Kinetics of the Inhibition of Human Leukocyte Elastase by Elafin, a 6-Kilodalton Elastase-Specific Inhibitor from Human Skin†

Qi-Long Ying and Sanford R. Simon*

Departments of Biochemistry and Pathology, State University of New York at Stony Brook, Stony Brook, New York 11794

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ABSTRACT: We have investigated the kinetics of inhibition of human leukocyte elastase by elafin, a small protein originally isolated from human skin. A single inhibitor molecule was found to bind to a single site on the protease, blocking the reactive serine at the enzyme's catalytic center. Association of the enzyme with the inhibitor proceeds via a single bimolecular process, with a second-order rate constant of 3.6×10^6 M^{-1} s^{-1} at pH 8.0 and 25 °C. Dissociation of the enzyme–inhibitor complex regenerates fully active enzyme with a first-order rate constant of 6.0×10^{-4} s^{-1}. The species of elafin which is released from the complex simultaneously with the enzyme was estimated to be at least 99.8% active, with association and dissociation kinetics identical to preparations of the inhibitor which had never been exposed to the enzyme. K_i, the equilibrium dissociation constant of the enzyme–inhibitor complex, decreases from 6.7×10^{-9} to 2.0×10^{-10} M as the pH is increased from 5.4 to 9.0. The effect of pH on the association rate constant reveals that the reaction rate is dependent on the concentration of the unprotonated form of a group with pK_a of 6.8, which we have assigned to the histidine which forms part of the catalytic triad in the enzyme's active site. On the basis of these findings, we conclude that elafin is a potent, substratelike, but fully reversible inhibitor of human leukocyte elastase.

Elafin is an acid-stable protein which was first isolated from scales of patients with psoriasis (Wiedow et al., 1990). The protein is a single peptide chain of 57 amino acid residues with molecular mass of 5999 daltons. The complete chemical synthesis of elafin has recently been reported (Tsunemi et al., 1992). Elafin inhibits human leukocyte elastase (HLE),[1] porcine pancreatic elastase, and proteinase 3, an elastin-degrading enzyme from neutrophils, but has no effect on trypsin, plasmin, α-chymotrypsin, and cathepsin G (Wiedow et al., 1990, 1991; Tsunemi et al., 1992). The specificity profile is essentially the same as that of the elastase-specific inhibitors found in bronchial mucous secretions (Hochstrasser et al., 1981; Kramps et al., 1985; Sallenave et al., 1991), and that of SKALP (skin-derived antileukoproteases), a group of base-stable proteins also isolated from psoriatic scales (Schalkwijk et al., 1991). Data from preliminary studies on primary structures and immunological cross-reactivities of these inhibitors are consistent with the hypothesis that elafin and these inhibitors are probably descended from a common precursor or a small family of ancestor molecules (Sallenave et al., 1992). The amino acid sequence of elafin shows 38% homology with that of the second domain of secretory leukoprotease inhibitor (SLPI, also known as mucous protease inhibitor, MPI) (Wiedow et al., 1992; Sallenave et al., 1992). Both elafin and SLPI belong to the four-disulfide core protein superfamily described by Drenth et al. (1980; Saheki et al., 1992), implying that they may also have similar tertiary structures. The biological function of elafin remains unclear. Its inhibitory specificity for a very limited number of cognate enzymes suggests that elafin may play a role in regulation of the turnover of connective tissue components. However, this speculation still awaits experimental verification. We have determined the kinetics of inhibition of HLE by elafin in order to elucidate the mechanism of inhibition and to assess its potential role in regulating the enzyme's activity.

MATERIALS AND METHODS

Reagents. HLE purified from azurophilic granules of human neutrophils was from Athens Research Technology (Athens, GA). Elafin prepared by complete chemical synthesis was from Peptides International (Louisville, KY). α_1-PI purified from human plasma was a generous gift of Cutter Biologicals (Berkeley, CA). MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA was from Bachem Bioscience (Philadelphia, PA), and MeO-Suc-Ala-Ala-Pro-Val-pNA was from Sigma (St. Louis, MO). [1,3-^{3}H]DFP (specific activity 4.4 Ci/mmol) was from NEN Research Products (Boston, MA). Other reagents were all of analytical grade.

Enzyme and Inhibitor Activity Assays. HLE was titrated by *N*-benzyloxycarbonyl-Ala-Ala-Pro-azaAla *p*-nitrophenyl ester (Enzyme System Products, Livermore, CA) (Powers et al., 1984). Assuming $\epsilon^{1\%}_{280nm} = 9.85$ (Babul & Stellwagen, 1969), and $M_r = 30\,000$ for HLE, the specific activity was 0.81 mol/mol of protein. Elafin was titrated by measuring its inhibition of the amidolytic activity of the previously titrated HLE preparation on a ThermoMax kinetic microplate reader (Molecular Devices, Palo Alto, CA) in 96-well microplates. Equal 50-μL aliquots of different concentrations of elafin and of 60 nM HLE in 0.1 M Tris buffer, pH 8.0, containing 0.15 M NaCl and 0.1 mg/mL Triton X-100 (hereafter referred to as pH 8.0 buffer) were combined, and complex formation was allowed to proceed at 25 °C for 20 min before 50 μL of 1.9 mM MeO-Suc-Ala-Ala-Pro-Val-pNA in pH 8.0 buffer containing 0.15 mL/mL Me_2SO was added to assay the

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* To whom correspondence should be addressed at the Department of Pathology, State University of New York at Stony Brook. Telephone: (516) 444-3007. FAX: (516) 444-7507.

[1] Abbreviations: DFP, diisopropyl fluorophosphate; HLE, human leukocyteelastase; MeO-Suc-Ala-Ala-Pro-Val-pNA, N^{α}-methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide; Meo-Suc-Lys(Pic)-Ala-Pro-Val-pNA, N^{α}-methoxysuccinyl-N^{ϵ}-2-picolinoyl-L-lysinyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide; M_2SO, dimethyl sulfoxide; α_1-PI, α_1-proteinase inhibitor; SLPI, secretory leukoprotease inhibitor.

