Functions of the N-Terminal Domain of Secretory Leukoprotease Inhibitor†

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ABSTRACT: Secretory leukoprotease inhibitor (SLPI) comprises two homologous domains: the C-terminal domain contains the reactive site, while the function of the N-terminal domain remains unknown. In order to elucidate the function of the N-terminal domain, we studied the kinetics of reactions of human leukocyte elastase with two recombinant forms of SLPI: the full-length inhibitor and the C-terminal domain alone. The reactions of elastase with the full-length inhibitor and the C-terminal domain share the same association rate constant, 2×10^6 M^{-1} s^{-1}, but the complex formed with the C-terminal domain is less stable, with a dissociation rate constant of 8×10^{-4} s^{-1}, 5 times higher than that of the complex with the full-length inhibitor. The binding of the full-length inhibitor to elastase is greatly accelerated by polyanions. In the presence of submicromolar concentrations (1 μg/mL) of heparin, the association rate constant is increased by more than 1 order of magnitude. The binding of the C-terminal domain alone to elastase shows much lower sensitivity to heparin; in the presence of 5 μM (25 μg/mL) heparin, association of the C-terminal domain with elastase reaches a maximum rate of 7×10^6 M^{-1} s^{-1}, about 3 times higher than the rate in the absence of heparin. Similar differential effects of heparin have been observed on the reactions of α-chymotrypsin with the two recombinant forms of SLPI. We also found that heparin has only a small effect on the binding of elastase with elafin, an elastase-specific inhibitor homologous to the C-terminal domain of SLPI. These data reveal two previously unrecognized functions of the N-terminal domain: stabilizing the elastase–inhibitor complex and mediating the activation of the inhibitor by heparin.

Secretory leukoprotease inhibitor (SLPI),[1] a 12-kDa protein synthesized by mucosal epithelial cells and other cells (Water et al., 1986; Franken et al., 1989; Sallenave et al., 1993), inhibits serine proteases including trypsin, α-chymotrypsin, human leukocyte elastase (HLE), and cathepsin G (Smith & Johnson, 1985; Fritz, 1988). In the lung, particularly in the lining fluids of upper airway epithelium, SLPI is a major physiological inhibitor of HLE (Gauthier et al., 1982; Piccioni et al., 1992), a neutrophil-derived protease with great tissue-destructive potential (Janoff, 1985; Hubbard et al., 1992). The inhibition of HLE by SLPI is a fast reaction with an association rate constant on the order of 10^6 M^{-1} s^{-1} as measured in vitro (Boudier & Bieth, 1989; Vogelmeier et al., 1990; Faller et al., 1992). The inhibition in vivo may proceed even faster since naturally occurring polyanions, including heparin (Faller et al., 1992) and DNA in airway secretions (Q.-L. Ying and S. R. Simon, data to be published), considerably accelerate the binding of SLPI and HLE. The accelerating effect of heparin has been ascribed to a conformational change in SLPI structure induced by the binding of heparin (Faller et al., 1992). SLPI comprises a single peptide chain of 107 amino acid residues evenly divided into two consecutive homologous domains (Seemüller et al., 1986; Thompson & Ohlsson, 1986; Grütter et al., 1988). The C-terminal domain contains the reactive site of SLPI, at which serine proteases bind the inhibitor (Grütter et al., 1988; Eisenberg et al., 1990; Kramps et al., 1990). The C-terminal domain alone, prepared by recombinant techniques or by mild acid hydrolysis, retains the ability to bind all of the cognate enzymes tested (Meckelein et al., 1991; Van-Seuningen & Davril, 1991; Renesto et al., 1993). The function of the N-terminal domain is unknown. This paper provides evidence that the N-terminal domain contributes to the stability of the elastase–SLPI complex, and is essential for activation of SLPI by heparin.

