Inhibition of neutrophil elastase by recombinant human proteinase inhibitor 9

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Abstract

Proteinase inhibitor PI9 (PI9) is an intracellular 42-kDa member of the ovalbumin family of serpins that is found primarily in placenta, lung and lymphocytes. PI9 has been shown to be a fast-acting inhibitor of granzyme B in vitro, presumably through the utilization of Glu340 as the P1 inhibitory residue in its reactive site loop. In this report, we describe the inhibition of human neutrophil elastase by recombinant human PI9. Inhibition occurred with an overall $K_i$ of 221 pM and a second-order association rate constant of $1.5 \times 10^5$ M$^{-1}$ s$^{-1}$, indicating that PI9 is a potent inhibitor of this serine proteinase in vitro. In addition, incubation of recombinant PI9 with native neutrophil elastase resulted in the formation of an SDS-resistant 62-kDa complex. Amino-terminal sequence analyses provided evidence that inhibition of elastase occurred through the use of Cys342 as the reactive P1 amino acid residue in the PI9 reactive site loop. Thus, PI9 joins its close relatives PI6 and PI8 as having the ability to utilize multiple reactive site loop residues as the inhibitory P1 residue to expand its inhibitory spectrum. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Human neutrophils provide host defense against bacterial infection and are important in the elimination of pathogenic organisms in the lower respiratory tract [1]. Neutrophil recruitment and accumulation, usually identified by an increase in the number of neutrophils in bronchoalveolar lavage, is associated with such lung diseases as pneumonia, cystic fibrosis, adult respiratory distress syndrome, pulmonary fibrosis, chronic bronchitis and asthma, and can result in tissue damage from the release of neutrophil proteinases, particularly elastase [1–7]. Human neutrophil elastase (HNE) is a 218-amino acid serine proteinase located in high concentration within the secretory granules of neutrophils, accounting for approximately 0.5% of the total cellular protein [8,9]. HNE demonstrates optimal proteolytic activity at neutral pH towards a wide variety of substrates, including coagulation factors, immunoglobulins, fibrin...
nogen, fibronectin, proenzymes, receptors, structural proteins, and selected proteinase inhibitors (reviewed in [10]).

Due to the broad substrate specificity and potency of HNE, serine proteinase inhibitors, or serpins, must be present to regulate its proteolytic activity. Serpins inactivate their target proteinases by mimicking the substrate sequence recognized by the active site of the proteinase to facilitate an initial loose interaction, followed by the formation of a tight 1:1 stoichiometric inhibitory complex which is, in most cases, resistant to denaturation [11]. During the interaction, cleavage occurs between the \( P_1 \) and \( P'_1 \) amino acid residues in the serpin reactive site loop (RSL), where the \( P_1 \) residue is the major determinant of the inhibitory specificity of the serpin, but adjacent residues also contribute to the rate and stability of the inhibition [12,13]. If the balance of proteinase activity and proteinase inhibition is perturbed, the resulting unregulated proteinase activity may be a causative factor in many pathological events, such as those listed above. Since the turnover of approximately \( 10^{11} \) neutrophils, containing approximately 50 mg of HNE, occurs daily, without clinical evidence of uncontrolled tissue degradation [9,14], the proteolytic activity of HNE must be tightly regulated by one or more serpins, in order to maintain tissue integrity. The three major inhibitors of elastase in vivo are \( \alpha_1 \)-proteinase inhibitor (\( \alpha_1 \)-PI), elastase inhibitor, and the low \( M_r \) inhibitors, including antileukoproteinase [15], secretory leukocyte proteinase inhibitor [16] and elafin [17]. \( \alpha_1 \)-PI, a major serine proteinase inhibitor in plasma, is constitutively synthesized in liver, as well as cells of epithelial origin [18,19]. Individuals with deficiencies of \( \alpha_1 \)-PI are usually at risk for the development of pulmonary emphysema from elastase-mediated degradation of lung extracellular matrix [10]. Human elastase inhibitor (EI), a 42-kDa member of the ovalbumin family of serpins found in monocytes, macrophages and neutrophils, is also a potent inhibitor of elastase [20,21]. EI utilizes Cys\(^{344}\) as the \( P_1 \) residue in its RSL to facilitate its interaction with HNE and the formation of a 66-kDa SDS-stable elastase-elastase inhibitor complex [22]. Finally, acid-stable inhibitors of elastase (\( M_r \approx 6-12 \) kDa) are synthesized by various secretory cells and are found in salivary gland secretions, cervical mucus, seminal plasma and bronchial mucus [15,16,23].

