Discriminating between the Activities of Human Neutrophil Elastase and Proteinase 3 Using Serpin-derived Fluorogenic Substrates* 

Human neutrophil elastase (HNE) has long been linked to the pathology of a variety of inflammatory diseases and therefore is a potential target for therapeutic intervention. At least two other serine proteases, proteinase 3 (Pr3) and cathepsin G, are stored within the same neutrophil primary granules as HNE and are released from the cell at the same time at inflammatory sites. HNE and Pr3 are structurally and functionally very similar, and no substrate is currently available that is preferentially cleaved by Pr3 rather than HNE. Discrimination between these two proteases is the first step in elucidating their relative contributions to the development and spread of inflammatory diseases. Therefore, we have prepared new fluorescent peptidyl substrates derived from natural target proteins of the serpin family. This was done because serpins are rapidly cleaved within their reactive site loop whether they act as protease substrates or inhibitors. The hydrolysis of peptide substrates reflects the specificity of the parent serpin including those from α-1-protease inhibitor and monocoyte neutrophil elastase inhibitor, two potent inhibitors of elastase and Pr3. More specific substrates for these proteases were derived from the reactive site loop of plasminogen activator inhibitor 1, proteinase inhibitors 6 and 9, and from the related viral cytokine response modifier A (CrmA). This improved specificity was obtained by using a cysteinyl residue at P1 for Pr3 and an Ile residue for HNE and because of occupation of protease S’ subsites. These substrates enabled us to quantify nanomolar concentrations of HNE and Pr3 that were free in solution or bound at the neutrophil surface. As membrane-bound proteases resist inhibition by endogenous inhibitors, measuring their activity at the surface of neutrophil membranes might be a great help in understanding their role during inflammation.

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¶ The abbreviations used are: HNE, human neutrophil elastase; Pr3, proteinase 3; PMN, polymorphonuclear leukocyte; RSL, reactive site loop; α1-PI, α1-protease inhibitor; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; MNEI, monocoyte neutrophil elastase inhibitor; Abz, ortho-aminobenzamide; AFC, 7-amino-4-trifluoromethyl-coumarin; Boc-AANva-SBzl, t-butyloxycarbonyl-Ala-Ala-Norvaline-thiobenzylester; CMK, chloromethyl ketone; CrmA, cytokine response modifier A; EDDnp, N-2,4-dinitrophenylethylendiamine; HPLC, high performance liquid chromatography; PAI-1, plasminogen activator inhibitor 1; MeO-Suc-, methoxysuccinyl.
Selective Measurement of Neutrophil Elastase and Proteinase 3

Based on the exposed RSL of lung serpins that are putative target for neutrophil neutral proteases, and we have used them as substrates for HNE and Pr3. This study was done to identify substrates for specifically measuring HNE and Pr3 activities at the surface of neutrophils where bound proteases resist inhibition by local inhibitors, thus favoring the development of inflammation.

The nomenclature used for the individual amino acid residues (e.g. P1, P2, and so on) of a substrate and corresponding residues of the enzyme subunits (e.g. S1, S2, and so on) is that of Schechter and Berger (17).

EXPERIMENTAL PROCEDURES

Materials—Human neutrophil elastase (EC 3.4.21.37), proteinase 3 (EC 3.4.21.78), α1-antichymotrypsin, and α1-PI were obtained from Athens Research and Technology (Athens, Georgia). Human cathepsin G (EC 3.4.21.20) was from ICN Pharmaceuticals. MoE-Suc-AAPV-pNA and Igepal CA-630 were from Sigma. MoE-Suc-AAPV-APC, Z-GLF- CMK, and MoE-Suc-AAPA-CMK were from Enzyme System Products (Livermore, CA). N,N-Dimethylformamide and acetonitrile were from Merck; C18 cartridges for reverse-phase chromatography were from Touzart et Matignon (Paris, France) and Interchim (Montluçon, France). Polymorphrep™ and Lymphoprep™ were from Nycomed Pharma (Oslo, Norway). Monoclonal mouse IgG1-FITC and CD63-FITC antibodies were from Beckman Coulter France (Roissy, France). All other reagents were of analytical grade.