MATERIALS AND METHODS

Materials. The reagents used in this study were from the following commercial sources: HLE isolated from sputum, Elastin Products (Pacific, MO); crystallized (three times) bovine α-chymotrypsin, Sigma (St. Louis, MO); elafin prepared by chemical synthesis, Peptides International (Louisville, KY); recombinant SLPI, R & D Systems (Minneapolis, MN); heparin ($M_r \approx 5000$, 50 USP units/mg, RD Heparin 5000), Calbiochem (La Jolla, CA). Recombinant C-terminal domain from Asp49 to Ala107 according to the sequence of SLPI with an additional Met residue at the N-terminus (cSLPI) was produced by the direct *Escherichia coli* expression system pALP1-02/JM103 and purified by chromatography on columns of SP-Sephadex C-25 and Superose 12 (Kemme et al., 1992). N-terminal sequencing of this preparation revealed the expected motif Met-Asp-Pro. Western blot analysis after electrophoresis of the preparation on polyacrylamide gel containing sodium dodecyl sulfate showed one predominant band, which reacted with a polyclonal rabbit antiserum against the C-terminal domain of SLPI. The active site concentrations of HLE and its inhibitors were titrated as

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[1] Abbreviations: HLE, human leukocyte elastase; MeO-Suc-Ala-Ala-Pro-Val-pNA, N^{α}-methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine p-nitroanilide; M_2SO, dimethyl sulfoxide; SLPI, secretory leukoprotease inhibitor; cSLPI, C-terminal domain of SLPI; Suc-Ala-Ala-Pro-Phe-pNA, N^{α}-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide; bis-Tris propane, 1,3-bis[[tris(hydroxymethyl)methyl]amino]-propane.

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described (Ying & Simon, 1993). The active site concentration of α-chymotrypsin was titrated by HLE-titrated α_1-proteinase inhibitor.

Kinetic Analysis. Kinetic parameters of protease–inhibitor reactions were evaluated by analyzing the time-dependent hydrolysis of protease substrates which were present along with the inhibitor in the reaction systems. All assays were performed in a pH 7.4 buffer consisting of 0.0358 M bis-Tris propane (Sigma), 0.1 M NaCl, 0.1 mg/mL Triton X-100, and 0.1 mL/mL Me_2SO, ionic strength of 0.15, at 25 °C. In the reactions of α-chymotrypsin, the buffer also contained 1 mM $CaCl_2$. The substrates used were MeO-Suc-Ala-Ala-Pro-Val-pNA (Sigma) for HLE and Suc-Ala-Ala-Pro-Phe-pNA (Sigma) for α-chymotrypsin. The reaction was initiated by the addition of 10 μL of enzyme solution into 990 μL of a preequilibrated mixture containing substrate and inhibitor, with or without heparin in the pH 7.4 buffer. The release of *p*-nitroaniline from the substrate was monitored at 405 nm in an LKB Ultraspec 4050 spectrophotometer equipped with reaction-rate software (Pharmacia-LKB, Piscataway, NJ). The data for progressive hydrolysis of the substrate were analyzed according to the reaction model in Scheme 1, where

Scheme 1

$$E + S \rightleftharpoons ES \longrightarrow P$$

$$+$$

$$I \underset{k_d}{\overset{k_a}{\rightleftharpoons}} EI$$

EI is the reversible complex formed from enzyme E and inhibitor I, and S, ES, and P are the substrate, Michaelis complex, and product, respectively. It was assumed that the presence of heparin did not change this fundamental reaction model (Faller et al., 1992). In the presence of heparin, however, k_a and k_d should be considered as the apparent association and dissociation rate constants, respectively. In measurements of inhibition of HLE, the assays were performed under conditions in which the initial concentrations of inhibitor were not in large excess over those of enzyme. The data for substrate hydrolysis were fit to eq 1, an integrated rate equation

$$[P] = v_s t + (v_0 - v_s)\frac{(\alpha - 1)}{y} \ln \frac{\alpha - e^{-yt}}{\alpha - 1} + [P_0] \qquad (1)$$

modified from that of Zhao and Tsou (1992), and mathematically equivalent to the equation derived by Williams et al. (1979) and Cha (1980) (for the derivation of eqs 1–4, see the Appendix). The fitting was done by a nonlinear regression data analysis program, Enzfitter (Elsevier-BIOSOFT, Cambridge, U.K.). In eq 1 [P] and $[P_0]$ are the concentrations of product at any time t and time zero, respectively, v_0 and v_s are initial and steady-state rates of substrate hydrolysis, respectively, y is an apparent rate constant for the transition from v_0 to v_s, and α is a temporary variable for the fitting as defined by eq 2, where $[E_0]$ and $[I_0]$ are the initial concentrations of enzyme and inhibitor, respectively. Each fitting yielded a set of curve parameters, v_0, v_s, and y, from which the dissociation rate constant, k_d, was calculated by using eq 3, and the association rate constant, k_a, was calculated from the slope of a linear plot according to eq 4.

