Demonstration of a Two-Step Reaction Mechanism for the Inhibition of Heparin-Bound Neutrophil Elastase by α_1-Proteinase Inhibitor

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ABSTRACT: Heparin decreases the rate of inhibition of neutrophil elastase by α_1-proteinase inhibitor as a result of its strong binding to the enzyme. Here, we used the slow-binding kinetic approach to decide whether the enzyme–inhibitor interaction proceeds *via* a two-step mechanism and to identify the step that is affected by heparin. The inhibition kinetics was assessed under pseudo-first-order conditions using conventional or stopped-flow spectrophotometry. In the absence of heparin, the pseudo-first-order rate constant of inhibition increased linearly with the inhibitor concentration indicating that within the experimental concentration range (≤ 6 μM) the enzyme–inhibitor association conforms either to a simple bimolecular reaction ($E + I \xrightarrow{k_{ass}} EI$ with $k_{ass} = 10^7$ M^{-1} s^{-1}) or to a two-step reaction ($E + I \overset{K_i^*}{\rightleftharpoons} EI^* \xrightarrow{k_2} EI$ with $K_i^* > 0.4$ μM and $k_2 > 4$ s^{-1}). In the presence of heparin, the rate constant of inhibition varied hyperbolically with the inhibitor concentration, indicating that the inhibition is a two-step process with $K_i^* = 80$ nM and $K_2 = 0.15$ s^{-1}. Thus, heparin has two opposite effects on the elastase + α_1-proteinase inhibitor interaction: it favors the association by decreasing K_i^* but impairs it by decreasing k_2. This rationalizes the previously demonstrated rate-depressing effect of the sulfated polymer. Heparin does not significantly alter the stability of the irreversible elastase–α_1-proteinase inhibitor complex.

Human polymorphonuclear leukocytes contain a number of proteolytic enzymes including neutrophil elastase (NE),[1] a 30-kDa glycoprotein that belongs to the class of serine proteinases. NE cleaves extracellular matrix proteins including elastin, interstitial collagen, proteoglycans, fibronectin, and laminin as well as plasma proteins such as antithrombin, fibrinogen, and components of the immune system. Uncontrolled release of this potent enzyme may lead to degenerative connective tissue diseases such as lung emphysema and rheumatoid arthritis (for a review see Bieth (1986)).

The concentration of NE in the azurophil granules of neutrophils is thought to be in the millimolar range (Campbell, 1986). An efficient anti-NE control system must therefore be present at sites where neutrophils are activated or where they die in order to prevent undesirable extracellular protein degradation. The antielastase control system is composed of three protein proteinase inhibitors: α_2-macroglobulin, α_1-proteinase inhibitor (α_1PI),[1] and mucus proteinase inhibitor. The two former proteins are present in plasma and in the interstitial fluid while the latter almost exclusively occurs in airway and genital tract secretions (for a review see Bieth (1986)).

The 53-kDa glycoprotein α_1PI is thought to be the most important NE inhibitor. It belongs to the serine proteinase inhibitors, the serpins (Carrel & Travis, 1985), a superfamily of proteins that have developed by divergent evolution over about 500 million years. Many of the serpins are present in plasma and interstitial fluid where they inhibit irreversibly neutrophil, coagulation, and complement serine proteinases by forming denaturant-stable complexes with them (Travis & Salvesen, 1983). Among all proteinases tested on α_1PI, NE was found to react with the highest second-order association rate constant ($k_{ass} \approx 10^7$ M^{-1} s^{-1}) (Beatty et al., 1980; Strauss et al., 1985; Braun et al., 1987; Frommherz et al., 1991). The high k_{ass} value, the elevated levels of α_1PI in plasma (~ 20 μM, see Travis and Salvesen (1983)) and in lung alveolar epithelial lining fluid (~ 4 μM, see Ogushi et al. (1988)), and the pseudoirreversible character of the binding of NE to α_1PI (Beatty et al., 1980; Beatty et al., 1984; Braun et al., 1987; Frommherz et al., 1991) render the inhibition process extremely fast and efficient. For example, the delay time of inhibition (Bieth, 1980, 1984), i.e., the time required to fully inhibit NE *in vivo*, is only 13 ms in plasma (Bieth, 1986) and 100 ms in the lung epithelial lining fluid (Ogushi et al., 1988). The anti-NE function of α_1PI is best illustrated by the well-known correlation between hereditary α_1PI deficiency and pulmonary emphysema (Bieth, 1986).