Proteinase inhibitor 9 (PI9) is another 42-kDa member of the ovalbumin family of serpins that shares 49% sequence identity with EI and has recently been shown to be a strong inhibitor of the amidolytic activity of granzyme B, an apoptosis-inducing serine proteinase contained within the granules of cytotoxic lymphocytes, as well as the bacterial endoproteinase, subtilisin [24–26]. PI9 is expressed primarily in placenta and lung, but PI9 mRNA has also been found in spleen, thymus, bone marrow, and lymphocytes, primarily cytotoxic lymphocytes and B-cells [24,25]. In this report, we describe the inhibition kinetics that characterize the inhibition of HNE by PI9, an interaction that occurs through the utilization of an alternative inhibitory residue in its reactive site loop.

2. Materials and methods

2.1. Materials

Porcine trypsin was obtained from Novo Nordisk (Bagsvaerd, Denmark). HEPES buffer, dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), and \( p \)-nitrophenyl \( p \)-guanidinobenzoate (\( p \)-NPGB) were purchased from Sigma (St. Louis, MO). HNE, MeO-Suc-Ala-Ala-Pro-Val-\( p \)-nitroanilide (MeOSuc-AAPV-\( p \)NA), and \( \alpha_1 \)-PI were purchased from Calbiochem-Novabiochem (La Jolla, CA). H-\( D \)-Ile-Pro-Arg-\( p \)-nitroanilide (S-2288) was purchased from Kabi Pharmacia Hepar, (Franklin, OH). Recombinant human PI9 was purified to homogeneity as described [24], and its mass concentration determined by amino acid analyses. Recombinant human neutrophil elastase inhibitor, expressed in insect cells, was generously provided by Dr. Eileen Remold-O’Donnell (The Center for Blood Research, Boston, MA). UltraFit 3.0 software for regression analysis was obtained from BioSoft (Ferguson, MO). IODO-GEN was purchased from Pierce (Rockford, IL), and \( Na^{125}I \) was from DuPont/NEN (Boston, MA). Rabbit anti-HNE IgG were purchased from New England Biolabs (Beverly, MA). 3,4-Dichloroisocoumarin were obtained from Boehringer Mannheim
Indianapolis, IN). Affi-Gel 10 was purchased from Bio-Rad (Hercules, CA). Human neutrophils were kindly provided by Dr. Larry Sklar (University of New Mexico, Albuquerque, NM).

2.2. General kinetic methods

The catalytically active concentration of porcine trypsin was determined by active-site titration with p-NPGB as described previously [27,28]. Subsequent titrations were performed by pre-incubating enzyme and inhibitor for 30 min at 37°C in 20 mM HEPES (pH 7.5)/0.15 M NaCl/0.01% BSA in wells of microtiter plates previously blocked with titration buffer containing 0.1% BSA, followed by the addition of substrate and measurement of the rate of substrate hydrolysis at 405 nm using a UVmax kinetic microtiter plate reader (Molecular Dynamics, Menlo Park, CA). Active-site titrated trypsin was used to titrate the reactive concentration of $K_{1-PI}$ using S-2288 as the substrate. Titrated $K_{1-PI}$ was then used to determine the catalytically active concentration of HNE using MeOSuc-AAPV-pNA as the substrate.