$$\alpha = \frac{[I_0]}{[E_0]}\left[\frac{v_0}{v_0 - v_s}\right]^2 \qquad (2)$$

$$k_d = y(v_s/v_0)\frac{[I_0] - (1 - v_s/v_0)[E_0]}{[I_0] - (1 - v_s/v_0)^2[E_0]} \qquad (3)$$

$$\beta = \frac{[I_0] - (1 - v_s/v_0)^2[E_0]}{y(1 - v_s/v_0)} = \frac{1}{k_a}\left(1 + \frac{[S]}{K_m}\right) \qquad (4)$$

The inhibitory reactions of α-chymotrypsin were studied under conditions of inhibitor excess, i.e., $[I_0] \gg [E_0]$. Data for progressive hydrolysis of the substrate were first fit to eq 5 to compute the initial hydrolysis rate, v_0 (Cha, 1975; Williams & Morrison, 1979), where k is an apparent first-order rate constant for the transition of v_0 to v_s. The meanings of the other symbols are the same as those defined above. By using the known v_0 value, the same progress curve data were then fit to eq 6 to directly compute the values of k_a and k_d (Baici & Gyger-Martazzi, 1982). We have previously demonstrated that if the data cover a sufficient wide range from the pre-steady state to the steady state, the fitting of individual progress curve data to eq 6 will provide accurate values of k_a and k_d (Ying & Simon, 1993).

$$[P] = v_s t - (v_s - v_0)(1 - e^{-kt})/k + [P_0] \qquad (5)$$

$$[P] = \frac{v_0 k_d}{k} t + \frac{v_0 k_a [I_0]}{(1 + [S]/K_m)k^2}(1 - e^{-kt}) + [P_0] \qquad (6)$$

Boudier and Bieth (1992) reported that SLPI possesses two reactive sites for α-chymotrypsin (as well as for trypsin and cathepsin G), one with a dissociation constant in the nanomolar range and the other with a dissociation constant in the micromolar range. They also suggested that both reactive sites might be located on the C-terminal domain of SLPI. In our kinetic assays, the concentrations of α-chymotrypsin and SLPI used were from 1 to 1.5 nM and from 25 to 50 nM, respectively. Under these conditions, the binding of α-chymotrypsin to the low-affinity site of SLPI was negligible. In the treatment and interpretation of the kinetic data below, we therefore considered only the reaction of α-chymotrypsin with the high-affinity site of the inhibitor.

All of the results reported in the text below, including those in the table and figures, are expressed as mean ± standard deviation (SD) (in the figures, SD values are represented by error bars), each of which is from three or more experimental determinations.

RESULTS

Inhibition of Elastase. Table 1 compares the kinetic constants for the inhibition of HLE by SLPI with those by cSLPI. The association rate constants for the two reactions, $(2.4 \pm 0.1) \times 10^6$ M^{-1} s^{-1} with SLPI and $(2.1 \pm 0.2) \times 10^6$ M^{-1} s^{-1} with cSLPI, are very close, and considering the experimental errors, the difference is insignificant. However, the dissociation rate constants for the two reactions differ considerably. The complex with the two-domain inhibitor dissociates 5 times more slowly than that with the single C-terminal domain of SLPI. As the recombinant preparation of the C-terminal domain lacks the N-terminal domain of SLPI, these data directly lead to the conclusion that the N-terminal domain of SLPI contributes to the stability of the HLE–SLPI complex.