NE forms tight complexes with heparin and other sulfated glycosaminoglycans (Marossy, 1981; Redini et al., 1988; Frommherz et al., 1991). This interaction is electrostatic in nature and probably involves some of the 19 arginine residues that are located in clusters on the surface of the enzyme (Bode et al., 1989; Navia et al., 1989). Mammalian heparins mostly consist of trisulfated disaccharide units formed of L-iduronic acid-2-sulfate linked to D-glucosamine-*N*,3,6-sulfate. These predominant regular sequences are variously interrupted by irregular sequences, the best known of which is the following: glucosamine-*N*,6-sulfate, iduronic acid, glucosamine-*N*,3,6-sulfate, iduronic acid-2-sulfate, and glucosamine-*N*,6-sulfate. This sulfated pentasaccharide tightly binds antithrombin with resultant acceleration of the rate of inhibition of thrombin and some other coagulation proteinases. This effect is the basis of the long known clinically relevant anticoagulant effect of heparin [for a review see Casu (1990)].

We have recently shown that the NE–heparin complex reacts much slower with α_1PI than does free NE (Frommherz et al., 1991). Unlike antithrombin and some other serpins (Huber & Carrell, 1989), α_1PI did not bind heparin, indicating that the observed rate-depressing effect was due to the NE–

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[1] Abbreviations: NE, human neutrophil elastase; α_1PI, α_1-proteinase inhibitor; Suc, succinyl; MeOSuc, methoxysuccinyl; *p*NA, *p*-nitroanilide.

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polysaccharide interaction. Commercially available "standard heparin" similar to the drug used in clinical care formed a very tight complex with NE ($K_d \approx 3$ nM) and decreased the second-order association rate constant k_{ass} from 1.3×10^7 M^{-1} s^{-1} (in the absence of heparin) to 4.6×10^4 M^{-1} s^{-1} in the presence of a saturating concentration of polymer. Heparin therefore increases the delay time of inhibition of NE (Bieth, 1980) by a factor of ~300 (i.e., the delay time is ~4 s in plasma and ~30 s in the alveolar lining fluid). These delay times are close to those calculated for the reaction of free NE with oxidized α_1PI, an *in vivo*-formed derivative that is ineffective in preventing NE-mediated proteolysis. As a consequence, heparin therapy may be harmful to patients with neutrophil activation, e.g., during septicemia or hemodialysis, because the released NE might not be inhibited fast enough by α_1PI to prevent proteolytic damage.

In our preceding work we measured the action of heparin on the NE + α_1PI system using second-order enzyme kinetics ($[E_o] = [I_o]$), the enzyme and the inhibitor being reacted for variable periods of time before addition of substrate (Frommherz et al., 1991). This method quantitates the enzyme–inhibitor interaction in terms of k_{ass}, the overall second-order association rate constant ($E + I \xrightarrow{k_{ass}} EI$), but does not allow one to decide whether the enzyme–inhibitor reaction is a simple bimolecular association process ($E + I \rightarrow EI$) or whether it proceeds *via* a two-stage binding mechanism ($E + I \rightleftharpoons EI^* \rightarrow EI$). The so-called slow-binding kinetic approach (Cha, 1975; Morrison, 1982; Morrison & Walsh, 1988) is apparently more promising. It consists of reacting enzyme with a mixture of inhibitor and substrate and analyzing the progress curves recorded with variable reactant concentrations. It has been successfully used to demonstrate that α_2-antiplasmin, another serpin, reacts with plasmin and chymotrypsin *via* a two-step mechanism (Longstaff & Gaffney, 1991). Here we describe the use of slow-binding kinetics to demonstrate that the NE–heparin complex reacts with α_1PI *via* a two-stage mechanism which explains why heparin decreases the overall rate of enzyme–inhibitor association.

EXPERIMENTAL PROCEDURES

Materials. Human NE was isolated and active-site titrated as described previously (Frommherz et al., 1991). Human α_1PI was purified from plasma using the procedure of Bruch & Bieth (1986). The molarities of inhibitor solutions were calculated using $\epsilon_{280nm} = 2.8 \times 10^4$ M^{-1} cm^{-1} (Pannell et al., 1974). Suc-Ala_3-*p*NA and MeOSuc-Ala_2-Pro-Val-*p*NA came from Bachem, Bubendorf, Switzerland. Heparin was a 5.1-kDa fragment purchased from Calbiochem, La Jolla, CA. It binds NE with a 1:1 stoichiometry and a dissociation constant K_d of 6 nM (Faller et al., 1992). A 5.05-kDa heparin fragment with a very low degree of polydispersity was prepared as described by Redini et al. (1988) and given to us by Dr. Maurice Petitou, Centre Choay-Sanofi, Gentilly, France. Unless otherwise stated, all experiments were done at 25 °C in 50 mM Hepes, 100 mM NaCl, pH 7.4, a solution that will be referred to as the "buffer" throughout the text.