The catalytic constants $K_m$ and $k_{cat}$ for HNE with the substrate MeOSuc-AAPV-pNA were determined at 25°C in a solution of 20 mM HEPES (pH 7.5)/0.15 M NaCl/0.01% BSA that contained 2% DMSO and 1% DMF to ensure substrate solubility during the reaction. Substrate hydrolysis was measured at 405 nm and the hydrolysis data were fitted to the Michaelis–Menten equation using UltraFit software to determine $K_m$ and $k_{cat}$.

2.3. Slow binding inhibition kinetics

Progress curves were produced from data obtained under pseudo first-order conditions by adding HNE (1 nM final concentration) to a solution containing MeOSuc-AAPV-pNA (1 mM final concentration) and PI9 at various concentrations. The concentrations of PI9 used to determine the kinetic constants were 4, 8, 12, 16, and 20 nM. Substrate hydrolysis was measured at 405 nm in UVT-acrylic cuvettes previously blocked with 20 mM HEPES (pH 7.5)/0.15 M NaCl/0.1% BSA in a Beckman DU-65 spectrophotometer equipped with a six-cell cuvette holder, allowing multiple reactions to be monitored simultaneously. Reactions were started within 30 s in 0.5 ml of the buffer used to determine the catalytic constants. Spontaneous substrate hydrolysis was measured in separate experiments and was negligible. The reactions were allowed to proceed at 25°C until steady-state velocity was attained. Data obtained from each reaction were fitted to the integrated rate equation for slow-binding inhibition [29]

$$A = v_o + (v_o - v_i) \frac{1-e^{-kt}}{k} + A_o$$  \hspace{0.5cm} (1)

by non-linear regression using UltraFit software to obtain values for the steady-state velocity ($v_s$), the initial velocity ($v_o$), and the apparent first-order rate constant ($k$) for the establishment of steady-state equilibrium of the proteinase–inhibitor complex. The data obtained from non-linear regression were used in graphical transformations to obtain values for the inhibition and rate constants for the interaction of PI9 with HNE [29–34].

2.4. Detection of PI9-elastase SDS-stable complexes

HNE was radiolabeled with Na$^{125}$I using the IODO-GEN transfer method [35] to an average specific radioactivity of 3.7 μCi/μg. $^{125}$I-HNE (0.5 pmol) was incubated with PI9 (0.7 nmol) for 30 min at 37°C, after which the reaction mixture was boiled in the presence of SDS and subjected to SDSPAGE under reducing conditions [36]. Following electrophoresis, proteins were transferred to a PVDF membrane by electroblotting for 45 min at 500 mA in 10 mM CAPS (pH 11) containing 10% methanol. The membrane was washed in TBS, dried, and subjected to autoradiography.

2.5. Amino acid sequencing

PI9 (500 pmol) and HNE (100 pmol) were incubated in PBS at 37°C for 30 min, followed by the addition of 3,4-dichloroisocoumarin or PMSF to a final concentration of 1 mM to neutralize uninhibited elastase. The reaction mixture was subsequently de-salted and washed by centrifugation on a ProSpin sample preparation cartridge (Perkin–Elmer/ABI; Foster City, CA), and sequenced in a Beckman LF3000 protein sequencer.
3. Results

3.1. Determination of kinetic constants

The $K_m$ and $k_{cat}$ for HNE with MeOSuc-AAPV-pNA were determined to be $233 \pm 31$ $\mu M$ and $28 \pm 1$ s$^{-1}$, respectively. The kinetic characterization of the inhibition of HNE by PI9 was performed using PI9 concentrations ranging from 4 to 20 times the molar concentration of HNE. A family of progress curves that are representative of the reaction between PI9 and HNE as a function of PI9 concentration are shown in Fig. 1. Data obtained from the progress curves were fitted to Eq. 1 by non-linear regression to obtain values for $v_o$, $v_s$, and $k'$. The results indicated that $v_o$ was inversely proportional to the inhibitor concentration at the selected PI9 concentrations. The observation was confirmed by a plot of $v_{max}/v_o$ as a function of PI9 concentration, which had a positive slope (data not shown). In addition, $k'$ appeared to increase as the PI9 concentration increased in the reaction mixtures. There are three mechanisms that can be used to describe the slow onset of inhibition by a tight-binding inhibitor [28]:

$$
P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PI \quad (\text{mechanism A})$$

$$
P \overset{k_{-1}}{\underset{k_{1} \text{ (slow)}}{\rightleftharpoons}} PI \overset{k_{2}}{\underset{k_{-2}}{\rightleftharpoons}} PI' \quad (\text{mechanism B})$$

$$
P \overset{k_{1} \text{ (slow)}}{\underset{k_{-1}}{\rightleftharpoons}} P' \overset{k_{2}}{\underset{k_{-2}}{\rightleftharpoons}} PI' \quad (\text{mechanism C})$$

Mechanism A describes the single-step formation of a complex of proteinase and inhibitor (PI). Mechanism B is distinguished by the formation of the initial PI complex, followed by the slow isomerization to a tight PI' complex. In mechanism C, the proteinase undergoes a slow isomerization to P' and then rapidly forms a tight PI' complex. Of the three mechanisms, only mechanism B describes an inhibitory mechanism in which the initial velocity is inversely proportional to the inhibitor concentration. Furthermore, mechanism B also suggests that $k'$ would increase as the inhibitor concentration increases. Because the observations that were made fit these criteria, the data obtained were treated in ways that would facilitate the determination of kinetic constants according to mechanism B.

The value for the equilibrium inhibition constant for the initial loose interaction was calculated by fitting the data obtained for the uninhibited control reaction velocity, $v_{max}$, as well as the values for $v_o$ and $k'$ to the equation [27,29]

$$
\frac{v_{max}}{v_o} = \frac{K_m[I]}{[S[K_i + K_m + 1]}
$$

which describes a linear relationship. Linear regression analysis yielded a value for $K_i$ of $8.1 \pm 0.8$ nM ($n=4$). To obtain the value for the overall equilibrium inhibition constant, $K'$, the equation

$$
\frac{v_{max}}{v_s} = \frac{K_m[I]}{[S[K_i' + K_m + 1]}
$$

was used [27,29]. Eq. 3 describes a linear relationship between $v_{max}/v_s$ and [PI9], with the value of $v_s$ being inversely proportional to the inhibitor concentration. The value for $K'$ was determined by linear regression to be $221 \pm 9$ pM ($n=4$). The second-order association rate constant was determined by calculating and
plotting values of \( \log([P_r]_3[P_t]) \) versus time for the initial 15 min of the reaction. The data obtained were fitted to the equation [30]

\[
\log([P_a] - [P_i]) = \log([P_a]) - \frac{0.43[I]k_{assoc}t}{1 + \frac{[S]}{K_m}}
\]

from which \( k_{assoc} \) was determined to be \( 1.5 \pm 0.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \) \( (n=4) \). The value for the rate constant for the reverse isomerization step, \( k_{-2} \), was calculated directly from the slope of a plot of \( k' \) versus \( v/v_{\max} \) [31] to be \( 1.6 \pm 0.2 \times 10^{-5} \text{ s}^{-1} \) \( (n=4) \), which indicates a half-life of 12 h for the PI9/HNE complex, according to the relationship \( t_{1/2} = 0.693/k_{-2} \). The value of the rate constant for the formation of the tight complex, \( k_2 \), was determined by fitting the data obtained for \( k' \) at selected PI9 concentrations to the equation

\[
k' = k_{-2} + k_2 \frac{[I]}{K_i} \frac{1}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_i}}
\]