Next we studied the effects of heparin on the binding of HLE with SLPI and cSLPI. Heparin and several other sulfated glycosaminoglycans are known to be inhibitors of

Table 1: Kinetic Constants of Inhibitory Reactions of Human Leukocyte Elastase and Bovine α-Chymotrypsin by Secretory Leukoprotease Inhibitor and Its C-Terminal Domain[a]

protease	kinetic constant	inhibitor SLPI	inhibitor cSLPI
elastase	$k_a \times 10^{-6}$ (M^{-1} s^{-1})	2.4 ± 0.1	2.1 ± 0.2
	$k_d \times 10^4$ (s^{-1})	1.5 ± 0.1	7.8 ± 0.6
	K_i (pM)	63	371
α-chymotrypsin	$k_a \times 10^{-5}$ (M^{-1} s^{-1})	2.6 ± 0.3	3.7 ± 0.3
	$k_d \times 10^5$ (s^{-1})	5.8 ± 1.3	9.3 ± 0.1
	K_i (pM)	223	251

[a] The assays were performed in 0.0358 M bis-Tris propane buffer, pH 7.4, containing 0.1 M NaCl, 0.1 mL/mL Me_2SO, and 0.1 mg/mL Triton X-100 (ionic strengths of 0.15) at 25 °C. In the assays for α-chymotrypsin, the buffer also contained 1 mM $CaCl_2$. The values of k_a and k_d are expressed as mean ± SD ($n \geq 3$); the values of K_i were calculated from the means of k_a and k_d according to $K_i = k_d/k_a$.

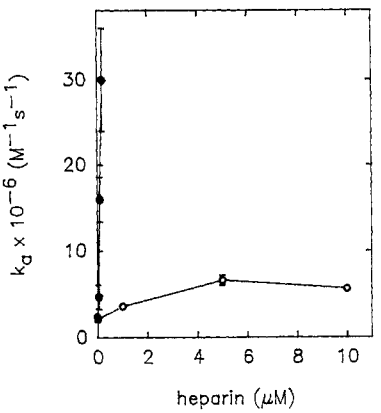

FIGURE 1: Effects of 5-kDa heparin on the rate constants of association of HLE with SLPI and cSLPI: closed circles, the reactions with SLPI in the presence of heparin of 0, 50, 100, and 200 nM (0, 0.25, 0.50, and 1 μg/mL); open circles, the reactions with cSLPI. The assays were carried out in pH 7.4 buffer at 25 °C with substrate MeO-Suc-Ala-Ala-Pro-Val-pNA. In these assays, HLE concentrations used were 5–6 nM, SLPI or cSLPI concentrations were 10–17.2 nM, and the substrate concentrations were varied from 239 to 3403 μM.

HLE (Baici et al., 1980; Marossy, 1981; Baici & Bradamante, 1984; Lentini et al., 1985; Redini et al., 1988; Walsh et al., 1991; Frommherz et al., 1991). The inhibitory strength of heparin against HLE depends on its chain length and the degree of sulfation (Redini et al., 1988; Walsh et al., 1991). In the present work, a preparation of heparin with an average molecular weight of 5000 from Calbiochem was chosen since its interaction with SLPI has been previously studied (Faller et al., 1992). We found that the inhibition of HLE by this heparin preparation contains both competitive and noncompetitive components. In our pH 7.4 buffer at 25 °C, K_m and k_{cat} for HLE-catalyzed hydrolysis of substrate MeO-Suc-Ala-Ala-Pro-Val-pNA were determined to be 132 ± 11 μM and 15 ± 1 s^{-1}, respectively. In the presence of 10 μM (50 μg/mL) heparin, a concentration at which HLE is nearly saturated by the polyanion, K_m is increased to 174 ± 13 μM, and k_{cat} declines to 12 ± 1 s^{-1}. The dissociation constant of the HLE–heparin complex is 216 ± 24 nM.