Kinetics of Inhibition of Free and Heparin-Bound NE by α_1PI. The rate of elastase inhibition by α_1PI was measured under pseudo-first-order conditions, i.e., $[I_o] \geq 10[E_o]$, by reacting free or heparin-bound NE with a mixture of α_1PI and MeOSuc-Ala_2-Pro-Val-*p*NA and recording the release of *p*-nitroaniline at 410 nm. A CARY 2200 spectrophotometer equipped with a thermostated cell holder was used for reactions that lasted more than 1 min. The reagents were mixed manually when the reaction time was longer than 15 min. A SFA-11 rapid mixing accessory with a dead time of ca. 1 s (High-Tech Scientific, Salisbury, U.K.) was used to mix the reagents when the experiments lasted less than 15 min. When the reactions were complete in 1 min or less, we used a High-Tech SF/PQ 53 stopped-flow apparatus equipped with a SU-40 amplifier and water circulation. This device has a dead time of ca. 1 ms. For both rapid mixing devices the syringes were filled with a buffered solution of either NE ± heparin or α_1PI + MeOSuc-Ala_2-Pro-Val-*p*NA. One hundred microliters of solution of each syringe were used per run, and 5–10 runs were performed for each kinetic experiment. The temperature was maintained at 25 °C.

The CARY spectrophotometer and the stopped-flow apparatus were on-line with an IBM PS/2 Model 30 microcomputer equipped with an ENZFITTER software or a Hewlett-Packard 9000 Model 300 microcomputer equipped with a Hi-Tech HS 1.1 software, respectively.

Influence of Heparin on the Stability of the NE–α_1PI Complex. A buffered mixture of 5 μM NE, 20 μM heparin, and 6.4 μM α_1PI was incubated for 15 min at 25 °C before being diluted 500-fold into a buffered mixture of 1.5 mM MeoSuc-Ala_2-Pro-Val-*p*NA and 20 μM heparin. The absorbance at 410 nm was then recorded at 25 °C for 25 h. A control experiment was done in a similar way except that the α_1PI concentration was 5.25 μM and that no heparin was included in the reaction media.

The stability of free and heparin-bound NE in the presence of substrate was studied in the following way. NE (5 μM) ± heparin (20 μM) was diluted 500-fold into a buffered mixture of 9.7 mM Suc-Ala_3-*p*NA ± 20 μM heparin, and the absorbance at 410 nm was recorded at 25 °C for 20 h.

All other technical details are given in the legends to the figures.

RESULTS

Kinetics of Inhibition of Free and Heparin-Bound NE by α_1PI. To measure the rate of inhibition of NE by α_1PI, the enzyme was added to a mixture containing the substrate and a 10-fold molar excess of inhibitor over enzyme, and the concentration of product (absorbance at 410 nm) was measured with time. NE and α_1PI form an SDS-stable irreversible complex (Travis & Salvesen, 1983). For such a system, the progress curve recorded under pseudo-first-order conditions should be a simple exponential (Tian & Tsou, 1982). This was, however, not the case. The upper panel of Figure 1 shows, for instance, that the progress curve does not reach its expected plateau but that the absorbance steadily increases with time. The value of this apparent steady-state rate was, however, poorly reproducible. On the other hand, when the stopped-flow apparatus was used, this apparent rate increased with the number of stopped-flow runs. We interpreted these data as indicating that part of the enzyme adsorbs on the walls of the spectrophotometer cuvettes and thus escapes inhibition by the macromolecular inhibitor while being still able to hydrolyze the small synthetic substrate. The tendency of NE to adsorb on glass is well documented (Gauthier et al. 1982; Straus et al., 1985). Although the rate due to cuvette-bound NE was small, i.e., 1–3% of the velocity recorded in the absence of α_1PI, it hindered the nonlinear regression analysis of the data since the experimental progress curve did not fit a simple exponential. We therefore corrected the progress curve as indicated in the legend to Figure 1.

The corrected curves could now be fitted to exponentials which were analyzed using the following relationship (Tian