which predicts a hyperbolic relationship between \( k' \) and [PI9]. Non-linear regression analysis using a \( K_i \) value of 8.1 nM yielded a value for \( k_2 \) of \( 6.6 \pm 0.2 \times 10^{-4} \text{ s}^{-1} \) \( (n=4) \). To verify that the inhibition of HNE by PI9 occurs according to mechanism B, a plot of \( 1/(k' - k_{-2}) \) versus \( 1/[\text{PI9}] \) was generated, as shown in Fig. 2. This plot is linear for both mechanisms A and B, but the two mechanisms can be distinguished by calculating the \( y \)-intercept of the plot, which will cross the positive \( y \)-axis for inhibition according to mechanism B. The plot shown in Fig. 2 was linear and crossed the positive \( y \)-axis at a point approximately equal to \( 1/k_2 \), which was calculated to be \( 4 \times 10^{-4} \text{ s}^{-1} \), and is reasonably close to the value calculated according to Eq. 5.

3.2. Detection of PI9-elastase SDS-stable complexes

PI9 was incubated with \(^{125}\text{I}\)-HNE at an approximate molar ratio of 1400:1, the reaction was heat-denatured with SDS, and electrophorized under reducing conditions, as shown in Fig. 3. The addition of PI9 to \(^{125}\text{I}\)-HNE resulted in the formation of an SDS-resistant complex that migrated with an apparent molecular mass of 62 kDa, along with an addi-
3.3. Determination of the PI9 RSL cleavage site

A 500-pmol amount of PI9 was incubated with 100 pmol of HNE for 30 min at 37°C, followed by the addition of 3,4-dichloroisocoumarin or PMSF to 1 mM. N-terminal amino acid sequence analysis of this reaction mixture yielded a sequence of Met-Glu-Ser-Gly-Pro, corresponding to amino acids 343–347 located within the reactive site loop of PI9, thus indicating that Cys\(^{342}\) is acting as the reactive center residue for the inhibition of HNE (Fig. 4). In one experiment where an active site inhibitor of elastase was not included in the reaction mixture following incubation of PI9 with HNE, a second minor sequence, Asn-Lys-Ala-Gly-Thr-Gln, corresponding to amino acids 78–83 of PI9, was also observed. The presence of this additional sequence suggests that PI9 undergoes limited proteolysis by HNE at an exposed site distinct from the reactive site loop during preparation of the sample for sequence analysis.

4. Discussion

In the present study, we describe the kinetic mechanism for the inhibition of HNE by recombinant PI9, which occurs through the use of an amino acid residue in the RSL distinct from the predicted P\(1\) Glu\(^{340}\) residue. PI9 inhibited HNE via a two-step mechanism with a \(K_i\) for the formation of the initial loose proteinase-inhibitor complex of 8.1 nM, which is similar to the \(K_i\) values of 8 and 6.6 nM for the inhibition of plasmin and chymotrypsin by \(\alpha_2\)-antiplasmin [31]. The overall equilibrium inhibition constant for this interaction was 221 pM, which is comparable to the \(K_i\) for the inhibition of chymotrypsin by \(\alpha_2\)-antiplasmin [31], as well as the \(K_i\) value reported for the inhibition of human thrombin by PI8 [37]. The second-order association rate constant for the inhibition of HNE by PI9 was calculated to be \(1.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\), which is comparable to the association rate constants reported for the inhibition of human thrombin by PI8 [37] and granzyme B by CrmA [38], but exceeded the rates reported for the inhibition of plasma kallikrein by Cl-inhibitor [39] and coagulation factor Xa by PI8 [37].

Amino acid sequence analysis provided suggestive evidence that the residue used as the reactive center in the reaction between PI9 and HNE was Cys\(^{342}\), and not Glu\(^{340}\), as predicted through primary sequence alignments and as implied in the inhibition of granzyme B, a serine proteinase that cleaves after acidic residues [24,25]. A P\(1\) Cys residue is also used to facilitate the inhibition of HNE by EI, another ovalbumin serpin [22]. In addition, both PI9 and EI utilize the reactive center P\(1\)–P\(1\)’ sequence of Cys-Met for the inhibition of HNE. PI9, however, does not share the potent inhibition of chymotrypsin that has been demonstrated with horse EI [40]. This apparent flexibility for the selection of P\(1\) residues in the reactive site loop may be a feature unique to PI6, PI8, PI9, and \(\alpha_2\)-antiplasmin [41]. PI6 uses Arg\(^{341}\) as its P\(1\) residue for the inhibition of trypsin and thrombin, but uses Met\(^{340}\) for the inhibition of chymotrypsin [42]. In this connection, PI8 utilizes Arg\(^{339}\) as its reactive center residue for the inhibition of human thrombin, in contrast to Ser\(^{341}\), which is used to inhibit chymotrypsin [43]. To our knowledge, these three serpins are the only ovalbumin-type serpins to