Figure 1 shows the effects of heparin on the association rate constants (k_a) of HLE with SLPI and cSLPI. The rate of binding of the two-domain inhibitor to HLE is sharply increased with submicromolar concentrations of heparin. At a heparin concentration of 0.2 μM (1 μg/mL), k_a rises to (3 ± 0.5) × 10^7 M^{-1} s^{-1}, 12.5 times higher than that in the absence of the polyanion. With higher concentrations of heparin, the k_a value is further increased as judged by the almost

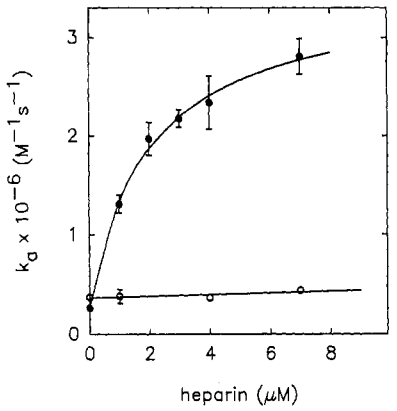

FIGURE 2: Effects of 5-kDa heparin on the rate constants of association of α-chymotrypsin with SLPI and cSLPI: closed circles, the reactions with SLPI (the curve is a theoretically calculated curve by fitting the data points to eq 11); open circles, the reactions with cSLPI. The assays were performed in pH 7.4 buffer at 25 °C with substrate Suc-Ala-Ala-Pro-Phe-pNA. In these reactions, α-chymotrypsin concentrations used were 1–1.5 nM, SLPI or cSLPI concentrations were 25–50 nM, and the substrate concentrations were varied from 481 to 1760 μM.

instantaneous approach to the steady state in our recorded progress curves for substrate hydrolysis. Unfortunately, these higher association rates exceed the upper limit that can be estimated with confidence by our assay method used in this study. Heparin also affects the association of HLE with the single C-terminal domain as shown by the data in Figure 1. However, at submicromolar levels of heparin, this effect is negligible; only at micromolar levels does the effect become significant. When the heparin concentration is around 5 μM (25 μg/mL), the association rate of HLE and cSLPI reaches a maximum value, (6.6 ± 0.6) × 10^6 M^{-1} s^{-1}, which is only 3 times higher than that in the absence of heparin (Table 1). These results indicate that the N-terminal domain plays an essential role in the activation of the two-domain inhibitor by heparin.

Over the concentration ranges tested (0–0.2 μM or 0–1 μg/mL for SLPI and 0–10 μM or 0–50 μg/mL for cSLPI), heparin shows no effect on the dissociation rate constants of the elastase–inhibitor complexes containing either the two-domain inhibitor or single C-terminal domain. At a heparin concentration of 0.2 μM (1 μg/mL), the dissociation rate constant of the HLE–SLPI complex is (1.6 ± 0.4) × 10^{-4} s^{-1}, while in the absence of heparin, it is (1.5 ± 0.1) × 10^{-4} s^{-1} (Table 1). Since in the presence of 0.2 μM (1 μg/mL) heparin, the association rate constant is increased by a factor of 12.5, the dissociation constant, K_i, drops from 63 pM in the absence of heparin to 5 pM in the presence of heparin.

Inhibition of α-Chymotrypsin. α-Chymotrypsin is one of several known cognate proteases of SLPI (Smith & Johnson, 1985). The crystal complex of α-chymotrypsin and SLPI is the only complex of SLPI for which the three-dimensional structure has been determined to near atomic resolution (Grütter et al., 1988). We have also studied the kinetics of the reactions of α-chymotrypsin with SLPI and cSLPI. Table 1 lists the kinetic constants for inhibition of this enzyme by SLPI and cSLPI. It can be seen that in the absence of heparin both the association and dissociation rates of α-chymotrypsin with the two-domain inhibitor are slightly lower than the corresponding values for the C-terminal domain. The ratios of the corresponding values for the single-domain and two-domain inhibitors are less than 2, which reflect differences below the level of functional significance. In contrast, the effects of 5-kDa heparin on the association rate constants of

α-chymotrypsin with SLPI and cSLPI differ considerably (Figure 2). Heparin almost has no effect on the association rate of α-chymotrypsin with the C-terminal domain; at a heparin concentration of 7 μM (35 μg/mL), k_a is (4.4 ± 0.2) × 10^5 M^{-1} s^{-1}, slightly higher than (3.7 ± 0.2) × 10^5 M^{-1} s^{-1}, the value of k_a in the absence of heparin (Table 1). The rate constant for association of α-chymotrypsin with the two-domain inhibitor increases hyperbolically with heparin concentration (Figure 2). At a heparin concentration of 7 μM (35 μg/mL), the association rate constant is (2.8 ± 0.2) × 10^6 M^{-1} s^{-1}, 10 times higher than that in the absence of heparin (Table 1).