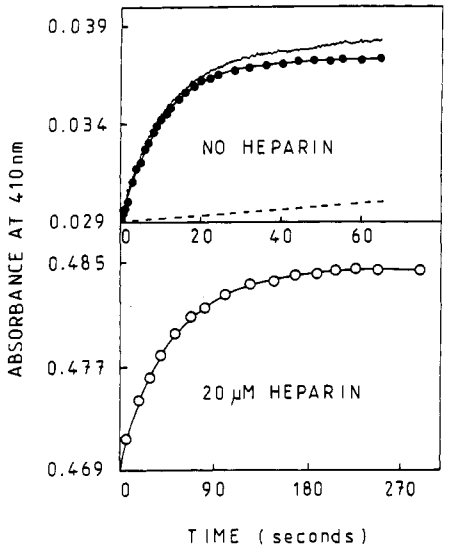


FIGURE 1: Pseudo-first-order kinetics of the inhibition of free and heparin-bound NE by α_1PI at pH 7.4 and 25 °C. The progress curves were recorded after mixing NE (20 nM) ● heparin (20 μM) with α_1PI (200 nM) + MeOSuc-Ala$_2$-Pro-Val-*p*NA (1.5 mM) dissolved in the buffer containing 2% (v/v) dimethylformamide. Upper curves: data collected in the absence of heparin: ( ) stopped-flow trace; (- - -) line parallel to the tail of the stopped-flow trace; (●) corrected absorbances, i.e., absorbances from the stopped-flow trace minus absorbance from the above parallel (—) theoretical curve generated using the best estimates of k and v_z (eq 1) calculated by nonlinear regression analysis. Lower curve: corrected progress curve in the presence of 20 μM heparin: (O) and (—) were calculated as described for the upper curve. Note that heparin increases both the absorbance at 410 nm (concentration of product) and the half-life of the reaction, two effects indicative of a decrease in the inhibition rate.

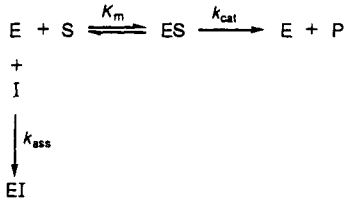
& Tsou, 1982):

$$P = \frac{v_z}{k}(1 - e^{-kt}) \qquad (1)$$

where P is the concentration of product (absorbance at 410 nm), v_z is the rate at $t = 0$, and k is the pseudo-first-order rate constant of NE inactivation. This constant was found to be 0.097 ± 0.01 s^{-1}. This inhibition experiment was repeated in the presence of 20 μM heparin which saturates 20 nM NE to the extent of 99.97% (K_d = 6 nM; Faller et al., 1992). The corrected progress curve could again be fitted to an exponential with $k = 0.024 \pm 0.003$ s^{-1}. Similar results were obtained with the 5.05-kDa heparin fragment having a very low degree of polydispersity.

The rate constant k decreased with the substrate concentration when the latter was varied between 0.75 and 6 mM (data not shown), indicating that substrate and inhibitor compete for the binding of the enzyme. This inhibition process may therefore be described by Schemes I or II (Tian & Tsou, 1982), where E stands for NE or the NE–heparin complex, I represents α_1PI, EI is the final irreversible complex, and EI* is an intermediate reversible complex whose concentration is governed by $K_i^* = k_{-1}/k_1$.

Scheme I



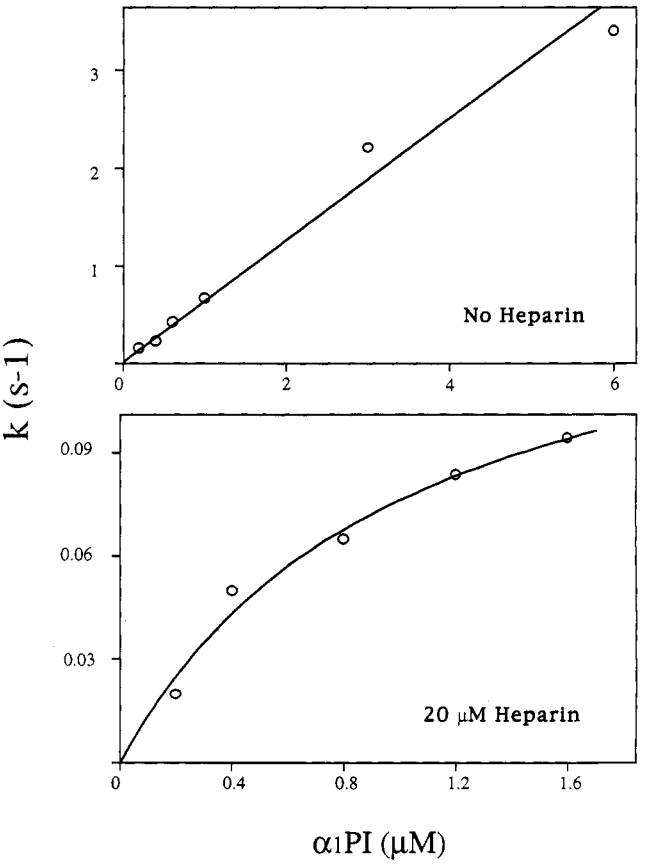
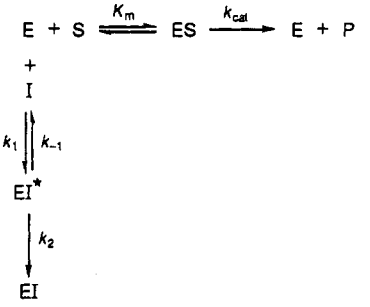


