[Q])e^{V[Q]} \quad (2)$$

where I_0 and I are the fluorescence intensites in the absence and presence, respectively, of a given concentration, [Q], of acrylamide. K_{sv}, the Stern–Volmer constant describing the collisional quenching process, is given by the product of k_q, the bimolecular quenching rate constant, and τ_m, the mean intensity-weighted lifetime in the absence of the quencher. V is the static quenching constant.

Binding Stoichiometry and Equilibrium Dissociation Constants for MPI–Heparin and NE–Heparin Complexes. The binding of heparin to MPI was monitored by measuring the enhancement of the fluorescence of the inhibitor with a Shimadzu RF-5000 spectrofluorometer equipped with a thermostated cell holder. The excitation and emission wavelengths were set to 295 and 350 nm, respectively. The binding experiments were performed by adding increasing concentrations of heparin to a constant concentration of MPI. The stoichiometries were determined using 1 μM MPI while the equilibrium dissociation constants, K_d, were measured using 0.1 μM MPI. K_d was calculated by a nonlinear least-squares fit of the data to the relationship:

$$\Delta F = \frac{([I_0] + [H_0] + K_d) - \sqrt{([I_0] + [H_0] + K_d)^2 - 4[I_0][H_0]}}{2[I_0]} \Delta F_{max} \quad (3)$$

where $[I_0]$ and $[H_0]$ are the total concentrations of MPI and heparin, respectively. ΔF and ΔF_{max} are the variation of fluorescence intensity at a given heparin concentration and at saturating heparin concentrations, respectively.

The binding stoichiometry and the equilibrium dissociation constant of the NE–heparin complex were determined enzymatically as described previously (Frommherz et al., 1991).

Enzymatic Methods

Kinetics of inhibition of NE by MPI were assessed using the progress curve method. NE ± heparin was added to a buffered solution of MPI, MeOSuc-Ala_2-Pro-Val-SBzl, and 5,5′-dithiobis(2-nitrobenzoic acid), a coupling reagent that transforms benzylthiol, the product of substrate hydrolysis, into a yellow-colored compound [ϵ_{412nm} = 13 600 M^{-1} cm^{-1} (Stein et al., 1987)]. The absorbance at 412 nm was recorded using a CARY 2200 spectrophotometer on-line with an IBM PS/2 Model 30 microcomputer. Nonlinear regression analysis of the progress curves was done using ENZFITTER software. The final reagent concentrations were as follows: NE, 10 nM; MPI, 100 nM; heparin, variable; substrate, 0.6 mM; 5,5′-dithiobis(2-nitrobenzoic acid), 0.2 mM; dimethylformamide, 1.8% (v/v). In some experiments, progress curves were recorded following addition of NE to a mixture of MPI, heparin, and substrate.

RESULTS

Binding of MPI to Heparin–Sepharose. Heparin–Sepharose affinity chromatography, done exactly as described earlier (Frommherz et al., 1991), showed that MPI binds to the matrix and elutes at 0.7 M NaCl. The NE–MPI complex elutes at 0.8 M NaCl.

Fluorescence Properties of Free and Heparin-Bound MPI. (*A*) *Steady-State Fluorescence.* The fluorescence spectrum of free MPI is characterized by a maximum emission wavelength of 336 nm. Heparin blue-shifts this wavelength to 330 nm. On the other hand, the very low quantum yield of free MPI dramatically increases in the presence of heparin (Table I).

(*B*) *Fluorescence Lifetimes.* Two exponentials were necessary and sufficient to describe the fluorescence decay curves for both free and heparin-bound MPI. The fluorescence decay of free MPI is characterized by a relatively short lifetime (≈1

Table I: Steady-State Fluorescence Parameters of Free and Heparin-Bound MPI[a]

	$\lambda_{em(max)}$ (nm)[a]	quantum yields at 280 nm	quantum yields at 295 nm
free MPI	336 ± 1	0.041 ± 0.002	0.045 ± 0.001
MPI–heparin complex	330 ± 1	0.15 ± 0.01	0.18 ± 0.010

[a] The measurements were done with at least three different samples. The results are expressed as mean ± standard error of the mean.

ns) associated with ca. 90% fractional fluorescence intensity. In contrast, the fluorescence of the MPI–heparin complex is dominated by a much longer lifetime (≈3 ns) associated with ca. 80% fractional intensity. The overall effect of heparin is to increase the mean intensity-weighted lifetime of MPI by a factor of 2.5 (see Table II).