Since the kinetic data for the two-domain inhibitor in Figure 2 show a saturation dependence on heparin concentration, we have analyzed the data as follows. It is assumed that there is a rapid equilibrium between the free inhibitor (I) and the heparin-binding inhibitor (IH) according to Scheme 2, where

Scheme 2

$$I + H \overset{K_D}{\rightleftharpoons} IH$$

K_D is the dissociation constant of the complex IH as defined by eq 7. Since in our assays the initial concentrations of SLPI, $[I_0]$, were at the nanomolar level (25–50 nM), while the initial

$$K_D = [I][H]/[IH] \quad (7)$$

concentrattions of heparin, $[H_0]$, were at the micromolar level (1–7 μM), the condition of $[H_0] \gg [I_0]$ was always maintained. By using the conservation equation, $[I_0] = [I] + [IH]$, and assuming $[H] \simeq [H_0]$ as $[H_0] \gg [I_0]$, eqs 8 and 9 can be derived. The observed association rate constant, $(k_a)_{obs}$, reflects

$$[I] = K_D[I_0]/(K_D + [H_0]) \quad (8)$$

$$[IH] = [I_0][H_0]/(K_D + [H_0]) \quad (9)$$

the sum of contributions from the free inhibitor and the heparin–inhibitor complex. If $(k_a)_f$ represents the association rate constant of α-chymotrypsin with the free inhibitor and $(k_a)_b$ the association rate constant with the heparin–inhibitor complex, then

$$(k_a)_{obs} = (k_a)_f \frac{[I]}{[I_0]} + (k_a)_b \frac{[IH]}{[I_0]} \quad (10)$$

Substituting eqs 8 and 9 into eq 10, eq 11 is obtained. Using the value of $(k_a)_f = 2.6 \times 10^5$ M^{-1} s^{-1} as determined previously (Table 1), and fitting the data in Figure 2 to eq 11, an

$$(k_a)_{obs} = \frac{(k_a)_f K_D + (k_a)_b[H_0]}{K_D + [H_0]} \quad (11)$$

association rate constant for the heparin–SLPI complex with α-chymotrypsin was computed to be 3.5 × 10^6 M^{-1} s^{-1}. This value should be the true association rate constant of the heparin–SLPI complex with α-chymotrypsin. From the fitting, K_D was computed to be 2 μM. In the determination of the association rate constant of HLE and SLPI in the presence of 0.2 μM (1 μg/mL) heparin, the enzyme and the inhibitor concentrations used were 5 and 10 nM, respectively. Thus, the condition of $[H] \simeq [H_0]$ is maintained, allowing us to use eq 11 to calculate an approximate estimate of the true association rate constant of HLE with the heparin–SLPI complex. With $(k_a)_{obs} = 30 \times 10^6$ M^{-1} s^{-1} (Figure 1), $(k_a)_f = 2.4 \times 10^6$ M^{-1} s^{-1} (Table 1), and K_D = 2 μM, $(k_a)_b$ was calculated to be 3.1 × 10^8 M^{-1} s^{-1}, a value falling in the range of the diffusion-controlled limit of bimolecular reactions.

The influence of heparin on the dissociation rate constants for the complexes formed from α-chymotrypsin with SLPI and cSLPI is also small (data not shown), as was the case for dissociation of the complexes with HLE.

These results show that the N-terminal domain of SLPI is not important for the stability of the complex of α-chymotrypsin and SLPI, but is also essential for activation of SLPI by heparin in association of the inhibitor with α-chymotrypsin.