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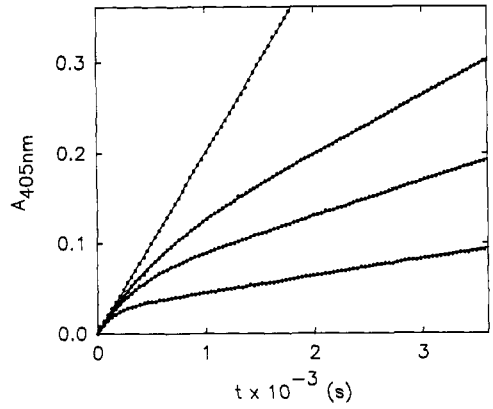

FIGURE 1: Progress curves for amidolysis of MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA by HLE in the presence of elafin. Reaction conditions: enzyme, 1 nM; substrate, 1208 μM; inhibitor (from top to bottom), 0, 30, 60, and 150 nM, in pH 8.0 buffer containing 0.1 mL/mL Me_2SO, at 25 °C. Reaction was initiated by addition of enzyme to a mixture of substrate and inhibitor. The points shown (t, A_{405nm}) are digitized experimental values; the solid lines are the theoretical curves computed by fitting the experimental data to eq 2.

remaining HLE activity. On a gravimetric basis, assuming M_r = 5999, the specific activity of the elafin preparation was found to be 0.75 mol/mol of protein. Since amino acid analysis revealed that the synthetic preparation contained 1.57 mol of methionine residues per mole of protein, compared with a theoretical value of 2 (Wiedow et al., 1990; Tsunemi et al., 1992), the titration data were consistent with a 1.04 (inhibitor):1 (enzyme) stoichiometry. The specific activity of α_1-PI was determined by the same method, and was found to be 0.82 mol/mol of protein. All substrate concentrations were determined spectrophotometrically in water containing 5 μL/mL Me_2SO, assuming ϵ_{316nm} = 12 800 M^{-1} cm^{-1} for peptide *p*-nitroanilides (Friberger, 1982).

Kinetic Measurements. The kinetics of binding of elafin to HLE were determined by an analytical procedure originally developed for slow-binding inhibition (Cha, 1975; Williams & Morrison, 1979; Morrison & Walsh, 1988). For this analysis, two types of progress curve data were collected. In experiments in which the progressive association of enzyme and inhibitor was monitored, elafin and the substrate MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA (Yasutake & Powers, 1981) were equilibrated at 25 °C in 990 μL of pH 8.0 buffer containing 0.1 mL/mL Me_2SO; 10 μL of HLE was then added to initiate the reaction. The reaction was also studied over the pH range from 5.4 to 9.0. Buffers of pH 5.4 and 6.0 contained 0.1 M sodium phosphate, while buffers covering the pH range from 6.4 to 9.0 contained 0.1 M 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane (Bis-Tris propane; Sigma). All buffers also contained 0.15 M NaCl, 0.1 mg/mL Triton X-100, and 0.1 mL/mL Me_2SO. Final concentrations of enzyme, substrate, and inhibitor in these experiments were 1–8 nM, 362–1208 μM, and 10–150 nM, respectively. In experiments in which the progressive dissociation of HLE from its complex was monitored, HLE (0.1 μM) and elafin (0.15 μM) in pH 8.0 buffer were incubated at 25 °C for 10 min; a 5-μL aliquot of the solution of complex was then removed and diluted into 995 μL of 1208 μM MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA in pH 8.0 buffer containing 0.1 mL/mL Me_2SO at 25 °C. In both cases, release of *p*-nitroaniline was monitored at 405 nm in an LKB Ultraspec-4050 spectrophotometer equipped with reaction-rate software (Pharmacia—LKB, Piscataway, NJ). All reactions were monitored at 25 °C for 1 h, and the absorbances at 405 nm were digitized into 99 values as a function of time for each progress curve. Absorbance values were converted, when necessary, into product concentrations, assuming ϵ_{405nm} = 9900 M^{-1} cm^{-1}. The digitized progress curves were analyzed by fitting data points to the appropriate equations (as shown under Results) by nonlinear regression, using a modified Marquardt (1963) algorithm (Enzfitter, Elsevier-Biosoft, Cambridge, U.K.).

Recovery of Elafin from Its Complex with HLE. In 100 μL of pH 8.0 buffer, elafin (4.0 μM) was first incubated with an excess of HLE (4.4 μM) at 25 °C for 10 min. A 100-μL aliquot of 13.2 μM α_1-PI was then added to trap all remaining free enzyme as well as any enzyme released by dissociation of the HLE–elafin complex. To ensure that dissociation of the HLE–elafin complex proceeded to completion, the reaction mixture was allowed to stand at 25 °C for 20 h. At the end of the incubation, 180 μL of the mixture was transferred into the reservoir of an MPS-1 ultrafiltration device (MPS-1 Micropartition System; Amicon, Danvers, MA) equipped with a Diaflo type YM10 membrane (M_r cutoff = 10 000). The device was placed in a 34° fixed-angle rotor and spun at 1500*g* for 30 min at 10 °C. The ultrafiltrate obtained was analyzed for anti-elastase activity by the procedures already described. Control experiments established that HLE, α_1-PI, and HLE–α_1-PI complex were totally retained by the YM10 membrane whereas free elafin passed through the membrane with 50–80% recovery.

Elafin Blocks the Incorporation of [^{3}H]DFP into Elastase. Elafin (3 μM) and HLE (1 μM) in 396 μL of 0.1 M sodium phosphate buffer, pH 7.3, containing 0.1 mg/mL Triton X-100 were incubated at 25 °C for 10 min. A 4-μL aliquot of 277 μM [^{3}H]DFP (2.2 mCi/μmol) in propylene glycol was then added, and the mixture was continuously incubated at 25 °C. At various times, 60-μL aliquots were removed from the reaction solution and diluted into 540 μL of 1 μM α_1-PI in the same pH 7.3 buffer to stop any further incorporation of DFP into HLE. Controls without elafin in the original solution were also run in parallel. The diluted solutions were exhaustively dialyzed in tubing with a molecular weight cutoff of 12 000–14 000 (Spectrapor; Spectrum Medical Industries, Los Angeles, CA) against 1 mM HCl at 4 °C until radioactivity in the dialysate reached background levels. The retentates were collected, and 200-μL aliquots were assayed for incorporated [^{3}H]DFP by liquid scintillation counting.