FIGURE 2: Effect of α_1PI concentration on k, the pseudo-first-order rate constant of NE inactivation at pH 7.4 and 25 °C. The rate constant k was measured using the progress curve method shown in Figure 1. Upper curve: data collected in the absence of heparin. Both α_1PI and NE concentrations were varied but the molar ratio of α_1PI to NE was 10 throughout: (O) experimental points; (—) line calculated by linear least-squares analysis. Lower curve: data collected in the presence of 20 μM heparin. The NE concentration was 20 nM throughout: (O) experimental points; (—) theoretical curve calculated using eq 3 and the best estimates of k_2 and K_i^* (1 + $[S_o]/K_m$) calculated by nonlinear regression analysis.

Scheme II



Schemes I and II predict eqs 2 and 3, respectively:

$$k = \frac{k_{ass}[I_o]}{1 + [S_o]/K_m} \qquad (2)$$

$$k = \frac{k_2[I_o]}{[I_o] + K_i^*(1 + [S_o]/K_m)} \qquad (3)$$

To decide between Scheme I and II, the rate constant k was measured as a function of α_1PI concentration (Figure 2). The concentration of NE–heparin complex was maintained constant while that of α_1PI was increased. This could not be done with free NE because the high concentrations of inhibitor used in this experiment (see Figure 2) yielded exponentials

with barely detectable amplitudes. The amplitude of the exponential described by eq 1 is given by v_z/k. It therefore decreases as k increases, i.e., as the inhibitor concentration increases (see eqs 2 and 3). To compensate for this decrease, we increased v_z by working with increasing concentrations of enzyme and keeping the inhibitor to enzyme ratio constant (see legend to Figure 2).

In the absence of heparin k increases linearly with α_1PI up to 6 μM, the highest inhibitor concentration compatible with a reliable measurement of the rate constant (Figure 2). Within this limit, the NE + α_1PI association is therefore described by Scheme I and eq 2. The slope of the curve shown in the upper part of Figure 2 is given by $k_{ass}/(1 + [S_o]/K_m)$, the apparent second-order association rate constant. The NE-catalyzed hydrolysis of MeOSuc-Ala_2-Pro-Val-*p*NA was investigated separately using our buffer. We found k_{cat} = 13 ± 1 s^{-1} and K_m = 85 ± 12 μM. Nakajima et al. (1979), who first described this substrate, reported k_{cat} = 17 s^{-1} and K_m = 140 μM in 100 mM Hepes, 500 mM NaCl, 9.8% dimethyl sulfoxide, pH 7.5, 25 °C. A k_{ass} value of (1.05 ± 0.15) × 10^7 M^{-1} s^{-1} was thus calculated using $[S_o]$ = 1.5 mM, K_m = 85 μM, and slope = (5.6 ± 0.8) × 10^5 M^{-1} s^{-1}.

In the presence of heparin k increases hyperbolically with the α_1PI concentration, suggesting that the association of NE–heparin with α_1PI is best described by Scheme II and eq 3 (Figure 2). Nonlinear regression analysis fitted the data to eq 3 and gave the best estimates of k_2 (0.15 ± 0.014 s^{-1}) and $K_i^*(1 + [S_o]/K_m)$ (1.04 ± 0.12 μM). The theoretical curve generated using these estimates closely fits the experimental points, confirming the validity of the model.

Steady-state kinetic analysis of the NE-catalyzed hydrolysis of MeOSuc-Ala_2-Pro-Val-*p*NA in the presence of heparin gave k_{cat} = 5.8 ± 0.6 s^{-1} and K_m = 125 ± 16 μM. K_i^* was thus found to be 80 ± 9 nM.

Influence of Heparin on the Stability of the NE–α_1PI Complex. Extensive dilution of a proteinase–inhibitor complex into substrate followed by recording of substrate hydrolysis is a way to demonstrate the reversibility of the inhibition process and to measure the rate constant of complex dissociation (Boudier & Bieth, 1989; Longstaff & Gaffney, 1991). We used this principle to study the influence of heparin on the stability of the NE–α_1PI complex.