(C) Acrylamide Fluorescence Quenching. Table III shows that heparin also alters the acrylamide-induced fluorescence quenching of MPI. The free inhibitor exhibits a high bimolecular quenching rate constant ($k_q = 2.2 \times 10^9$ M^{-1} s^{-1}) and a rather low static component ($V = 0.36$ M^{-1}). Heparin decreases k_q about 4-fold and slightly increases the static component.

Binding Characteristics of the MPI–Heparin and NE–Heparin Complexes. Figure 1 shows the effect of increasing concentrations of heparin on the intrinsic fluorescence of constant concentrations of MPI. The concaved titration curve obtained with 0.1 μM inhibitor was used to determine the equilibrium dissociation constant K_d of the complex while the straight titration curve obtained with 1 μM inhibitor and shown in the inset of Figure 1 was used to determine the stoichiometry of binding. Thus, 1 mol of heparin binds 1 mol of MPI with a K_d of 50 ± 9 nM. On the other hand, enzymatic measurements (Frommherz et al., 1991) show that 1 mol of heparin binds about 1 mol of NE with a K_d of 6 ± 2 nM (data not shown). Heparin is known to be heterogeneous in size distribution and sulfation pattern. Even heparin fragments such as those used in the current study likely exhibit some polydispersity and charge heterogeneity. We nevertheless analyzed our binding data assuming our preparation contained heparin chains with a uniform 5.1-kDa molecular mass and an even charge distribution. The 1:1 binding stoichiometry found for both MPI and NE appears to validate this assumption.

Influence of Ionic Strength on the K_d of the Heparin–MPI Complex. The K_d was determined in the presence of 0.1–0.3 M NaCl. The data were analyzed using the theory of protein–polyelectrolyte interactions (Record et al., 1978; Olson et al., 1991), according to which, the binding of a protein (MPI) to a polyelectrolyte (heparin) in the presence of a monovalent salt (NaCl) may be described by the equilibrium shown in Scheme I where Z is the number of ionic interactions that MPI makes with heparin which result in the displacement of $Z\psi$-bound Na^+ ions from the polymer, ψ being the fraction of Na^+ bound to heparin per unit charge, a dimensionless number that depends only upon the axial charge density of the polymer.

Scheme I

$$MPI^{Z+} + \text{heparin} \overset{K_d}{\rightleftharpoons} \text{MPI–heparin} + Z\psi Na^+$$

The observed equilibrium dissociation constant, K_d, characterizing Scheme I is related to the nonionic equilibrium dissociation constant, $K_{d(nonionic)}$, through the relationship:

$$\log K_d = \log K_d(\text{nonionic}) + Z\psi \log [Na^+] \quad (4)$$

Figure 2 shows that the plot of log K_d vs log $[Na^+]$ is linear as predicted from eq 4. The value of Z calculated from the slope of the curve and using $\psi = 0.8$ (Olson et al., 1991) was found to be 7.2 ± 0.2. Thus, seven ionic interactions are involved in the binding of heparin to MPI. For $[Na^+] = 1$ M, log K_d = log $K_{d(nonionic)}$ and $K_{d(nonionic)} = 68$ mM. The binding of heparin to MPI is thus mainly governed by electrostatic forces. In the presence of 0.1 M NaCl, the ionic strength used throughout this paper, $K_d = 50$ nM while $K_{d(nonionic)} = 68$ mM. Comparison of the logarithms of these numbers, i.e., −7.3 and −1.17, respectively, indicates that 84% of the heparin–MPI binding energy is accounted for by ionic interactions.

Influence of Heparin on the Inhibition of NE by MPI. The progress curve method (Morrisson & Walsh, 1988) was used to follow the time course of inhibition of NE by MPI. Enzyme ± heparin was added to a mixture of inhibitor and substrate, and the concentration of product (absorbance of 412 nm) was measured with time. Figure 3 shows typical progress curves recorded in the absence of heparin and in the presence of a saturating concentration of the polymer. In each case, a pre-steady-state release of product precedes the steady-state of the reaction. Heparin dramatically decreases the concentration of product released during the pre-steady-state and considerably shortens the duration of the latter. The curves were analyzed assuming Scheme II where E is NE or NE–heparin, I is MPI or MPI–heparin, S and P are the substrate and the product, respectively, k_{ass} is the second-order rate constant for the formation of the EI complex, and k_{diss} is the first-order rate constant for the decomposition of this complex. Neither I nor S is depleted to a significant extent during the progress of the reaction since $[I_0] = 10[E_0]$ and since the highest [P] is much lower than $[S_0]$ (see Figure 3). Hence, the progress curves shown in Figure 3 can be described by the following integrated equation (Morrison & Walsh, 1988):

$$[P] = v_s t + \frac{v_z - v_s}{k}(1 - e^{-kt}) \quad (5)$$

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. The progress curves were fitted to eq 5 by nonlinear least-squares analysis to obtain the best estimates of v_z, v_s, and k. Figure 3 shows that the fit between the theoretical and the experimental curves is excellent in the absence of heparin and fair in the presence of the polymer.