RESULTS

Reversibility of the Reaction. Figure 1 shows the progressive inhibition of HLE by elafin in the presence of the substrate MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA. The rate of substrate amidolysis declines exponentially from an initial maximal value to a steady-state value. The steady-state value does not reach zero even in the presence of a 150-fold molar excess of elafin (Figure 1). Zhou et al. (1989) have proposed two kinetic mechanisms which are consistent with this pattern of progress curves: (1) the reaction of enzyme and inhibitor is reversible, so free enzyme always exists in the reaction solution; or (2) enzyme and inhibitor combine irreversibly to form a complex in which the bound enzyme retains residual activity. The second mechanism can be eliminated from the results in Figure 1. If the enzyme–inhibitor complex retained some residual activity, the residual activity should depend on the concentration of enzyme, but not on the concentration of inhibitor as long as saturation of enzyme with inhibitor had been reached. In contrast, Figure 1 shows that the steady-state velocity decreases with increasing concentrations of elafin when the HLE concentration is maintained constant.

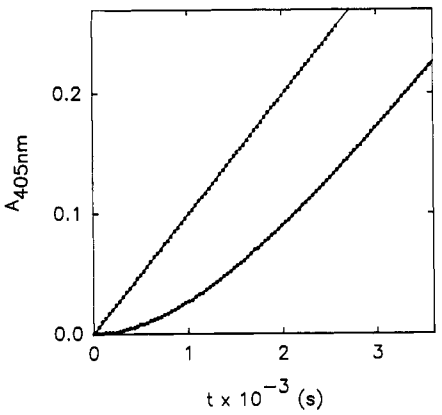

FIGURE 2: Regeneration of HLE activity by dissociation of the HLE–elafin complex. HLE (0.1 μM) and elafin (0.15 μM) were incubated at 25 °C for 10 min; a 5-μL aliquot of the mixture was removed and diluted into 995 μL of 1208 μM MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA in pH 8.0 buffer/0.1 mL/mL Me_2SO, at 25 °C, and the change of absorbance at 405 nm was recorded. The points shown (t, A_{405nm}) are digitized experimental values as in Figure 1. The straight line represents the fit to the data for the control without inhibitor in the original incubation solution. The curved solid line was computed by fitting the experimental data to eq 1.

Table I: Kinetic Constants for the Inhibition of Human Leukocyte Elastase by Elafin[a]

conditions	method	k_1 ($\times 10^6$ M^{-1} s^{-1})	k_{-1} ($\times 10^{-4}$ s^{-1})	K_I ($\times 10^{-10}$ M)
pH 8.0 buffer,[b] 25 °C	eq 3	3.6 ± 0.5 [4]	5.8 ± 1.2 [4]	
	eq 4		6.0 ± 0.3 [33]	1.7
	eq 1		5.8 ± 1.5 [3]	
pH 8.0 buffer + 0.85 M NaCl, 25 °C	eq 3	3.2 ● 0.7 [3]	2.8 ± 0.4 [3]	
	eq 4		4.0 ± 0.8 [15]	1.3

[a] Results are expressed as mean ± SD; numbers of determinations are indicated in brackets. [b] Buffer components: 0.1 M Tris, 0.15 M NaCl, 0.1 mg/mL Triton X-100, and 0.1 mL/mL Me_2SO.

Recovery of enzyme activity by reversible dissociation of the HLE–elafin complex is confirmed by the experiments in Figure 2. In these experiments, HLE was first incubated with a slight excess of elafin; an aliquot of the solution was then diluted into a large volume of buffer containing the substrate. The return of amidolytic activity was monitored by recording the release of *p*-nitroaniline (for details of the experiments, see Materials and Methods). After dilution, the concentration of elafin was still 4.4 times greater than its K_i (K_i = 0.17 nM, see Table I). However, since the concentration of substrate in the solution was as high as 86 times the K_m for HLE (K_m = 14 μM), reassociation of any HLE released from the initially formed enzyme–inhibitor complex with free elafin was assumed to be minimal. This assumption is verified by the value of the final velocity of substrate amidolysis (v_f) in the solution after dilution, and by the first-order rate constant of the return of HLE activity (k_{-1}). Both these values were computed by fitting the progress curve data in Figure 2 to eq 1, as derived from Williams and

$$P = v_f - v_f(1 - e^{-k_{-1}t})/k_{-1} + v_r t + P_0 \quad (1)$$

Morrison (1979), where P and P_0 represent the absorbance values at 405 nm at any time t and zero time, respectively, and v_r is the residual enzyme activity in the diluted solution at zero time. From three independent determinations, v_f was found to be (1.1 ± 0.1) × 10^{-2} μM s^{-1}, as compared to the rate of amidolysis in the absence of elafin (control in Figure 2) of (1.0 ± 0.2) × 10^{-2} μM s^{-1}. The value of k_{-1} computed from these data is also identical to the values determined by other methods (Table I). Thus, the reaction is fully reversible with respect to the recovery of active enzyme. We present evidence below that the reaction is also reversible with respect to the recovery of active inhibitor.

Classification of the Inhibition Mode. To define the mode of inhibition of HLE by elafin, the initial rate (v_0) and the steady-state rate (v_s) of substrate hydrolysis were determined under conditions in which amidolysis was initiated by addition of enzyme to a mixture of substrate and inhibitor. These velocities were computed by nonlinear least-squares fits of progress curve data to eq 2 as shown in Figure 1 (Cha, 1975;

$$P = v_s - (v_s - v_0)(1 - e^{-kt})/k + P_0 \quad (2)$$

Williams & Morrison, 1979). In this expression, k is an apparent first-order rate constant for the exponential decline of v_0 to v_s; the definitions of P and P_0 are the same as in eq 1. A set of reciprocal plots of $1/v_s$ vs $1/[S]$ ([S], substrate concentration) at different fixed elafin concentrations is shown in Figure 3. The intersection of the straight lines on the y-axis is consistent with a model of competitive inhibition. The linearity of a replot of the slopes of these lines as a function of the elafin concentrations, shown in the inset to Figure 3, indicates that inhibition by elafin is caused by binding of a single inhibitor molecule to a single site on HLE which overlaps the enzyme's extended substrate binding domain. The 1:1 stoichiometry deduced from this kinetic analysis is consistent with the results of the active-site titration of elafin by HLE (see Materials and Methods).

Since MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA is a large substrate which extends over at least five subsites of the enzyme's extended substrate binding domain (Yasutake & Powers, 1981), competition by elafin for any one of these subsites could be sufficient to generate a set of results like those shown in Figure 3. Thus, from the kinetic analysis alone, it cannot be concluded that elafin competes with this substrate for the enzyme's catalytic center. To examine if the catalytic center is involved in binding, the effect of elafin on the incorporation of [^{3}H]DFP into HLE was studied (see Materials and Methods). Figure 4 shows that elafin effectively blocks the incorporation of [^{3}H]DFP into HLE. As DFP specifically modifies the active-site serine of serine proteases, the results in Figure 4 strongly support the interpretation that at least part of the binding domain for elafin on HLE includes the enzyme's catalytic center.