Since highly concentrated complex had to be used for this experiment, we first titrated NE with α_1PI using high protein concentrations in order to know exactly the enzyme–inhibitor equivalence point under these conditions and hence to avoid the presence of a significant excess of free enzyme or inhibitor during the dissociation experiment. Figure 3 shows the linear titration curves obtained upon reacting 5 μM NE with increasing concentrations of α_1PI. In the absence of heparin, the titration curve intercepts the abscissa at a molar ratio of α_1PI to NE of 1.05. In contrast, 1.28 mol of α_1PI is required to block 1 mol of NE when the complex is formed in the presence of heparin. A heparin-induced increase in the apparent enzyme–inhibitor binding stoichiometry has been demonstrated previously with the thrombin–antithrombin system (Danielsson et al., 1986).

Figure 4A shows the progress curves recorded following a 500-fold dilution of 5 μM NE–α_1PI complex (prepared using the above titration data) into the sensitive substrate MeOSuc-Ala_2-Pro-Val-*p*NA ($[S_o]$ = 1.5 mM ≈ 18K_m). The shape of these curves suggests a progressive release of free or heparin-bound NE from the corresponding enzyme–inhibitor complexes. The reaction rates calculated from the progress curves indicate, however, that the release of enzyme is exceedingly

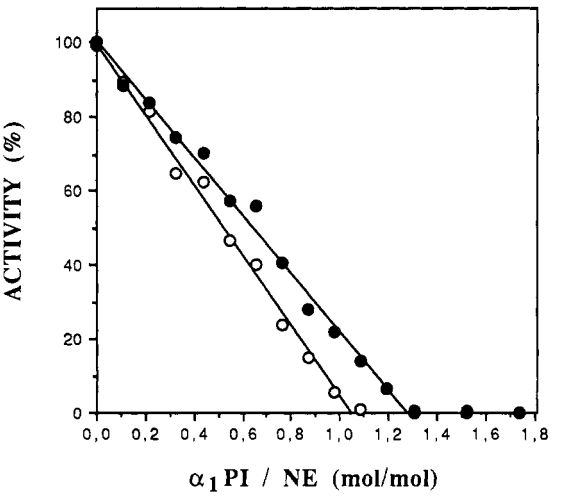


FIGURE 3: Titration of NE by α_1PI in the presence and absence of heparin at pH 7.4 and 25 °C. Buffered mixtures formed of constant amounts of NE (5 μM) and heparin (20 μM), and variable amounts of α_1PI were incubated for 15 min at 25 °C before being diluted 100-fold into buffered solutions of 20 μM heparin and 1.5 mM MeOSuc-Ala_2-Pro-Val-*p*NA. The residual enzymic activities (●) were then measured at 410 nm and 25 °C. A similar titration experiment was done without heparin (○). The titration curves were calculated by linear regression analysis.

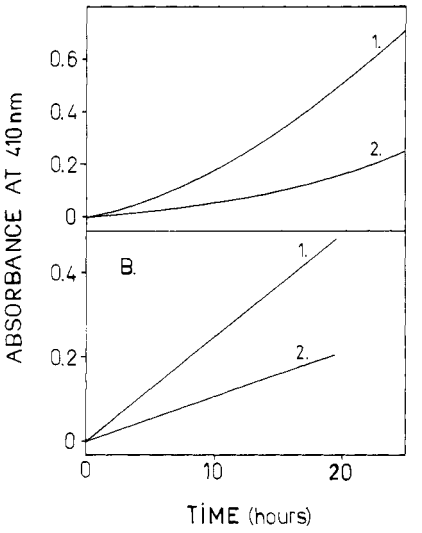


FIGURE 4: (A) Dissociation of the NE–α_1PI complex by dilution into substrate. The complex was prepared in the absence (curve 1) or presence (curve 2) of heparin and diluted 500-fold into substrate ± heparin as described in the Experimental Procedures. The release of product (absorbance at 410 nm) was then recorded as a function of time. (B) Stability of free NE (curve 1) and heparin-bound NE (curve 2) in the presence of substrate.

small. For instance, in the absence of heparin, there is 0.39% and 1.87% free enzyme present at t = 0 and t = 25 h, respectively. These levels are not much different in the presence of heparin: there is 0.55% and 2.53% relaxed enzyme at t = 0 and t = 25 h, respectively. The great stability of the NE–α_1PI complex in the absence of heparin has been demonstrated previously (Beatty et al., 1984; Padrines et al., 1989).

In order to check whether free or heparin-bound NE is stable during the dissociation experiment, we mixed 10 nM enzyme with the less sensitive substrate Suc-Ala_3-*p*NA and recorded the release of product for 20 h. Suc-Ala_3-*p*NA was used at a relative concentration identical to that of MeOSuc-Ala_2-Pro-Val-*p*NA used above, i.e., $[S_o] \approx 18K_m$ so that in both experiments most of the enzyme was saturated with

substrate. The very low k_{cat} of the NE-Suc-Ala$_3$-*p*NA system (k_{cat} = 0.15 s^{-1}, see Lestienne & Bieth (1980)) allowed the enzymatic reaction to be followed without substrate depletion. Figure 4B shows that both free and heparin-bound NE yielded linear absorbance vs time curves indicating that the two enzyme species are remarkably stable. The very small enzyme activity measured during the dissociation experiment is therefore not an artifact due to inactivation of released enzyme.