Isolation of Blood PMN—Human neutrophils were purified from 8-ml samples of peripheral blood collected from volunteers into tubes containing EDTA. Aliquots (4 ml) of blood were layered over 4-ml Polymorphrep and centrifuged at 2,000 × g for 20 min at 20 °C. The neutrophil-enriched bands (75% PMNs and 25% lymphocytes) were harvested, diluted with an equal volume of half-strength PBS, and brought to 6 ml with PBS. These samples were layered over 3-ml Lymphoprep and centrifuged at 1,000 × g for 20 min at 20 °C. All of the resulting fractions with the exception of the neutrophil band at the bottom of the gradient were carefully removed. Any erythrocytes remaining in the neutrophil fraction were removed by hypotonic lysis. Purified PMNs were pelleted at 500 × g for 5 min, and contaminating erythrocyte membranes were removed. The PMNs were suspended in PBS at ~3 × 10^6 cells/ml and used immediately or kept at 4 °C under gentle stirring.

Flow cytometry was performed on a Coulter Epics Elite ESP flow cytometer equipped with a 488-nm argon laser. The forward and side scatters of each sample were measured for at least 10,000 events. Samples contained >96% neutrophils, no monocytes, and <1% lymphocytes. CD63 surface expression was measured incubating 5 × 10^5 cells/ml with PBS at 37 °C with 5 μM Igepal CA-630 and diluted to 0.5 mM with 50 mM Hepes buffer, pH 7.4.

Design and Synthesis of Quenched Fluorescent Substrates—All quenched fluorescent substrates were prepared by solid-phase synthesis with the Fmoc (N-(9-fluorenyl)methoxycarbonyl) methodology using a multiple automated peptide synthesizer (PSSM-8, Shimadzu) (15, 18, 19). Glutamine was the C-terminal residue in all of the peptides because of a requirement of the synthesis strategy (19). Substrate purity was checked by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry (ToSpec-E, Matrixscience) and by reverse-phase chromatography on a C18 column eluted at 2 ml/min with a 10-min linear gradient of acetonitrile (0–60%) in 0.05% trifluoroacetic acid as solvent.

Stock substrate solutions (2–5 mM) were prepared in 30% (v/v) N,N-dimethylformamide and diluted to 0.5 mM with 50 mM Hepes buffer, pH 7.4.

Enzyme Assays—Experimental conditions were optimized for each enzyme, taking into account the great propensity of dilute solutions to stick to plastic and glass surfaces. Assays were carried out at 37 °C in 50 mM Hepes buffer, pH 7.4, 0.75 mM CaCl2, 0.05% Igepal CA-630 (v/v) for HNE and Pr3 and in 50 mM Hepes buffer, pH 7.4, 0.1 mM CaCl2, 0.01% Igepal CA-630 (v/v) for cathepsin G. The hydrolysis of Abz-peptidyl-EDDnp substrates was followed by measuring the fluorescence at λex = 320 nm and λem = 420 nm in a Hitachi F-2000 spectrophotometer. The system was standardized using Abz-FR-OH prepared from the total tryptic hydrolysis of an Abz-FR-pNA solution; its concentration was determined from the absorbance at 410 nm assuming ε410 nm = 8,800 M^-1 cm^-1 for p-nitroaniline. The concentrations of Abz-peptidyl-EDDnp substrate were determined by measuring the absorbance at 365 nm using ε365 nm = 17,500 M^-1 cm^-1 for EDDnp.

Specificity constants (Kcat/Km) were determined using first-order conditions using a substrate concentration far below the Km (1–8 μM depending on the enzyme). Final enzyme concentrations were 10–50 mM for HNE and Pr3. Under these conditions, the Michaelis-Menten equation is reduced to v = kcat · [S] / Km. Integrating this equation over time gives ln[S]/[S] + ln[S]/[S] with [S] and [S] being the substrate concentrations at time 0 and time t, respectively. Because Vm = kcat / [E]t gave the kcat/Km ratio. The kcat for the first-order substrate hydrolysis was calculated by fitting experimental data to the first-order law using Enzfitter software (Elsevier Science Publishers, Amsterdam). All assays with Pr3 were titrated with α1-PI, the titer of which had been determined using bovine trypsin titrated with p-nitrophenyl-p-gua- nidino benzoate (20). Cathepsin G was titrated with α1-antichymotryp- sin (21).