The x-axis intercept of the replot of the slopes of the reciprocal plots in Figure 3 vs [I] as shown in the inset to the figure gives a measurement of K_i, the equilibrium dissociation constant of the enzyme–inhibitor complex. By this method of analysis, however, the value of the x-intercept is too close to the origin to provide more than an estimate of K_i < 1 nM.

Kinetic Mechanism. In his original analysis of the progressive hydrolysis of a substrate by an enzyme in the presence of the enzyme's inhibitor, Cha (1975, 1976) proposed four kinetic mechanisms for reversible enzyme inhibition, all of which produce progress curves with the profile described by eq 2. Among the four mechanisms, the most frequently cited one in studies of enzyme inhibition is mechanism "b"; i.e., inhibitor and enzyme first form a loose complex which subsequently undergoes a slow isomerization to produce a tight, but still dissociable complex [for a review, see Morrison and Walsh (1988)]. The manifestation of this mechanism in progress curves of hydrolysis of substrate is that the initial rate, v_0, decreases with increasing inhibitor concentration. This mechanism also predicts that the value of the pseudo-

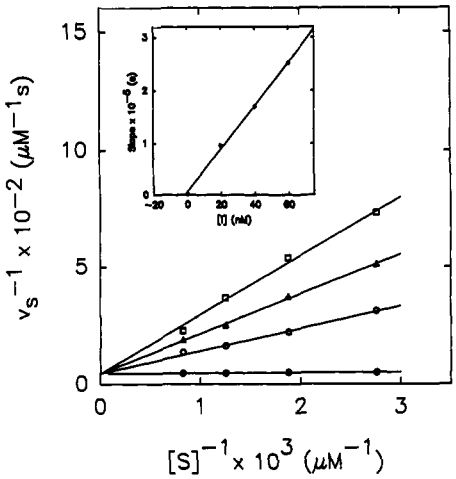

FIGURE 3: Plots of $1/v_s$ vs $1/[S]$. Reaction conditions were similar to those in Figure 1. HLE, 1 nM; elafin (from top to bottom), 60, 40, 20, and 0 nM; the concentrations of substrate were as indicated. The inset shows a replot of slopes of the straight lines vs elafin concentrations.

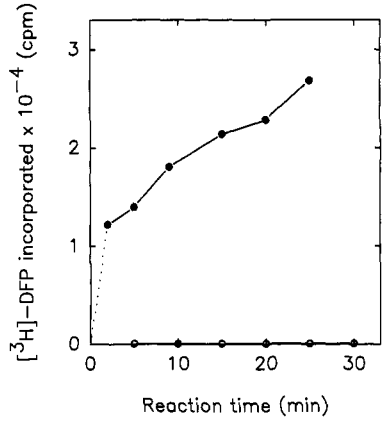

FIGURE 4: Effect of elafin on incorporation of [^{3}H]DFP into HLE. For details of the experiments, see Materials and Methods. Open circles, radioactivity incorported into HLE in the presence of elafin; closed circles, radioactivity incorporated into HLE in the absence of elafin.

first-order rate constant, k, should increase hyperbolically with inhibitor concentration (Cha, 1975, 1976). In the reaction of elafin and HLE, however, v_0 does not vary with [I]. In a set of experiments at pH 8.0, we used constant enzyme and substrate concentrations: 1 nM and 1208 μM, respectively. In the absence of elafin, the rate of amidolysis was $(1.95 \pm 0.07) \times 10^{-2}$ μM s^{-1} ($n = 3$), while in the presence of a range of elafin concentrations from 20 to 150 nM, the mean value of v_0 was $(2.00 \pm 0.14) \times 10^{-2}$ μM s^{-1} ($n = 15$). The difference between the mean values of v_0 in the absence and in the presence of elafin is not significant. Moreover, the standard deviation among individual v_0 values determined in the presence of elafin is small, with a random distribution of residuals which is independent of elafin concentration. The absence of an accumulating initial intermediate is further supported by the linearity of the plot of k vs [I], as shown in Figure 5.

The positive slope of the plot of k vs [I] (see Figure 5) also excludes a mechanism in which two conformations of an enzyme are in slow equilibrium, but only one of the two conformations reacts with inhibitor [Cha's mechanism "c" (1975)]. If this conformational equilibrium was present in HLE, and if only one of the two putative conformations could be inhibited by elafin, k would decrease with increasing [I] (Cha, 1975, 1976; Duggleby et al., 1982). We have demonstrated that the steady-state inhibition data for elafin are

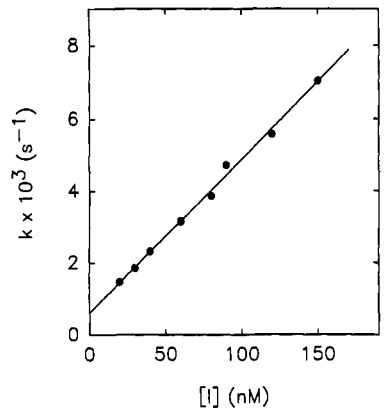

FIGURE 5: Dependence of the exponential phase of progress curves for inhibition of HLE-catalyzed amidolysis on elafin concentration. The constant of exponentiation, k, was determined by fitting progress curve data to eq 2 as outlined under Materials and Methods. Experimental conditions were the same as in Figure 1.

Scheme I

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI$$

most consistent with a model of simple competitive inhibition, eliminating Cha's mechanism "d", in which the inhibition is noncompetitive. Only mechanism "a" among the four schemes originally proposed by Cha is consistent with all our observations (Scheme I) It should be noted that while our experimental data are accommodated by this mechanism, other mechanisms are not excluded. Two quasi-irreversible mechanisms discussed below are also consistent with our results. However, these two quasi-irreversible schemes can be excluded by additional experiments.