Inhibition by Elafin. Elafin is an elastase-specific inhibitor originally isolated from psoriatic epidermis (Wiedow et al., 1990) and also found in airway mucous secretions (Sallenave et al., 1992). Elafin is composed of a single peptide chain of 57 amino acid residues, the sequence of which is 38% homologous with the C-terminal domain of SLPI (Wiedow et al., 1990; Sallenave & Ryle, 1991; Saheki et al., 1992). Furthermore, both elafin and the C-terminal domain of SLPI conserve the same four disulfide connections, and the same alignment of five proline residues (Wiedow et al., 1990; Saheki et al., 1992; Tsunemi et al., 1992), suggesting that their tertiary structures may be very similar. These data also imply that the two inhibitors may be descended from a common ancestor in the evolutionary tree, but elafin lacks an N-terminal domain analogous to that of SLPI. We have studied the effects of 5-kDa heparin on the kinetics of reaction of HLE with elafin. Four concentrations of heparin were used: 0, 1, 4, and 10 μM (0, 5, 20, and 50 μg/mL). The corresponding rate constants are 4.3 ± 0.1, 4.3 ± 0.4, 6.7 ± 0.5, and 5.5 ± 0.6 (unit 10^6 M^{-1} s^{-1}) for association, and 4.0 ± 0.2, 4.3 ± 0.1, 4.3 ± 0.1, and 4.3 ± 0.2 (unit 10^{-4} s^{-1}) for dissociation, respectively. Clearly, heparin has only minor effects on the interaction of elafin with HLE.

DISCUSSION

The kinetic data with HLE reported here demonstrate two previously unknown functions of the N-terminal domain of SLPI: stabilizing the HLE–SLPI complex and mediating the activation of SLPI by heparin. Our data also reveal that the domain stabilizes the HLE–SLPI complex by two mechanisms: in the absence of heparin, it decreases the dissociation rate, and in the presence of heparin, it further increases the association rate. The overall effect is a great reduction of K_i, the dissociation constant of the HLE–SLPI complex. The role of the N-terminal domain in the activation of SLPI by heparin, which considerably accelerates binding of SLPI to HLE, appears to be a unique functional property of this domain alone. According to the theory developed by Bieth (1980, 1984), the in vivo potency of an inhibitor depends not only on the stability of the enzyme–inhibitor complex but also on the association rate of the inhibitor with enzyme. A fast-acting inhibitor even at low concentrations may effectively prevent the access of an enzyme to its natural substrates [for a discussion directly related with the physiological significance of the accelerated association of HLE with SLPI by heparin, see Faller et al. (1992)].

The data for α-chymotrypsin differ in several aspects from the results for HLE. First, α-chymotrypsin and the C-terminal domain alone of SLPI form a complex with a dissociation rate constant only slightly higher than that of the complex with the complete two-domain inhibitor (Table 1). The contribution of the N-terminal domain to the stability of the α-chymotrypsin–SLPI complex is therefore negligible in the absence of heparin. Second, heparin almost has no effect on the rate

of the association of α-chymotrypsin with the single C-terminal domain (Figure 2), while heparin does accelerate the association of HLE with the C-terminal domain of SLPI somewhat (Figure 1). Finally, the association rate of HLE with the two-domain inhibitor increases sharply with heparin concentration (Figure 1), but in the reaction with α-chymotrypsin, the response to heparin is less pronounced (Figure 2). Therefore, in the case of α-chymotrypsin, the major role of the N-terminal domain is reduced to a single function: mediation of the activation of SLPI by heparin. In the crystal structure of the complex of α-chymotrypsin and SLPI reported by Grütter et al. (1988), both the C- and N-terminal domains of the inhibitor make contacts with the enzyme. The amino acid residues of the C-terminal domain involved in the contacts include eight residues (Thr67 to Leu74) in the primary binding segment, two residues (Met96 and Cys97) in the secondary binding segment, and one residue (Val102) in the C-terminal segment. In the N-terminal domain, only one amino acid residue, Trp30, makes direct contact with the enzyme. From our kinetic data, it seems that the contact via Trp30 does not significantly affect the association and dissociation rates in the reaction of α-chymotrypsin with SLPI, since very similar rate constants were obtained for the reactions with the two-domain inhibitor and C-terminal domain alone (Table 1). However, Trp30 might be one of the residues in the N-terminal domain which are involved in the activation of SLPI by heparin. This possibility is implied by the study of Faller et al. (1992) who found that binding by heparin induces a conformational change in SLPI that brings the indole ring of Trp30 into a more hydrophobic environment. Further studies are needed to relate these discrete results together to find how the N-terminal domain mediates the activation of SLPI by heparin.