The Km for the hydrolysis of Abz-peptidyl-EDDnp substrates by HNE and Pr3 was too high (>10 μM) to be determined by measuring the rates of hydrolysis at different substrate concentrations and fitting the to hyperbolic Michaelis-Menten rate equation. Therefore, we used two competing substrates whose hydrolysis products could be measured independently. Each substrate acts as a competitive inhibitor of the other under these mixed alternative substrates conditions. Ki,values, values of intramolecularly quenched fluorogenic substrates were obtained by determining the dissociation constant (Kt) toward the su- rintronic acid substrate. Assays were carried out by adding 10 mM HNE or Pr3 to a mixture of 0.1–1 mM MoE-Suc-AAPV-pNA whose Km are 0.085 (22) and 0.6 mM, respectively, and 0–100 μM fluorogenic Abz- peptidyl-EDDnp derivative. The hydrolysis of MoE-Suc-AAPV-pNA was monitored at 410 nm with <5% of substrate hydrolyzed. The velocity of the enzyme action is described by the following equation:

\[ \frac{v}{v_c} = \frac{(K_0 + S)}{(K_0 + K_t + S)} \]  
(Eq. 1)

with v being the initial velocity at a given substrate concentration with Abz-peptidyl-EDDnp competitor, v_c being the initial velocity at the same substrate concentration without competitor, K_0 being the Michaelis-Menten constant of the substrate, S being the chromogenic substrate concentration, and I being the Abz-peptidyl-EDDnp substrate concentration. The K_t value corresponds to the observed Michaelis-Menten constant (Kt,values) of Abz-peptidyl-EDDnp substrate as competitor.

Oxidation of Abz-peptidyl Substrates—The methionyl residues in Abz-peptidyl-EDDnp substrates were selectively oxidized to methionyl sulfoxide with N-chlorosuccinimide (23). The peptides (30 nmol) were treated with a 5–10-fold molar excess of N-chlorosuccinimide in 0.1 M Tris-HCl buffer, pH 8.5, for 20 min at room temperature. Unreacted N-chlorosuccinimide was removed by HPLC on a C18 car- tidge (2.1 × 30 mm, Brownlee, or 2 × 33 mm, Upchurch) at a flow rate of 0.3 ml/min with a linear gradient (0–60%, v/v) of acetonitrile in 0.01% trifluoroacetic acid over 20 min.

Enzyme Activity at the Surface of PMN—PMNs (5 × 10^5 to 5 × 10^6) were incubated in 50 mM Hepes buffer, pH 7.4, 150 mM NaCl, 0.05% Igepal CA-630 (v/v) with 5 μM Abz-peptidyl-EDDnp substrate in micro- plate wells at 37 °C. The fluorescence was recorded continuously using a microplate fluorescence reader (Spectra Max Gemini, Molecular De- vices) under continuous stirring. The enzyme activity in supernatants obtained by washing PMNs in PBS was also assayed. The elastase inhibitor EPI-HNE4 developed by Debiopharm S.A. (Lausanne, Swit- zerland) was used at a final concentration of 0.1 μM and incubated for 15 min at 37 °C with ~10^5 cells before measuring residual elastase activity. Peptidyl-CMK inhibitors (2 mM stock solution in 30% N,N-di- methylformamide) were used at a final concentration of 10^-4 to 10^-5 M and incubated for 60 or 120 min with ~10^6 neutrophils or purified 10^-9 M HNE.

Chromatography and Analysis of Peptide Products—Fluorogenic substrates (4–8 μM final) were incubated with HNE or Pr3 (10–100 nM) at 37 °C in reaction buffer. The reaction was stopped by adding 4 volumes of absolute ethanol and incubating for 15 min on ice. Precipi- tated protein was removed by centrifugation at 10,000 × g for 10 min. The supernatant containing the hydrolysis products was dried under vacuum and dissolved in 200 μl of 0.01% trifluoroacetic acid (v/v). Hydrolysis fragments were purified by reverse-phase chromatography on a C18 column (2.1 × 30 mm, Brownlee, or 2 × 33 mm, Upchurch)
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Using ThermoSeparation product P200 pump and a Spectrasytem UV3000 detector (ThermoSeparation product) at a flow rate of 0.3 ml/min with a linear gradient (0–60%, v/v) of acetonitrile in 0.01% trifluoroacetic acid over 20 min. Eluted peaks were monitored at three wavelengths (220, 320, and 360 nm) simultaneously, which allowed the direct identification of EDDnp-containing peptides prior to sequencing. Cleavage sites were identified by N-terminal sequencing using an Applied Biosystems 477A pulsed liquid sequencer with the chemicals and program recommended by the manufacturer. Phenylthiohydantoin derivatives were identified with an on-line model 120A analyzer.