The following relationships were used to calculate k_{ass} and k_{diss} from v_z, v_s, and k (Morrison & Walsh, 1988):

$$k = \frac{k_{ass}[I_0]}{1 + [S_0]/K_m} + k_{diss} \quad (6)$$

$$k_{diss} = k(v_s/v_z) \quad (7)$$

k_{ass} and k_{diss} were found to be 2.8×10^6 M^{-1} s^{-1} and 1.2×10^{-4} s^{-1} in the absence of heparin and 7.7×10^7 M^{-1} s^{-1} and 4×10^{-3} s^{-1} in the presence of a saturating concentration of the polymer. Thus, heparin increases k_{ass} about 30-fold but virtually does not change K_i ($K_i = k_{diss}/k_{ass} \sim 5 \times 10^{-11}$ M). When the progress curves were recorded following addition of NE to a mixture of MPI, heparin, and substrate, similar rate constants were observed, indicating that the equilibrium between heparin, MPI, and NE establishes rapidly.

Table II: Fluorescence Lifetimes of Free and Heparin-Bound MPI[a]

	τ_1 (ns)	f_1 (%)	τ_2 (ns)	f_2 (%)	τ_m (ns)	χ^2
free MPI	0.94 ± 0.05	89 ± 3	2.1 ± 0.4	11 ± 3	1.06 ± 0.05	1.15 ± 0.02
MPI–heparin complex	1.40 ± 0.10	23 ± 1	3.13 ± 0.03	77 ± 1	2.72 ± 0.02	1.03 ± 0.02

[a] The excitation wavelength was 295 nm. τ_1, τ_2 = component lifetimes; f_1, f_2 = fractional fluorescence intensities; τ_m = mean intensity-weighted lifetime. For error calculations, see Table I.

Table III: Acrylamide Quenching Parameters for Free and Heparin-Bound MPI[a]

	K_{sv} (M^{-1})	k_q ($\times 10^{-9}$ M^{-1} s^{-1})	V (M^{-1})
free MPI	2.35 ± 0.10	2.2 ± 0.2	0.36 ± 0.04
MPI–heparin complex	1.50 ± 0.06	0.55 ± 0.06	0.65 ● 0.07

[a] K_{sv} = Stern–Volmer constant, k_q = bimolecular quenching rate constant, and V = static quenching constant. For error calculations, see Table I.

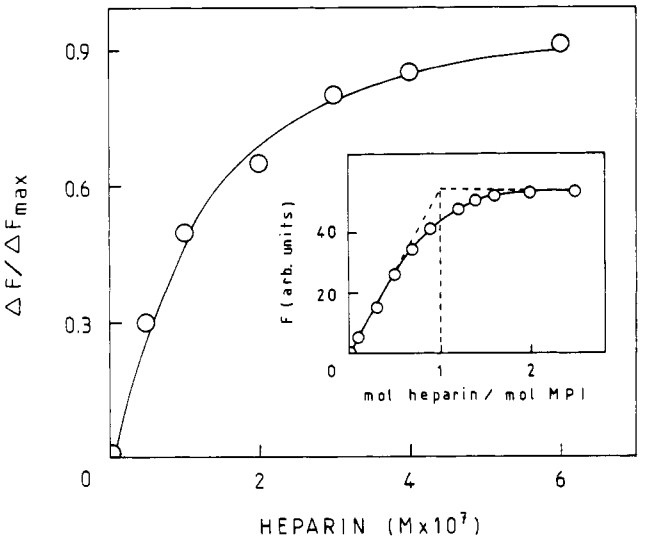

FIGURE 1: Determination of the stoichiometry and the equilibrium dissociation constant (K_d) of the heparin–MPI complex at pH 7.4 and 25 °C. K_d was measured by reacting constant concentrations of MPI (0.1 μM) with increasing concentrations of heparin, recording the fluorescence intensities of the mixtures, and fitting the data to eq 3 by nonlinear regression analysis. (○) Experimental points; (—) theoretical curve generated using the best estimate of K_d (50 nM). The inset shows a similar titration experiment done with 1 μM MPI and used to infer the MPI/heparin binding stoichiometry. Note the large heparin-induced increase in fluorescence intensity.