Evaluation of Rate Constants. The association rate constant, k_1, and dissociation rate constant, k_{-1}, in Scheme I can be determined from a plot of k vs [I] (Figure 5) according to eq 3 (Cha, 1975, 1976).

$$k = k_{-1} + \frac{k_1[I]}{1 + [S]/K_m} \quad (3)$$

Another method for calculating k_{-1} employs the values of k, v_0, and v_s determined from each individual progress curve according to eq 4. Theoretically, the value of $k(v_s/v_0)$ should

$$k_{-1} = k(v_s/v_0) \quad (4)$$

be independent of the concentrations of enzyme, inhibitor, and substrate. In our experiments in which the amidolytic reaction was initiated by addition of the enzyme to a mixture of substrate and inhibitor, we collected data for 33 separate progress curves, in which the concentrations of HLE, elafin, and MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA were 1 nM, 10–150 nM, and 362–1208 μM, respectively. The k_{-1} values calculated according to eq 4 range from a minimum of 5.2×10^{-4} s^{-1} to a maximum of 6.6×10^{-4} s^{-1}, with a mean ± SD of $(6.0 \pm 0.3) \times 10^{-4}$ s^{-1} ($n = 33$). The value of k_{-1} has also been determined independently according to eq 1 by measuring the release of enzyme from the enzyme–inhibitor complex (Figure 2). All results for these different methods of determining k_{-1} are reported in Table I. It can be seen that the values of k_{-1} from the three methods are almost identical. We have employed the value of k_{-1} from eq 4, along with the value of k_1 obtained from the plot of k vs [I] (Figure 5), to calculate $K_i = k_{-1}/k_1$.

To examine the effect of ionic strength on the association and dissociation of elafin, k_1 and k_{-1} were determined in pH

Scheme II

$$E + I \xrightarrow{k_1} EI \xrightarrow{k_2} E + I'$$

Scheme III

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E + I'$$

8.0 buffer containing a total concentration of 1.0 M NaCl. The results of these determinations are also given in Table I.

Exclusion of Other Possible Kinetic Models. We have considered several quasi-irreversible mechanisms which generate progress curves virtually identical with those generated by the reversible mechanisms presented by Cha. Two such quasi-irreversible mechanisms, in which there is no hypothetical rapidly and transiently accumulating initial complex (Schemes II and III), are consistent with all the kinetic data presented above. In Schemes II and III, an irreversible decomposition of the EI complex by a first-order process with the rate constant k_2 results in liberation of an inactivated form of inhibitor, I'. It can be seen that if $k_1 \gg k_2$ in Scheme II, or $k_1 \gg k_{-1} + k_2$ in Scheme III, the complex EI will accumulate, progressively reducing the concentration of free enzyme available to hydrolyze a substrate from the maximum rate observed at the initiation of the reaction. When the rate at which the free enzyme is removed due to association with the inhibitor is equal to the rate at which free enzyme is regenerated from decomposition of the enzyme–inhibitor complex, the rate of substrate hydrolysis will reach a steady state. If the quantities of inhibitor and substrate in the reaction solution are sufficiently great, the steady-state velocity can be maintained for a protracted time. These two mechanisms are fully consistent with the progress curves in Figure 1. The association rate constant, k_1, is equivalent in the reversible mechanism in Scheme I and the two irreversible mechanisms in Schemes II and III. The dissociation rate constant, k_{-1}, is equivalent to the single constant, k_2, in Scheme II, and to the sum of the two dissociation constants, $k_{-1} + k_2$, in Scheme III.

Two sets of experiments have been designed to rule out these quasi-irreversible mechanisms. In the first set of experiments, dissociation of the HLE–elafin complex was allowed to proceed in the presence of an excess of α_1-PI, which would be expected to trap any HLE released from the complex. The species of elafin dissociated from the complex was recovered, and other reactants and products in the solution were removed by ultrafiltration. The procedure has been described in detail under Materials and Methods. The elastase inhibitory activity of the elafin in the ultrafiltrate was titrated, and the kinetic constants for its reaction with HLE were determined as described above. The association rate constant for the recovered elafin was found to be $(3.1 \pm 0.2) \times 10^6$ M^{-1} s^{-1} ($n = 3$), as determined from plots of k vs [I]. The dissociation rate constant for the recovered inhibitor was found to be $(5.8 \pm 0.3) \times 10^{-4}$ s^{-1} ($n = 12$), as determined from the ratio $k(v_s/v_0)$. Inspection of the results in Table I shows that the recovered elafin is kinetically identical to native elafin. These results indicate that active elafin is released from dissociation of the HLE–elafin complex, thereby eliminating Scheme II, which postulates that decomposition of the complex yields only inactive inhibitor.

The results from these experiments, however, do not rule out the mechanism of Scheme III. Although the recovery of sample volume in the ultrafiltrates was as high as 89–97% of the initial volume, the recoveries of anti-elastase activity in the ultrafiltrates ranged only from 58% to 76%. A similar range of incomplete recoveries of active elafin was observed in control experiments in which samples containing inhibitor in the absence of HLE were subjected to ultrafiltration. Since this range of recovered activities is relatively large and some inhibitor is apparently lost in the ultrafiltration process even when enzyme is absent, we cannot exclude the possibility that some of the elafin recovered in the ultrafiltrates was in fact inactivated by decomposition of the EI complex, as indicated in Scheme III. From the results of the ultrafiltration experiments, we can conclude only that if Scheme III is correct, the majority of the inhibitor released from breakdown of the EI complex is active, i.e., $k_{-1} > k_2$.

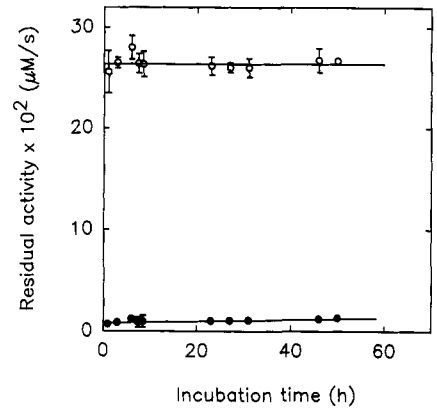

FIGURE 6: Residual enzyme activity in equilibrium mixtures of HLE and elafin submitted to long-term incubation. A mixture of 25.3 nM elasfin and 20 nM HLE was incubated in pH 8.0 buffer at 25 °C, and aliquots were removed over the next 54 h for amidolytic assay of residual enzyme activities as described in the text. In control experiments, HLE alone was incubated for the same extended times. Open circles, control in which HLE was incubated alone; closed circles, residual HLE activities in a mixture of HLE and elafin. Each point with an error bar represents a mean ± SD ($n = 3$).