![PI9 REACTIVE SITE LOOP SEQUENCE](BBAMCR_14521_3-9-99)

Fig. 4. Multiple reactive centers in the reactive site loop of PI9. Cleavage of the P\(1\)–P\(1\)’ scissile bond occurs in the positions indicated by the arrows. Amino acid residues in PI9 which act as reactive centers (P\(1\) amino acids) are circled. The putative P\(1\) residue used in the inhibition of granzyme B is Glu\(^{340}\), while the putative P\(1\) residue used for the inhibition of HNE is Cys\(^{342}\).
demonstrate this flexibility, suggesting that they may represent a unique sub-family of ovalbumin serpins evolutionarily similar to α2-antiplasmin that demonstrate a broadened inhibitory specificity through the use of multiple reactive centers in their reactive site loop.

A second minor cleavage in PI9 occurred at a site distinct from the RSL following incubation of PI9 with HNE. This second sequence was observed in our initial RSL cleavage experiment, where an active-site HNE inhibitor was not included after the 30-min incubation period. Although not known with certainty, this minor cleavage is probably unrelated to the inhibitory mechanism, and most likely represents an artifact related to the preparation of the sample for sequence analysis as a result of cleavage of either free PI9 or the PI9–HNE complex by free HNE. Of potential relevance, cleavage at this site would decrease the apparent molecular mass of the PI9–HNE complex by approximately 8 kDa, which may explain the minor 54 kDa band seen in SDS-PAGE/autoradiography following incubation of [125I-HNE] and PI9 (Fig. 3, lane 2) without the subsequent addition of an HNE inhibitor.

The physiological significance of elastase inhibition by PI9 is unclear. SDS-PAGE and immunoblot analyses of human neutrophil cytosol preparations using affinity-purified rabbit anti-recombinant human PI9 IgG indicated the presence of a 42-kDa immunoreactive band. Subsequent studies revealed that the affinity-purified anti-PI9 IgG preparation cross-reacted weakly with recombinant human elastase inhibitor (W. Kisiel, unpublished data), a 42-kDa serpin present in neutrophils that shares 49% sequence identity with PI9, suggesting that the 42-kDa band observed in immunoblots may represent a mixture of EI and PI9. PI9 has been reported to be synthesized by IL-2-stimulated peripheral blood mononuclear cells [25], and it is possible that PI9, in concert with EI, may play a role in the regulation of deleterious intracellular proteolysis caused by aberrantly released HNE in these cells. In addition, more recent studies in our laboratory revealed the presence of PI9 antigen and transcript in human umbilical vein endothelial cells (HUVECs). Of potential importance, PI9 transcript levels were markedly upregulated (~10-fold) in these cells following their stimulation with the inflammatory mediator, phorbol myristate acetate (W. Kisiel, unpublished data). PI9, like PI8, has two mRNA transcripts and shares a great deal of primary sequence identity with the internal hydrophobic signal sequences found in ovalbumin and PAI-2 [24,44]. Thus, it is conceivable that, in an inflammatory state, some PI9 may be secreted by the endothelial cell into the vascular basement membrane to protect these cells from elastase-mediated degradation of the matrix during neutrophil or tumor cell extravasation. In this regard, the ability of PI9 to be secreted by HUVECs is currently under investigation in this laboratory, and these studies may yield insight into the possible regulation of HNE proteolysis by PI9 extracellularly.

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