DISCUSSION

The serine proteinase inhibitors that belong to the serpin superfamily of proteins share the following properties: (i) they form 1:1 denaturation-resistant complexes with proteinases, (ii) they are proteolyzed and undergo a major conformational change during the inhibition reaction, and (iii) when reacted with their target proteinases under second-order conditions ([E$_o$] = [I$_o$] = 1 – 100 nM) they form enzyme–inhibitor complexes that are essentially irreversible from a kinetic viewpoint (Travis & Salvesen, 1983; Beatty et al., 1980; Loebermann et al., 1984; Bruch et al., 1988). On the other hand, the serpins differ from each other with respect to their ability to bind heparin or other sulfated glycosaminoglycans. For example, heparin forms tight complexes with antithrombin, heparin cofactor, protein C inhibitor, proteinase nexin (Huber & Carrell, 1989), and plasminogen activator inhibitor type I (Ehrlich et al., 1991). As a rule, the sulfated polymer enhances the rate of proteinase inhibition by these serpins. The ~2000-fold increase in the rate of association of thrombin with antithrombin best illustrates this effect (Olson & Shore, 1982). Although α_1PI, the archetype of the serpins, does not bind heparin, its biological activity is modulated by the sulfated polymer. Its rate of reaction with neutrophil elastase and cathepsin G is greatly depressed due to a strong binding of heparin with the two neutrophil proteinases (Frommherz et al., 1991). We have previously used second-order conditions ([E$_o$] = [I$_o$] = 100 nM) to study the effect of heparin on the NE + α_1PI association. This method showed that the maximal rate-depressing effect of high and low molecular weight heparin was related to the degree of saturation of NE by the polymer. However, this enzymatic procedure did not allow us to gain insight into the mechanism by which heparin decreases the rate constant k_{ass} for the NE + α_1PI association (Frommherz et al., 1991). We therefore investigated the NE and NE–heparin + α_1PI association under pseudo-first-order conditions ([I$_o$] ≥ 10 [E$_o$]) in the presence of substrate and variable α_1PI concentrations (Tian & Tsou, 1982; Morrisson & Walsh, 1988) in order to see whether the enzyme-inhibitor binding is a multistep reaction and to detect the step that is affected by heparin.

Reaction of NE or NE–heparin with α_1PI in the presence of substrate yielded a pre-steady-state release of product followed by a steady-state breakdown of substrate (Figure 1). The latter suggests that NE and α_1PI form a reversible enzyme–inhibitor complex (Morrisson & Walsh, 1988). We hypothesized, however, that this steady-state velocity was entirely due to adsorption of enzyme to the spectrophotometer cells, a well-known phenomenon (Gauthier et al., 1982; Straus et al., 1985). Accordingly, we subtracted it from the progress curves to easy the data analyses. We feel that this correction is fully appropriate since the α_1PI–NE and the α_1PI–NE–heparin complex do not undergo significant dilution- and substrate-induced dissociation over a period of 25 h (Figure 4A), indicating that the enzyme–inhibitor binding is essentially irreversible. All data were therefore analyzed using equations for irreversible enzyme–inhibitor association (Tian & Tsou, 1982).

In the absence of heparin, k, the pseudo-first-order rate constant of NE inhibition, increases linearly with the α_1PI concentration indicating that up to 6 μM, the highest inhibitor concentration that could be used, the enzyme–inhibitor binding conforms to a simple bimolecular reaction (Scheme I). The second-order rate constant for the inhibition of NE by α_1PI (k_{ass} = 1 × 10^7 M^{-1} s^{-1}) is, however, at least 2 orders of magnitude lower than the maximum rate constant for a bimolecular diffusion-controlled reaction (Alberty & Hammes, 1958). It may therefore be assumed that the NE + α_1PI association involves an intermediate EI* (Scheme II) even if the latter is not seen kinetically. As a consequence, K_i^* (1 + [S$_o$]/K_m) must be higher than 6 μM, i.e., K_i^* must be greater than 0.4 μM. On the other hand, comparison of eqs 2 and 3 indicates that if [I$_o$] is lower than K_i^* (1 + [S$_o$]/K_m), the second-order association rate constant k_{ass} will be equal to k_2/K_i^*. Hence, the isomerization of EI* into EI must occur with a first-order rate constant k_2 greater than 4 s^{-1} ($t_{1/2}$ > 173 ms).