In order to gain a better estimate of the fraction of elafin which retains inhibitory activity after dissociation of the EI complex, we have incubated HLE with a slight excess of inhibitor for an extended period of time and have attempted to detect the reappearance of active HLE which would gradually accumulate if in fact some of the complex decomposed to release inactive inhibitor, I', as indicated in Scheme III. A mixture of 25.3 nM elafin and 20 nM HLE was incubated for an extended period in pH 8.0 buffer at 25 °C; at various times, 290-μL aliquots of the reaction mixture were removed and combined with 10 μL of 12 mM MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA in Me_2SO to assay residual enzyme activities. The release of *p*-nitroaniline was monitored at 405 nm for 1 min; during this short period, dissociation of HLE–elafin complex would be negligible. In control experiments, HLE alone was incubated for the same extended times to detect any loss of enzyme activity which might occur as the result of autolysis, denaturation, or other causes. As shown by the results of the control experiments in Figure 6 no loss of the amidolytic activity of HLE in this buffer could be detected over an incubation period of 50 h. The amidolytic activity of HLE after 1 h of incubation in the absence of elafin was found to be 0.256 ± 0.021 μM s^{-1}, while after 50-h incubation, it was 0.267 ± 0.001 μM s^{-1} (mean ± SD; $n = 3$). The residual enzyme activity in the mixture of HLE and elafin showed an increase from 0.007 ± 0.001 μM s^{-1} after 1-h incubation to 0.013 ± 0.001 μM s^{-1} after 50-h incubation. The absolute magnitude of the increase is small but could be significant as the value increases monotonically with time (Figure 6). Assuming that this increase in activity reflects an increase in the concentration of free enzyme, we can calculate the decrease of the concentration of active elafin

which would account for this small increase in the level of uninhibited HLE. The total concentration of active inhibitor, $[I]_0$, in a reaction solution at any time is expressed by eq 5,

$$[I]_0 = K_i\frac{[E]_0}{[E]} + [E]_0 - [E] - K_i \qquad (5)$$

where $[E]_0$ is the total concentration of enzyme in an equilibrium mixture and [E] is the concentration of free enzyme. The equation was derived from the relationship $K_i = [E][I]/[EI]$ and the conservation equations $[E]_0 = [E] + [EI]$ and $[I]_0 = [I] + [EI]$. Using the previously determined value of $K_i = 0.17$ nM (Table I), and $[E]_0 = 20$ nM, $[I]_0$ in the reaction solution after 1 h of incubation was calculated to be 25.4 nM, which is identical to the concentration of elafin we added to the reaction solution at time zero, 25.3 nM. $[I]_0$ after 50 h of incubation was calculated to be 22.3 nM, giving a total loss of active elafin of 3.1 nM after 49 h of incubation. Assuming that any inactivation of elafin arises from decomposition of the enzyme–inhibitor complex, EI, according to the mechanism in Scheme III, the value of k_2 can be roughly estimated to be 9×10^{-7} s^{-1}, which is only $0.0015k_{-1}$ ($k_{-1} = 6.0 \times 10^{-4}$ s^{-1}; see Table I), and lies within the range of experimental errors of determination of k_{-1}. These results indicate that the reaction is not only fully reversible with respect to enzyme, as demonstrated previously, but also fully reversible with respect to inhibitor within the limits of experimental error. The form of elafin which is released from the enzyme–inhibitor complex is at least 99.8% active over each cycle of association and dissociation. The reversible mechanism described by Scheme I, therefore, is most consistent with the kinetics of elafin binding to HLE.

Effect of pH on Inhibition. We have measured the values of k_1, k_{-1}, and K_i for inhibition of HLE by elafin at pH values from 5.4 to 9.0. We have assumed that the fundamental kinetic mechanism of the reaction which we elucidated at pH 8.0 does not change over this pH range but that the individual kinetic constants may be quantitatively altered. On the basis of this assumption, these rate constants were computed from least-squares fits of eq 6 to the digitized data from individual

$$P = \frac{v_0k_{-1}}{k'}t + \frac{v_0k_1[I]}{(1 + [S]/K_m)k'^2}(1 - e^{-k't}) + P_0 \qquad (6)$$

progress curves obtained at different pH values (Baici & Gyger-Marazzi, 1982) where k' is an apparent first-order rate constant and the definitions of other symbols are the same as those in eq 2 and 3. The results from this method are in excellent agreement with those from the preceding methods. For example, at pH 7.9, we obtained values of $k_1 = (2.6 \pm 0.4) \times 10^6$ M^{-1} s^{-1} and $k_{-1} = (5.7 \pm 0.2) \times 10^{-4}$ s^{-1} ($n = 3$) by this method; these values are both in good agreement with the values listed in Table I, determined at pH 8.0. In Figure 7, panels A, B, and C show the effects of pH on k_1, k_{-1}, and K_i, respectively. As the pH is increased from 5.4 to 9.0, k_1 increases 13-fold, from $(2.0 \pm 0.3) \times 10^5$ M^{-1} s^{-1} to $(2.6 \pm 0.2) \times 10^6$ M^{-1} s^{-1} ($n = 3$), while k_{-1} decreases 2.5-fold, from $(1.3 \pm 0.1) \times 10^{-3}$ s^{-1} to $(5.3 \pm 0.2) \times 10^{-4}$ s^{-1}, resulting in a decrease of K_i by a factor of 33. The diminution of K_i occurs mainly within the pH range from 5.4 to 7.5; beyond pH 7.5, changes of K_i are more modest.

The shape of the pH dependence of K_i found in this study resembles those observed in the reactions of bovine basic pancreatic trypsin inhibitor (Kunitz), porcine pancreatic secretory trypsin inhibitor (Kazal), and eglin c with various