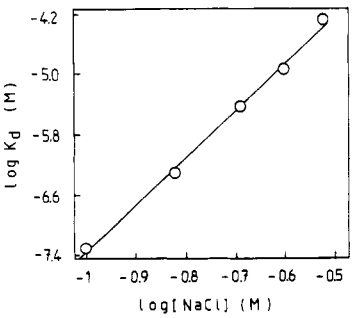

FIGURE 2: Influence of ionic strength on the K_d of the heparin–MPI complex measured at pH 7.4 and 25 °C (50 mM Hepes buffer). K_d values were plotted as a function of [Na^+] in accordance with eq 4, and the data were analyzed by linear regression. K_d = 50 ± 9 nM, 0.50 ± 0.08 μM, 3.6 ± 0.4 μM, 11 ± 2 μM, and 50 ± 7 μM for [NaCl] = 0.1, 0.15, 0.2, 0.25, and 0.30 M, respectively.

Progress curves were also recorded with nonsaturating concentrations of heparin. Under these conditions, the reaction media contain both free and heparin-bound NE and MPI (see Table IV), predicting a complex kinetic behavior. Surprisingly, however, all progress curves could be well fitted to eq

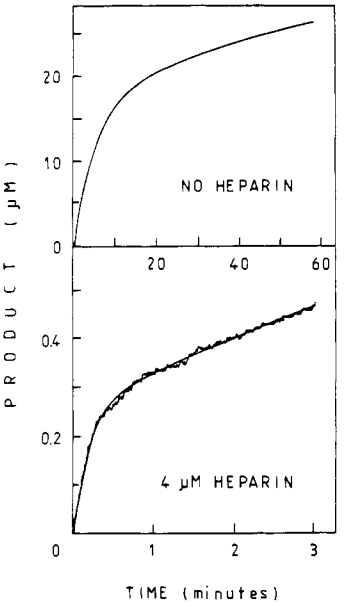

FIGURE 3: Progress curves for the inhibition of 10 nM NE by 100 nM MPI at pH 7.4 and 25 °C in the absence and presence of 4 μM heparin, a concentration sufficient to saturate both NE and MPI. The experimental curves were fit to eq 5 by nonlinear regression analysis. The smooth lines represent the theoretical curves corresponding to the best fit. The experimental and theoretical curves are superimposed in the upper panel.

Scheme II

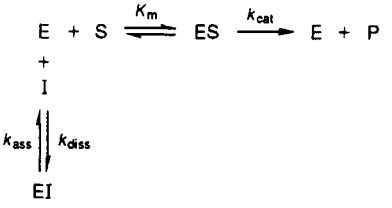

Table IV: Effect of Heparin on the Second-Order Rate Constant (k_{ass}) for the Inhibition of NE by MPI at pH 7.4 and 25 °C[a]

	k_{ass}[b]		% heparin bound	
[heparin] (M)	M^{-1} s^{-1}	rel values	NE[c]	MPI[c]
none	2.8×10^6	1.0	0	0
2.5×10^{-8}	4.9×10^6	1.6	54	12
5.0×10^{-8}	2.1×10^7	7.5	74	25
1.0×10^{-7}	3.3×10^7	13.0	88	47
1.0×10^{-6}	6.3×10^7	22.0	99	95
4.0×10^{-6}	7.7×10^7	27.0	~100	~100

[a] The k_{ass} was determined as described in Figure 3. [b] Mean value for $n \geq 3$, the individual values varying by ≤15%. [c] Calculated using the K_d values from Table IV and a published program for computing distribution diagrams for multiple equilibria (De Stefano, 1989).

5 (data not shown) so that a "k_{ass}" value could be calculated in each case. Table IV shows that this value sharply increases with the concentration of heparin. The relative k_{ass} was linearly related to the percentage of heparin-bound MPI but not to that of heparin-bound NE (Figure not shown). This strongly suggests that the activation of k_{ass} by heparin is due to the binding of the polymer to MPI. The strong effect of heparin on k_{ass} probably explains why mixtures containing

significant amounts of free MPI did not yield a complex kinetic behavior.