RESULTS

Inhibitory Serpin-derived Substrates for HNE and Pr3—At least two serpin inhibitors, α1-PI (serpin A1) and MNEI (serpin B1), are potent inhibitors of HNE and Pr3. α1-PI is the main extracellular inhibitor of HNE in the lung (24). Its deficiency or inactivation by proteolytic cleavage or oxidation is clearly associated with the pathogenicity of several inflammatory lung diseases including emphysema (25, 26). MNEI is an elastase inhibitor first found in the cytosol of human lung macrophages and blood monocytes (27). It has since been identified as a serpin inhibitor of elastase-like and chymotrypsin-like proteases with two cleavage sites at Cys344 and at the adjacent Phe residue in its RSL (28–30). We first used fluorescent peptides of different lengths derived from the RSL of these inhibitors as substrates for HNE and Pr3 for measuring specificity constants (k_{cat}/K_{m}) (Fig. 1).

α1-PI-derived substrates were rapidly cleaved by HNE and Pr3 and to a lesser extent by cathepsin G (Table I). However, the k_{cat}/K_{m} for HNE was approximately one order of magnitude greater than that for Pr3, although the cleavage site was the same (Met-Ser bond) and identical to that in the RSL of the parent serpin. α1-PI-derived substrates that differ by the length of their peptide segments on the prime side had similar k_{cat}/K_{m} values regardless of the protease used with the exception of Pr3, which preferentially cleaved the shorter substrate (Table I). This observation suggests that residues beyond P3 have little influence on the interaction between these proteases and their substrates.

It was recently shown that MNEI uses either a Cys residue or a Phe residue at P1 in its reactive center loop (30). We prepared two substrates from the RSL of MNEI, one being shortened on both sides of the peptide chain (Table I). HNE and Pr3 cleaved both substrates at the same C–M bond as in the parent molecule, although the shorter substrate, Abz-TF-CMLQ-EDDnp, was hydrolyzed more slowly. However, Abz-TFCMLQ-EDDnp is the first sensitive substrate to be reported that is cleaved more efficiently by Pr3 than by HNE. Cathepsin G also cleaves MNEI-derived substrates but at the F–C bond in agreement with its substrate specificity. However, Abz-GHAT-FCMLPMEQ-EDDnp is cleaved more efficiently than Abz-TF-CMLQ-EDDnp (Table I). It is possible that the sensitive bond within the shorter substrate is too close to the N terminus to allow efficient cleavage by cathepsin G (16).

The oxidation of Met⁶⁰⁰ in the RSL of α1-PI reduced its capacity to inactivate HNE ~2,000-fold (31). This phenomenon probably contributes to the protease-inhibitor imbalance at inflammatory sites where neutrophils release reactive forms of oxygen (3). We looked at the effects of oxidizing the Met residues in α1-PI and MNEI-derived substrates, which have a Met residue at P1 and P1', respectively, on the hydrolysis by HNE and Pr3. The hydrolysis of α1-PI-derived and MNEI-derived substrates was dramatically altered when they were oxidized with N-chlorosuccinimide. The k_{cat}/K_{m} values were reduced by more than one order of magnitude (Table I). This finding suggests that the oxidative microenvironment at inflammatory sites also inactivates MNEI when this inhibitor is present locally. Adding native (unoxidized) substrate to the reaction mixture restored full activity, demonstrating that the trace amounts of N-chlorosuccinimide remaining in the oxidized substrate after HPLC did not interfere with the rate of hydrolysis.

A Selective Substrate for Pr3—Several intracellular serpins in addition to MNEI have a Cys residue in their RSL, which makes them putative targets for HNE and Pr3. P19 is found in the lung and other tissues and is reported to be a potent inhibitor of HNE that uses a cysteinyl residue at its reactive site (32). P19 also inhibits granzyme B (33) and caspase1 (34) but uses a different site in its RSL for this purpose. The predicted reactive site in CrmA, a closely related viral homologue of P19 that also inhibits caspase 1 and granzyme B, is an Asp or a Cys, even though the Asp residue is most certainly involved in the inhibition of these proteases (35, 36). A Cys residue is also present in the RSL of the intracellular serpin inhibitor P16, which is found in monocytes and granulocytes, and is a potent inhibitor of cathepsin G (37).