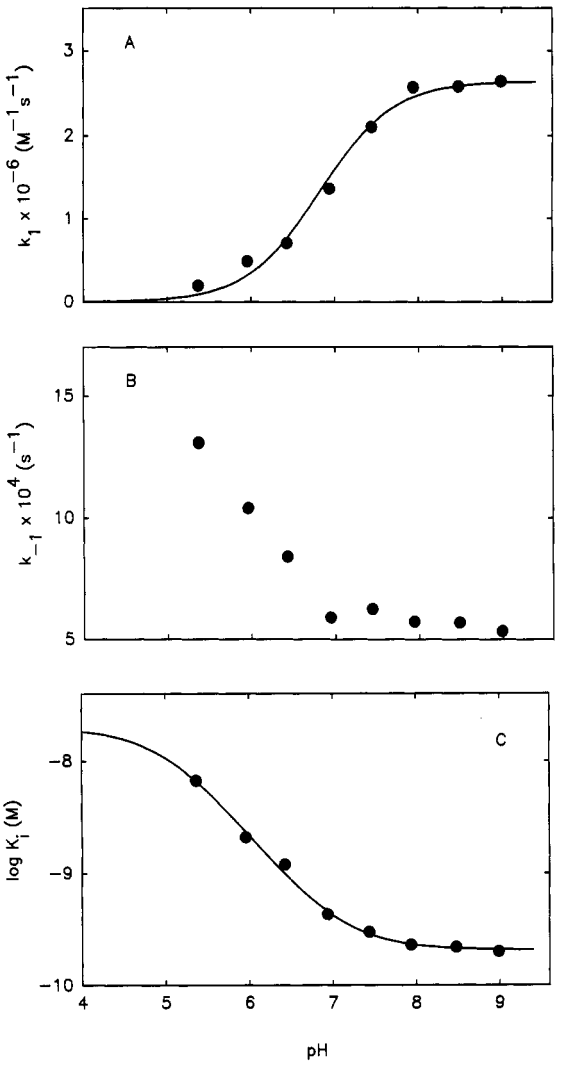

FIGURE 7: Effect of pH on the kinetic parameters of the reaction between elafin and HLE. (A) Effect of pH on the association rate constant, k_i; the curve represents the least-squares fit of the data to eq 8. (B) Effect of pH on the dissociation rate constant, k_{-1}; the data in the low-pH range do not extend sufficiently below the apparent pK_a to permit fitting. (C) Effect of pH on the equilibrium dissociation constant, K_i; the curve represents the least-squares fit of the data to eq 7. Each point in these graphs represents a mean of three assays.

serine proteinases (Vincent & Lazdunski, 1973; Antonini et al., 1983; Menegatti et al., 1984, 1986; Ascenzi et al., 1986, 1988). The decrease of K_i with pH can be interpreted by a model in which binding of enzyme and inhibitor leads to a shift to lower pH of the pK_a value for a single ionizable group (which theoretically could be on the enzyme or on the inhibitor), the protonation state of which determines the association rate of the enzyme and the inhibitor and the dissociation rate of their complex (Antonini et al., 1983; Menegatti et al., 1984, 1986; Ascenzi et al., 1986, 1988). By employing the linkage concept originally developed for the Bohr effect in hemoglobin (Wyman, 1964), the pK_a values of this proposed group in the mixture of free enzyme and inhibitor (pK_1) and in the enzyme–inhibitor complex (pK_2), respectively, can be determined according to eq 7 (Antonini et al., 1983).

$$\log K_i = \log(K_i)_{lim} + \log\frac{10^{-pK_1} + 10^{-pH}}{10^{-pK_2} + 10^{-pH}} \qquad (7)$$

In this expression, $(K_i)_{lim}$ is the limiting value of K_i at alkaline pH. By fitting data in Figure 7C to eq 7, we have obtained

$pK_1 = 7.0 \pm 0.06$ and $pK_2 = 5.1 \pm 0.02$ ($n = 3$) for the reaction of elafin and HLE. These values fall in the range of those reported for the reactions of serine proteinases with the other proteinase inhibitors mentioned above (Antonini et al., 1983; Menegatti et al., 1984, 1986; Ascenzi et al., 1986, 1988). However, the pH range over which we have determined our progress curves is not sufficiently extensive to place high confidence on both these computed pK_a values even though the apparent fits to the experimental data are excellent (cf. Figure 7C), since the value of pK_2 calculated by least-squares fit is lower than the lowest pH at which we have made measurements. We could not extend our determinations to lower pH values because HLE slowly but significantly loses its catalytic activity in solutions of pH <5.

The sigmoidal dependence of k_1 on pH (Figure 7A) is consistent with the assumption that the rate at which elafin associates with HLE is dependent on the concentration of the unprotonated form of a single ionizable group in the free enzyme or free inhibitor. By fitting the data to eq 8 (Fersht,

$$k_1 = \frac{(k_1)_{lim}}{1 + 10^{pK_a - pH}} \quad (8)$$

1985), we have computed a value of pK_a for the proposed group of 6.8 ± 0.1 ($n = 3$). In this expression, $(k_1)_{lim}$ is the limiting value of k_1 at alkaline pH. The pK_a of 6.8 estimated from the pH dependence of k_1 is in good agreement with the pK_1 value determined from the pH dependence of K_i for the free reactants and is also consistent with the value reported for the active-site histidine of most serine proteinases. We have already noted the limitations in obtaining experimental data on neutrophil elastase below pH 5 and the relatively small pH dependence of k_{-1}. Because of these considerations, we cannot provide better verification of the hypothesis that the approximate pK_a of 5 calculated for the ionizable group in the HLE–elafin complex and the pK_a of the group which contributes to the pH dependence of k_{-1} are in fact the same.

DISCUSSION

The kinetic data presented in this study support a mechanism in which both binding of elafin to HLE and dissociation of the enzyme–inhibitor complex proceed via a single step. The association rate constant, k_1, has been determined to be 3.6×10^6 M^{-1} s^{-1} at pH 8.0 and 25 °C. This value falls in the range of many other rate constants reported for bimolecular reactions between enzymes and substrates or substratelike inhibitors (Fersht, 1985), but is about 3 orders of magnitude slower than the diffusion-controlled limit. We have considered two possible explanations which might account for the relatively low k_1. One possibility is that binding is impeded by unfavorable electrostatic interactions. Both elafin and HLE are basic proteins with $pI = 9.7$ (Wiedow, 1990) and 8.8–9.2 (Taylor, 1978), respectively, so that at pH 8.0, both proteins would be expected to be positively charged. Theoretical analysis predicts that if the net charges of two reactants bear the same sign, and if the product of the values of the net charges is around 6, the rate of association of the reactants can be expected to be retarded by about 3 orders of magnitude below that of the diffusion-controlled encounter frequency (Alberty & Hammes, 1958). To examine this possibility, we determined the association and dissociation rate constants in the presence of high salt. If electrostatic repulsions influenced the association rate, such charge effects should be effectively shielded by counterions in a medium of high ionic strength, and the rate would be expected to increase in high salt (Alberty & Hammes, 1958). However, as shown in the results listed in Table I, when the concentration of NaCl in the reaction medium is increased from 0.15 to 1 M, k_1 remains unchanged, with a slight decrease of k_{-1}. Electrostatic effects, therefore, cannot be verified in this system. Examination of the three-dimensional structure of HLE reveals that the positive charges in this enzyme are clustered in a few patches on the surface some distance from the extended substrate binding site, suggesting that the contributions of electrostatic effects to binding of a competitive inhibitor would not be expected to be significant (Wei et al., 1988; Navia et al., 1989).