This is the first attempt to demonstrate a two-step mechanism for the reaction of NE with α_1PI. In a previous study we have used the proflavin-displacement method and stopped-flow spectroscopy to detect an intermediate between chymotrypsin and α_1PI: K_i^* and k_2 were found to be 0.19 mM and 252 s^{-1}, respectively (Bruch & Bieth, 1989). On the other hand, Olson and Shore (1982) used the *p*-aminobenzamidine displacement method and stopped-flow fluorimetry to demonstrate a two-step mechanism for the inhibition of thrombin by antithrombin: K_i^* and k_2 were 1.4 mM and 10.4 s^{-1}, respectively. It was possible to measure such very high K_i^* values because the ligands used to assess the enzyme–inhibitor binding were enzyme inhibitors and not an enzyme substrate as in the present study. The design of a fluorescent NE inhibitor should allow us to measure precisely K_i^* and k_2. A two-step reaction mechanism has also been demonstrated for the interaction of plasmin and chymotrypsin with α_2-antiplasmin: for plasmin, K_i^* and k_2 were found to be 8 nM and 6 × 10^{-3} s^{-1}, respectively, whereas for chymotrypsin, these parameters were 6.6 nM and 9 × 10^{-3} s^{-1}, respectively (Longstaff & Gaffney, 1991). The three above-mentioned serpins, therefore, share a common reaction pathway characterized by an initial fast-equilibrating EI* complex that isomerizes into a more stable EI complex. The kinetic behavior of α_1PI and antithrombin is, however, considerably different from that of α_2-antiplasmin: the two former serpins form very loose EI* complexes with proteinases, but these complexes decay very rapidly into EI, while α_2-antiplasmin forms tight EI* complexes that isomerize very slowly into EI. Serpins may therefore use variable combinations of kinetic parameters (K_i^*, k_2) to achieve similar overall efficiencies (k_2/K_i^*). This behavior might have physiological bearing.

Heparin-bound NE reacts with α_1PI through a two-step mechanism (Scheme II), K_i^* and k_2 being equal to 80 nM and 0.15 s^{-1}, respectively. Thus, K_i^* is at least 5-fold lower in the presence of heparin than in its absence while the sulfated polymer decreases k_2 by a factor of at least 27. Through its binding to NE, heparin therefore favors the association of NE with α_1PI by lowering K_i^* but impairs this association by decreasing k_2. Equation 3 predicts that the overall effect of heparin will be a decrease in the rate of inhibition of NE by α_1PI. The present data therefore provide a rationale for the previously-observed deleterious effect of heparin on the apparent second-order rate constant k_{ass} for the NE + α_1PI binding (Frommherz et al., 1991). It is noteworthy that heparin also decreases K_i^* and k_2 for the reaction of thrombin

with antithrombin, a heparin-binding serpin (Olson & Shore, 1982). However, in that case, K_i* decreases 2800-fold whereas k_2 decreases by a factor of only 2. As a result, significant rate acceleration occurs (see eq 3).

The heparin-promoted decrease in the NE + α_1PI association rate contrasts with the polymer-induced acceleration of the inhibition of a number of serine proteinases by other serpins (Huber & Carrell, 1989; Ehrlich, et al., 1991). This divergent effect is probably related to the ability of these serpins (i.e., antithrombin, heparin cofactor, protein C inhibitor, protease nexin, and plasminogen activator inhibitor I) to tightly bind heparin, a property that is not shared by α_1PI (Frommherz et al., 1991). In this context it is worth notice that the proteolysis-modulating effect of heparin is not unique to serpins. For example, the rate of inhibition of NE by the mucus proteinase inhibitor, a nonserpin protein, is also increased by heparin as a result of a tight heparin–inhibitor binding (Faller et al., 1992). This effect thus resembles that observed on the above heparin-binding serpins. In contrast, heparin decreases the rate of inhibition of NE by eglin c, another nonserpin inhibitor, which differs from the mucus proteinase inhibitor by its inability to bind the sulfated glycosaminoglycan (Frommherz et al., 1991). This effect therefore resembles that observed on the NE + α_1PI association. Thus, some serpins and some nonserpin inhibitors apparently behave in a similar way with respect to their sensitivity to heparin: their reaction rate with proteinases will either be enhanced or depressed depending upon whether they are able or not to bind heparin. This common behavior somewhat favors the recent belief that serpins and nonserpin inhibitors share a common mechanism of action (Shieh et al., 1989; Longstaff & Gaffney, 1991; Matheson et al., 1991; Mast et al., 1991).

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