The hydrolysis of fluorogenic substrates covering the RSL of P19 (Abz-VAECQQ-EDDnp), P16 (Abz-MMRCACQ-EDDnp), and CrmA (Abz-VAECQQ-EDDnp) by HNE, Pr3 and cathepsin G were monitored. Cathepsin G did not cleave any of these substrates. The P19-derived substrate was cleaved very efficiently by HNE and Pr3 (Table II) but HNE cleaved at the C–Q bond, whereas Pr3 cleaved at the C–C bond. Nevertheless, the specificity constants were similar (Table II). The P16-derived substrate was preferentially cleaved by Pr3 with a single cleavage site at the C–A bond, but HNE also caused significant hydrolysis at the A–Q bond; the ratio k_{cat}/K_{m}(Pr3)/k_{cat}/K_{m}(HNE) was ~2.8 (Table II). The CrmA-derived substrate, Abz-VAECQQ-EDDnp, which also has the Cys-Ala pair in its reactive loop, was cleaved very efficiently by Pr3 but significantly more slowly by HNE (Table II). The ratio k_{cat}/K_{m}(Pr3)/k_{cat}/K_{m}(HNE) was ~30. The difference depended mainly on k_{cat} values, because the K_{m} values are similar (Table III). The difference in k_{cat}/K_{m} ratio is high enough to selectively measure Pr3 activity in a biological sample containing similar concentrations of HNE and Pr3.

A Selective Substrate for HNE—PAI-1 is a member of the serpin family involved in the regulation of fibrinolysis (38). It is cleaved at a P1 = Arg residue in its RSL upon interaction with urokinase-type and tissue-type plasminogen activators (39). It is also cleaved and inactivated by HNE. The cleavage site was identified as the P4-P3 (V–S) bond in intact PAI-1 (40). The substrate covering the PAI-1 RSL from P5 to P10' (Abz-IVSARMAPEEIMDRQ-EDDnp) was rapidly hydrolyzed by...
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Table I

| Serpin          | Substrate                                      | Proteinase 3 | Elastase | Cathepsin G |
|-----------------|------------------------------------------------|--------------|----------|-------------|
| α1-PI           | Abz-Elam-PepVFK-NQn-EDDnp                      |              |          |             |
|                 | Abz-EIamPepVFK-NQn-EDDnp                      |              |          |             |
|                 | Abz-EIamPepVFK-NQn-EDDnp                      |              |          |             |
|                 | Abz-EIamPepVFK-NQn-EDDnp                      |              |          |             |
| MNEI            | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |

n.s.h., no significant hydrolysis; n.d., not determined.

Table II

| Serpin          | Substrate                                      | Proteinase 3 | Elastase | Cathepsin G |
|-----------------|------------------------------------------------|--------------|----------|-------------|
| α1-PI           | Abz-VAECQ-EDDnp                                |              |          |             |
|                 | Abz-VAECQ-EDDnp                                |              |          |             |
|                 | Abz-VAECQ-EDDnp                                |              |          |             |
|                 | Abz-VAECQ-EDDnp                                |              |          |             |
| MNEI            | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |

Values are the means ± S.D. of three experiments.

Table III

| Serpin          | Substrate                                      | Proteinase 3 | Elastase | Cathepsin G |
|-----------------|------------------------------------------------|--------------|----------|-------------|
| α1-PI           | Abz-VAECQ-EDDnp                                |              |          |             |
|                 | Abz-VAECQ-EDDnp                                |              |          |             |
|                 | Abz-VAECQ-EDDnp                                |              |          |             |
|                 | Abz-VAECQ-EDDnp                                |              |          |             |
| MNEI            | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |
|                 | Abz-EDDnp                                      |              |          |             |

Values are the means ± S.D. of four experiments.

Values are the means ± S.D. of three experiments.