DISCUSSION

Heparin binds numerous proteins including macromolecules of the clotting cascade (Danielsson et al., 1986; Hogg & Jackson, 1990; Stein et al., 1989) and the neutrophil proteinases NE and cathepsin G (Frommherz et al., 1991; Marrossy, 1981). We show for the first time that it also forms a complex with MPI, an 11.7-kDa cationic serine proteinase inhibitor present in mucus secretions. Heparin strongly enhances the intrinsic fluorescence of MPI, a property that could be used to measure the stoichiometry and the equilibrium dissociation constant of the heparin–MPI complex. The observed fluorescence enhancement led us to study in detail the fluorescence properties of free and heparin-bound MPI in order to ascertain whether or not this enhancement is related to a conformational change of the protein. MPI has only one tryptophan residue, Trp30 located in the N-terminal domain, and two tyrosine residues, one per domain. This eases the interpretation of the spectroscopic data.

Steady-state fluorescence studies indicate that free MPI exhibits a Trp fluorescence (λ_{ex} = 295 nm) with a maximum emission wavelength close to 336 nm. This value is significantly lower than that observed with Trp in water, which indicates that the immediate surrounding of Trp30 is less polar than water. The structural data of Grütter et al. (1988) show that Trp30 lies on the surface of the protein but is in van der Waals contact with the aromatic ring of Phe79, the β-CH_2 of Asp29, and the hydrophobic part of Lys6. Thus, a significant part of the indole ring is embedded in a hydrophobic environment. This accounts for the low value of the maximum emission wavelength. The surface exposure of Trp30 also explains the very high bimolecular quenching rate constant observed upon reacting acrylamide with free MPI. Reaction of the inhibitor with heparin further blue-shifts the maximum emission wavelength to 330 nm. This, together with the 4-fold drop in the acrylamide quenching rate constant, indicates that heparin renders the environment of Trp30 highly hydrophobic.

The quantum yield of Trp30 in free MPI is among the lowest ever reported in proteins (Chen, 1990). Disulfide linkages, peptide bonds, or protonated histidines are internal quenchers that may decrease the quantum yield of Trp in proteins. MPI has no histidine residue, and the closest disulfide bond is distant by about 8 Å from Trp30, eliminating it as a candidate for internal quenching. Interestingly, however, five peptide bonds of the protein backbone are in van der Waals contact with the indole ring of Trp30 and are thus probably responsible for its low quantum yield. Heparin increases the latter by a factor of 4, indicating that within the heparin–MPI complex the quenchers have been significantly removed from Trp30.

The biexponential fluorescence decay of free MPI is dominated by a relatively short lifetime, confirming that Trp30 lies mainly in an environment with strong quenching properties. Heparin increases the mean intensity-weighted lifetime by a factor of 2.5, indicating that it strongly decreases the dynamic quenching of Trp30. This increase in lifetime, however, is significantly lower than the enhancement in quantum yield. Such a discrepancy has been observed for other systems and may be ascribed either to a decrease in static quenching which increases the quantum yield without affecting the lifetime or to an increase in radiative lifetime associated with a change in the dipolar environment of the indole ring (Hutnik et al., 1990). In our case, the latter possibility may be excluded as heparin only slightly decreases the maximum emission wavelength of Trp30. The polymer therefore decreases both the dynamic and the static quenching of Trp30.

In conclusion, heparin brings Trp30 in a highly hydrophobic environment as evidenced by the important decrease of the maximum emission wavelength and the acrylamide quenching rate constant. This increase in hydrophobicity cannot be due to a simple shielding effect of heparin on the indole ring because the heparin molecule is very hydrophilic. The increase in the hydrophobicity of the indole's environment together with the strong decrease in both static and dynamic quenching of the chromophore is more likely indicative of a heparin-induced change in the conformation of MPI.

The heparin–MPI binding is very sensitive to ionic strength as are other heparin–protein interactions (Frommherz et al., 1991; Marossy, 1981; Olson & Björk, 1991; Ehrlich et al., 1991): the K_d of the complex increases 1000-fold when the ionic strength increases from 0.1 to 0.3. Analysis of the ionic strength dependence of K_d according to the theory of protein–polyelectrolyte interactions (Record et al., 1978; Olson et al., 1991) indicates that at μ = 0.1 about 85% of the heparin–MPI binding energy is due to ionic interactions and that about seven ionic interactions are involved in heparin–MPI binding. The heparin fragment used here is composed of about eight disaccharide units [one unit has a molecular mass of about 0.6 kDa; refer to Casu (1990)]. If we assume that each disaccharide unit bears an average of 3.5 negatively charged group (sulfates + carboxylates) (Lindhal et al., 1984) and that 80% of them are ionized [ψ = 0.8; see Olson et al. (1991)], we may conclude that 1 mol of heparin carries about 22 negatively charged groups that are able to interact with the positively charged groups of MPI. This result, compared to the seven ionic interactions calculated above, indicates that about one-third of the ionized groups of heparin are involved in the binding of MPI. On the other hand, MPI carries 20 positively charged groups [15 lysines and 5 arginines; see Thompson and Ohlsson (1986)]. Hence, the heparin–MPI interaction also involves about one-third of the inhibitor's charged groups.