An alternative possibility to be considered is that the enzyme and inhibitor might first combine in a very fast bimolecular process (which we have failed to resolve by our experimental procedure) to form a loose complex which then isomerizes to a tight complex (Fersht, 1985). If the dissociation constant of the loose complex is much larger than the highest inhibitor concentration used to measure the apparent association kinetics, this mechanism may not be demonstrable by the slow-binding analysis technique (Morrison & Walsh, 1988). In this work, the highest elafin concentration used for kinetic analysis was 0.15 μM, i.e., $\sim 1000K_i$. If an initial "loose complex" intermediate were formed, its dissociation constant would be at least ≥1 μM to account for our failure to detect it. Consequently, the isomerization rate constant for conversion of this putative loose complex to the final tight complex would be ≥4 s^{-1} in order to account for the apparent second-order rate constant we have observed. This rate is much faster than most of the published isomerization rates determined by the slow-binding analysis technique [see the references cited by Morrison and Walsh (1988)], but it is close to the rates of conformational changes in enzymes induced by substrates (Fersht, 1985). At present, we are unable to confirm or exclude this kind of initial intermediate experimentally. On the basis of current results, we must conclude that association of HLE and elafin is consistent with a single-step process until new data which might support a more complex sequential scheme are obtained.

The pH dependence of k_1 offers some interesting insight into the roles of amino acid side chains in the association of HLE with elafin. The association rate appears to be affected by the concentration of a deprotonated form of a group with pK_a of 6.8. The contribution of a group with this pK_a in one of the reactants can also be inferred from the pH dependence of K_i. Several lines of evidence indicate that this group may very well be the imidazole group of the active-site histidine of HLE. A number of studies on the pH dependence of equilibrium binding of substrates and inhibitors to several different serine proteases implicate a common ionizable group with a pK_a value around 6.8, which has been assigned to the imidazole group of the histidine residue that forms part of the catalytic triad in all these enzymes (Antonini et al., 1985; Menegatti et al., 1984, 1986; Ascenzi et al., 1986, 1988; Polgár, 1989). There are no histidines in the amino acid sequence of elafin (Wiedow et al., 1990; Tsunemi et al., 1992), so if the group with the pK_a of 6.8 in our pH dependence studies is indeed an ionizable imadazole side chain, it must belong to a histidine in HLE. From our experiments on [^{3}H]DFP incorporation, we have established that the active site of HLE is at least sterically blocked by binding of elafin, supporting the possibility that the group of pK_a 6.8 may be located in the vicinity of the active site of the enzyme. Examination of the structure of the extended substrate binding domain of HLE around the catalytic triad shows that there are no ionizable side chains with pK_a values ~7 in the vicinity of the subsites

of the substrate binding domain except for the histidine in the triad itself (Wei et al., 1988; Navia et al., 1989). The pK_a of the ionizable group in HLE which participates in catalysis has been reported to be 7.18 ± 0.04 (Stein, 1983). By determining the pH dependence of the value of k_{cat}/K_m for the amidolysis of MeO-Suc-Lys(Pic)-Ala-Pro-Val-pNA by HLE, we have computed a pK_a of 6.91 ± 0.06 for an ionizable group in the free enzyme which participates in binding (data not shown); this value is very close to that for an ionizable group in the mixture of free reactants determined from the pH dependence of k_1 and K_i in the studies on inhibition of HLE by elafin reported here. These considerations implicate the unprotonated form of the active-site histidine of HLE as a participant in the association of the enzyme with elafin. It is generally accepted that the unprotonated form of the active-site histidine in serine proteases functions as a general base to catalyze the acylation and deacylation steps in hydrolysis of various substrates (Polgár, 1989). The classic hypothesis for the catalytic mechanism includes a complete transfer of a proton from Ser-195(O_γ) to His-57(N_ϵ), followed by the formation of a tetrahedral adduct. Analysis of the crystal structures of complexes of serine proteases with small protein inhibitors has failed to confirm formation of a fully developed tetrahedral adduct, but is more consistent with a partial transfer of the proton, or a strengthening of the hydrogen bond between Ser-195(O_γ) and His-57(N_ϵ) (Marquart et al., 1983). The acid pK_a shift of the ionizable group implicated in the formation of complexes between small protein inhibitors and serine proteases has been attributed to the strengthening of this hydrogen bond (Antonini et al., 1983; Menegatti et al., 1984, 1986; Ascenzi et al., 1986, 1988). This interpretation may also be applicable to the acid pK_a shift of the ionizable group which participates in the reversible inhibition of HLE by elafin. However, further data will be required to substantiate the mechanistic significance of the pH dependence of the inhibitory reaction and its relationship to potential involvement of the active-site histidine in the enzyme.

The association rate constant we have determined for elafin and HLE at pH 7.4 is 2.1×10^6 M^{-1} s^{-1}, a value which is very close to that reported for association of the homologous four-disulfide core inhibitor SLPI and HLE (Boudier & Bieth, 1989; Faller et al., 1992). Whereas elafin was first described as an inhibitor of HLE in skin, SLPI has been generally regarded as a major inhibitor of HLE in the upper airways (Gauthier et al., 1982; Hubbard & Crystal, 1992). Although a comprehensive study of the tissue distribution of elafin has not yet been undertaken, a recent study has confirmed that this inhibitor is present along with SLPI in bronchial mucous secretions (Sallenave et al., 1992). HLE inhibitory activity distinct from SLPI in bronchial mucous secretions which has been described in a number of earlier reports appears to be from intact elafin or its proteolytic degradation products which retain activity (Hochstrasser et al., 1981; Kramps & Klasen, 1985; Stockley et al., 1986; Boudier et al., 1987; Morrison et al., 1987). Although elafin and SLPI have comparable association rates under the assay conditions we and others have usually employed, in the environment of the airways the inhibitory potential of SLPI may dominate over that of elafin. Most reports have concluded that the levels of SLPI and of α_1-PI in the airways account for the great majority of the anti-elastase activity [reviewed in Hubbard and Crystal (1992)], although a significant fraction of heretofore unidentified inhibitory activity has also been reported (Stockley et al., 1986). We have recently found that a glycoprotein-rich fraction of tracheobronchial mucus which consists primarily of strongly negatively charged mucins greatly accelerates the association of SLPI with HLE but has no effect on the association of elafin with this protease. These preliminary finding suggest that polyanions in tracheobronchial mucus may provide a specialized environment which enhances the anti-elastase activity of SLPI over that of elafin. In tissues in which such polyanions are less abundant or in which levels of SlPI are low, elafin may still be an HLE inhibitor of major physiologic importance.

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