HNE at the two Ile residues close to the C terminus but also by Pr3 at the A–R bond on the N-terminal end. There was no cleavage at the V–S bond as in the native serpin (40). The kcat/Km values for the two proteases were similar in the 10-5 M·s⁻¹ range (Table IV). A shorter substrate, in which the N-terminal peptide sequence containing the Pr3 cleavage site and one Ile residue were deleted (Abz-APEEIMDRQ-EDDnp), was cleaved at a single Ile site by HNE. Unexpectedly, Pr3 also cleaved at the same site to some extent. However, the kcat/Km for HNE was 50-fold higher than that for Pr3, which allows almost selective measurement of HNE activity, even in the presence of Pr3. The specificity constant is also higher than that for the currently used HNE substrates MeO-Suc-AAPV-pNA (kcat/Km = 182,000 M⁻¹·s⁻¹) (41) and MeO-Suc-AAPV-FC (kcat/Km = 139,000 M⁻¹·s⁻¹) as determined in this study.

The Km values for HNE and Pr3 on Abz-APEEIMDRQ-EDDnp were determined by competition experiments using MeO-Suc-AAPV-pNA as a competing substrate. Under these conditions, the experimental Ks value corresponds to the Ks for Abz-APEEIMDRQ-EDDnp and was 12-fold higher for Pr3 than for HNE (Table III). Thus, both Ks and kcat contribute to make the specificity constant for Pr3 less than that for HNE. The oxidation of the P1 Met residue in Abz-APEEIMDRQ-EDDnp resulted in a 6.1-fold decrease in the specificity constant for HNE and a 9.1-fold decrease in that for Pr3 (Table IV).

The Abz-VSARQ-EDDnp substrate, which includes both the HNE cleavage site in native PAI-1 (Val-Ser) (40) and the Pr3 cleavage site in the longer PAI-1-derived substrate described above (Ala-Arg), was not cleaved at a significant rate by HNE or Pr3 (Table IV).

Measurement of HNE and Pr3 Activities at the Surface of PMNs—Part of the neutral proteases released from activated PMNs remains bound to the cell membrane. This is an important form of active proteases, because these enzymes are more resistant to inhibition by local protease inhibitors (1, 2). Cell attachment of proteases amplifies tissue injury by extending the time and efficiency of uncontrolled proteolysis (reviewed in Ref. 3). The relative contributions of HNE and Pr3 to this membrane-bound proteolytic activity have not been investigated to date, because no substrate that discriminates between these proteases when they are both present in a solution has been available. Campbell et al. (1) attempted to overcome this problem by studying the catalytic activity of exogenous Pr3 bound to unstimulated cells. We used Abz-VAECQ-EDDnp and Abz-APEEIMDRQ-EDDnp to measure proteolytic activity at the surface of purified blood neutrophils that had undergone minimal activation during purification as judged from the low percentage of cells (<5%) expressing CD63 by flow cytometry. Both substrates were rapidly hydrolyzed by suspensions of freshly prepared neutrophils, demonstrating that the two proteases are present and catalytically active at their surface. Only a weak activation was observed when cells were diluted to ~10³ cells/µl and kept at 6°C under gentle stirring, and <15% cells expressed CD63 after overnight storage in these conditions. However, keeping undiluted purified neutrophils on ice for 3 h (>10³ cells/µl) resulted in the activation of 10–40% of the neutrophil population as deduced from surface CD63. There was a parallel increase in proteolytic activity on both substrates, indicating greater HNE and Pr3 expression at the membrane surface of partially activated neutrophils. Proteolytic activity remained after the cells were washed with PBS, centrifuged, and suspended in the reaction buffer, whereas there was no significant activity in the supernatant of freshly prepared or diluted stock cell suspensions. Approximately 5% of the total activity can be released into the supernatant of partially activated cells. HPLC analysis of the soluble fraction from cells incubated with Abz-VAECQ-EDDnp and Abz-APEEIMDRQ-EDDnp showed that both substrates were cleaved at a single site identical to those of Pr3 and HNE (Fig. 2). This finding indicates that HNE is the main protease cleaving Abz-APEEIMDRQ-EDDnp and Pr3 is the main protease cleaving Abz-VAECQ-EDDnp. This hypothesis was reinforced by the observation that the Abz-VAECQ-EDDnp-hydrolyzing activi-
ity was released more rapidly than that cleaving Abz-APEEIMDRQ-EDDnp from freshly prepared cells washed with buffer containing 1.5 mM NaCl for 10 min at 37 °C (Fig. 3). This finding is in agreement with the fact that Pr3 has a more acidic pl and is therefore released more readily from the cell surface than is HNE (1). Further confirmation was provided using the low M₄ specific inhibitor of HNE, EPI-HNE4, developed by Debiopharm S.A. that inhibits both free and membrane-bound HNE. 3 EPI-HNE4 specifically and totally inhibits Abz-APEEIMDRQ-EDDnp-hydrolyzing activity but not Abz-VADCAQ-EDDnp-hydrolyzing activity when incubated at a final concentration of 0.1 mM with 10⁴ neutrophils. By comparison, the peptidyl-chloromethylketone elastase and Pr3 inhibitor MeO-Suc-AAPA-CMK totally inhibited membrane-bound proteolytic activity on both Abz-APEEIMDRQ-EDDnp and Abz-VADCAQ-EDDnp, whereas the chymotrypsin-like Z-GLF-CMK inhibitor had no effect on Abz-VADCAQ-EDDnp-cleaving activity and inhibited ~20% of the Abz-APEEIMDRQ-EDDnp-hydrolyzing activity under the experimental conditions used. Because of the possible release of oxidants from neutrophils, we checked that the methionyl residue in Abz-APEEIMDRQ-EDDnp was not oxidized during the reaction, thus reducing significantly its rate of hydrolysis (Table IV). This analysis was done by comparing the HPLC elution times of the substrate and its hydrolysis products upon hydrolysis by membrane-bound proteases with that of Abz-APEEIM(O)DRQ-EDDnp, which is eluted earlier than the native substrate. No peak eluting with the retention times of Abz-APEEIM(O)DRQ-EDDnp or its hydrolysis product M(O)DRQ-EDDnp was observed (data not shown).