The results in Figure 1 show that both the two-domain inhibitor and C-terminal domain alone can be activated by heparin, although the magnitudes of the activations differ remarkably. In contrast, heparin only weakly affects the activity of elafin, a homologue of the C-terminal domain. These findings support our hypothesis (Ying & Simon, 1993) that the two inhibitors, SLPI and elafin, may perform their antielastase function in different environments. The specialized environment where SLPI functions with greatest efficacy may be on the surface of polyanions, which include sulfated glycosaminoglycans in the tissue, and DNA fragments from disintegrated inflammatory cells in airway mucous secretions (Q.-L. Ying and S. R. Simon, data to be published). Elafin may function in an environment where such polyanions are less abundant. The differences in the responses of the two inhibitors to heparin might reflect divergent evolution in these different environments.

Recombinant SLPI has been suggested as a potential therapeutic agent for the treatment of patients with inflammatory lung diseases in which excess HLE activity is considered to be a causative factor (Rice & Weiss, 1990). Aerosolized recombinant SLPI has been employed in clinical trials, and some benefits to patients have been reported (McElvaney et al., 1992, 1993; Gillissen et al., 1993). The recombinant C-terminal domain has been proposed as another candidate for the same purpose (Renesto et al., 1993). The small one-domain inhibitor may have an advantage over the two-domain inhibitor in that it may diffuse more rapidly into the interstitium of inflammatory tissue. Data presented in this study show that the one-domain inhibitor lacks some functional properties associated with the N-terminal domain. This functional deficiency should be taken into account if the single C-terminal domain is tested as a therapeutic agent.

APPENDIX: DERIVATION OF EQUATIONS 1–4

By assuming that the steady-state assumption applies throughout to the reaction of substrate with enzyme and that depletion of the substrate is negligible during the reaction, Zhao and Tsou (1992) analyzed a general model which includes components of both competitive and noncompetitive inhibition. In the case of competitive inhibition as described by Scheme 1, the concentration of product [P] at any time t can be expressed by eq A1,a slightly modified form of eq 19

$$[P] = v_s t + \frac{v_0}{A[E_0]} \ln \frac{\alpha - e^{-yt}}{\alpha - 1} + [P_0] \qquad (A1)$$

of Zhao and Tsou (1992). The meanings of the symbols used here and below are the same as those in the text. A, y, and α are defined by eqs A2–A4 (Zhao & Tsou, 1992). In order

$$A = k_a \frac{K_m}{K_m + [S]} \qquad (A2)$$

$$y = [[A([I_0] - [E_0]) + k_d]^2 + 4k_d A[E_0]]^{1/2} \qquad (A3)$$

$$\alpha = \frac{A([I_0] + [E_0]) + k_d + y}{A([I_0] + [E_0]) + k_d - y} \qquad (A4)$$

to reduce the number of variables in eq A1, we cite the expression of Zhao and Tsou (1992) for $[E_s]$, the total concentration of enzyme, both in the free state and in the Michaelis complex at steady state:

$$[E_s] = \frac{-[A([I_0] - [E_0]) + k_d] + y}{2A} \qquad (A5)$$

Equation A5 may be rewritten as

$$\frac{v_s}{v_0} = \frac{[E_s]}{[E_0]} = \frac{-[A([I_0] - [E_0]) + k_d] + y}{2A[E_0]} \qquad (A6)$$

By algebraic rearrangement, eqs A7 and A8 can be derived from eqs A3–A6. Substituting eqs A7 and A8 into eq A1 gives eq 1 in the text. This integrated rate equation is

$$\alpha = \frac{[I_0]}{[E_0]} \left[\frac{v_0}{v_0 - v_s}\right]^2 \qquad (A7)$$

$$y = A\left[\frac{[I_0]}{(1 - v_s/v_0)} - [E_0](1 - v_s/v_0)\right] \qquad (A8)$$

mathematically equivalent to eq 6 of Williams et al. (1979) and eq 39 of Cha (1980). From eqs A6 and A8, eq 3 (in the text) can be derived, and from eqs A2 and A8, eq 4 is obtained.

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