We have recently shown that the NE–heparin complex reacts much slower with α_1-proteinase inhibitor or eglin c than free NE. It thus appeared surprising that heparin increases the rate of inhibition of NE by MPI. Since α_1-proteinase inhibitor and eglin c do not bind heparin, it is likely that this increase is due to the ability of the polymer to bind MPI and to change its structure in a way that brings the inhibitor's active-site loop in a conformation that is more suitable for the binding of NE. When both NE and MPI are saturated with heparin, k_{ass} increases by a factor of 27 but k_{diss} increases by about the same factor so that K_i remains essentially constant. Heparin therefore does not alter the stability of the NE–MPI complex but simply decreases the time required for the enzyme–inhibitor system to reach the equilibrium.

The effect of heparin on the NE–MPI system is reminiscent of that observed by others on the thrombin/antithrombin III pair: heparin changes the conformation of antithrombin (Olson & Shore, 1981) and accelerates the inhibition of thrombin by this inhibitor (Jordan et al., 1979). There are, however, quantitative differences in the effect of heparin on the two inhibitors: (i) the intrinsic fluorescence of antithrombin increases by 40% only (Olson & Shore, 1981); (ii) the k_{ass} for thrombin–antithrombin association is increased 2300-fold (Jordan et al., 1979).

We believe our finding has important pathophysiological implications. MPI and α_1-proteinase inhibitor are the physiologic NE inhibitors that protect the lung against the tissue-destructive function of accidentally liberated NE (Bieth, 1986). This protecting function may be expressed in a quantitative way through dt, the delay time of inhibition, i.e., the time required to almost fully inhibit NE in vivo: $dt = 5/k_{ass}[I_0]$ where $[I_0]$ is the in vivo inhibitor concentration (Bieth, 1980). The lower dt, the less substrate hydrolysis during the inhibition process, hence, the more efficient the anti-elastase protection. MPI and α_1-proteinase inhibitor have about equal k_{ass} values in the absence of heparin (Frommherz et al., 1991; Boudier & Bieth 1989). On the other hand, the molar ratio of the former to the latter is about 0.1 in the lower respiratory tract (Gast et al., 1990). Hence, dt(MPI) $\approx$ 10dt(α_1-proteinase inhibitor), so that MPI appears to play a minor anti-elastase function in the lower respiratory tract as compared to α_1-proteinase inhibitor. Heparin increases k_{ass}(MPI) by a factor of 27 and decreases k_{ass}(α_1-proteinase inhibitor) by a factor of 40 [see references in Frommherz et al. (1991)]. Thus, if heparin were present in the lung at a concentration sufficient to saturate both NE and MPI, one would have dt(MPI) $\approx$ 0.1dt(α_1-proteinase inhibitor); i.e., MPI would very favorably compete with α_1-proteinase inhibitor for the binding of NE, and would therefore be the most efficient NE inhibitor of the lower respiratory tract despite its low concentration. Heparin is abundant in the lung since this organ is a commercial source of the polymer (Clark et al., 1983). Lung mast cells which secrete heparin (Clark et al., 1983) are found in large quantities in the alveolar walls, the sites where NE attacks lung matrix proteins to induce emphysematous lesions (Janoff, 1988). MPI, which is commonly thought to play an anti-elastase function in the upper respiratory tract only (Janoff, 1988), might therefore be an important anti-elastase of the lower respiratory tract too.

Recombinant MPI is being tested as a potential drug against destructive lung diseases in which NE is involved (Volgelmeier et al., 1990; Lucey et al., 1990; Rudolphus et al., 1991). Our data suggest that heparin or heparin-like molecules could be helpful adjuvants of such an anti-elastase therapy.

ACKNOWLEDGMENT

We thank Synergen, Boulder, CO, for the gift of recombinant MPI, Dr. Markus Grütter, Ciba-Geigy, Basel, for interatomic distance measurements on MPI and fruitful discussions, and Dr. Bernard Spiess, Université Louis Pasteur de Strasbourg, for computer assistance.

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