Suspensions containing 5 × 10³ to 5 × 10⁴ cells/200 μl were enough to observe significant hydrolysis within minutes because of the great sensitivity of these two substrates. We calculated that membrane-bound HNE and Pr3 were present at similar concentrations of 0.05–0.5 pg/cell (mean value 0.29 pg/cell, n = 13 experiments) using titrated HNE and Pr3 as standards. Similar values were found for cathepsin G at the cell surface.

An overall serine protease activity can also be measured using as few as 1,000 cells and the nonspecific MNEI-derived substrate, Abz-GIATFCML-MPEQ-EDDnp, that is cleaved by HNE, Pr3, and cathepsin G with specificity constants that are all greater than 2 × 10⁶ M⁻¹ s⁻¹. However, protease activity could not be quantified under these conditions because of differences in the kinetic parameters for each protease.

### DISCUSSION

Elastase, proteinase 3, and cathepsin G are all released from the primary granules of activated PMNs when they are recruited and activated at an inflammatory site and probably act in concert at this site. The fate of each protease may differ depending on how they are partitioned between soluble and membrane-attached forms and on their sensitivity to inhibitors or other local controlling agents. The role of each neutral protease from PMN primary granules has not been yet elucidated because there is no substrate that allows the specific measurement of each one, especially at the cell surface or in a complex medium. For example, the currently used Pr3 substrate Boc-AANva-SBzl is hydrolyzed nine times faster by HNE (8), which makes it impossible to measure Pr3 activity in the presence of HNE. Furthermore, using peptidyl-SBzl substrates requires a coupled assay with a thio-disulfide reagent that may react with any reactive SH groups present (e.g. at the cell surface) and interfere with the colored reaction. Sensitive cathepsin G substrates that provide an alternative to the use of peptidyl-SBzl substrates have only recently become available (16). The catalytic activity of Pr3 bound to the membranes of PMN or in the sputum of patients with cystic fibrosis has been studied using indirect methods. They were based on the susceptibility of Pr3 to protein inhibitors, especially secretory leukocyte protease inhibitor (42), or on the use of exogenous Pr3 bound to unstimulated neutrophils (1). HNE and Pr3 are structurally and functionally very similar. Both cleave at small aliphatic amino acid residues such as Val, Ala, and Ser at P1, but HNE also accommodates Cys and Leu residues at that position (9, 43). Both proteases also cleave Met residue at P1, a feature shared by cathepsin G (44), which explains why all three proteases are inhibited by α1-PI. HNE and Pr3 have an extended site of interaction with their substrate that extends from S4 to S3′ as first suggested for HNE by Lestienne and Bieth (45) and later shown by x-ray crystallography (14, 46). New substrates of greater specificity for these proteases can be developed by investigating their Pᵣ specificity. We used serpin RSLs because they are mobile and flexible segments that are easily cleaved; however, they interact with the target protease. Serpins may interact with target enzymes to form an almost irreversible SDS-resistant complex or to inactivate the inhibitor by cleavage of its RSL without further binding to the protease. Some serpins that do not inhibit a given protease may also be used as a substrate for this protease, thus making them inefficient for their target enzyme. Therefore, separate residues in RSLs can be used to broaden the inhibitory specificity (32, 47) or to inactivate the serpin by proteolytic attack.

Substrates related to a serpin reactive site were first developed by Nakajima et al. (44) using the reactive loop of α1-PI as substrates for HNE and cathepsin G. But these chromogenic p-nitroanilide substrates were not suitable for

| Serpin | Substrate | Proteinase 3 | Elastase | Cathepsin G |
|--------|-----------|-------------|----------|-------------|
| PAI-1  | Abz–IVSARMAPEEIMDRQ–EDDnp | 614 ± 3³ | 421 ± 6³ | n.s.h. |
|        | Abz–VSARQ–EDDnp     | 1.5 ± 0.4³ | 1.6 ± 0.3³ | n.s.h. |
|        | Abz–APEEIMDRQ–EDDnp | 14.6 ± 1.3³ | 683 ± 75³ | n.s.h. |
|        | Abz–APEEIM(O)DRQ–EDDnp | 1.6 ± 0.7³ | 112 ± 4³ | n.d. |

³ Values are the means ± S.D. of two experiments.

⁴ Values are the means ± S.D. of four experiments.

⁵ Values are the means ± S.D. of three experiments.

n.s.h., no significant hydrolysis; n.d., not determined.
investigating the P' side of the cleavage site despite the importance of S' subsites in substrate binding by these proteases. Since then, we have developed substrates with intramolecularly quenched fluorescence, some of which are based on the reactive loop of serpin inhibitors and are more sensitive and specific than currently used fluorogenic or chromogenic substrates (16, 48).

We first used sequences derived from the reactive loop of PI-1 and MNEI, because both serpins inhibit HNE, Pr3, and to a lesser extent cathepsin G. These substrates are all cleaved by the three neutral proteases from PMN primary granules but at different rates. The MNEI-derived substrate is cleaved more rapidly by Pr3 unlike currently used Pr3 substrates, which are all better cleaved by HNE. The cleavage sites are identical at a P1 Cys residue for the two proteases as in the parent molecule (28, 29). Oxidation of the methionyl residue at P1 or at P1' in these substrates considerably slowed the rate of degradation regardless of the substrate or the protease used, which demonstrates the importance of the P1' residue and agrees with the decreased capability of oxidized serpins to inhibit elastase-like proteases (49).

The RSL of the ov-serpins PI6 and PI9 and their viral homologue CrmA all contain a Cys residue as does MNEI. PI9 is an intracellular serpin of the ovalbumin family that is found mainly in the lung and placenta. It was first reported as an inhibitor of caspase 1 and granzyme B (33, 34, 50) and later found to inhibit HNE through cleavage at a Cys site (32). The PI9-derived substrate is cleaved by both HNE and Pr3 but at different sites. PI6 has not yet been reported to be an elastase inhibitor but has been shown to be a cathepsin G inhibitor (37). Its RSL-derived substrate is preferentially cleaved by Pr3 again at a Cys residue. Whether this cleavage occurs in the native serpin, thus preventing it from inhibiting cathepsin G, remains to be investigated. The viral serpin CrmA functions very similar to PI9; it inhibits caspase 1 and granzyme B using a characteristic Asp residue at P1 (35, 36). Cowpox virus uses this serpin to prevent the proteolytic activation of the proinflammatory cytokine interleukin-1β, thus facilitating viral infection (35). The CrmA RSL-derived substrate is cleaved 30-times faster by Pr3 than by HNE, which makes it a discriminating substrate for measuring Pr3 activity, even in the presence of HNE. Pr3 also activates the precursor of interleukin-1β, representing an alternative pathway for the production of proinflammatory cytokines, particularly in the context of local inflammatory processes (5, 12).

PAI-1 is a potent inhibitor of fibrinolysis that can be inacti-
the activity of these proteases selectively at the membrane surface will be of great help in testing the activity of exogenous inhibitors developed to regulate proteolytic activity at inflammatory sites where the protease/inhibitor imbalance greatly influences the self-perpetuating inflammation characteristic of chronic inflammatory diseases and especially airway inflammation.

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