Influence of Charge Distribution at the Active Site Surface on the Substrate Specificity of Human Neutrophil Protease 3 and Elastase

A KINETIC AND MOLECULAR MODELING ANALYSIS*

The biological functions of human neutrophil protease 3 (Pr3) differ from those of neutrophil elastase despite their close structural and functional resemblance. Although both proteases are strongly cationic, their sequences differ mainly in the distribution of charged residues. We have used these differences in electrostatic surface potential in the vicinity of their active site to produce fluorescence resonance energy transfer (FRET) peptide substrates for investigating individual Pr3 subsites. The specificities of subsites S5 to S3 were investigated both kinetically and by molecular dynamic simulations. Subsites S2, S1′, and S2′ were the main definers of Pr3 specificity. Combinations of results for each subsite were used to deduce a consensus sequence that was complementary to the extended Pr3 active site and was not recognized by elastase. Similar sequences were identified in natural protein substrates such as NFκB and p21 that are specifically cleaved by Pr3. FRET peptides derived from these natural sequences were specifically hydrolyzed by Pr3 with specificity constants $k_{cat}/K_m$ in the $10^6$ M$^{-1}$ s$^{-1}$ range. The consensus Pr3 sequence may also be used to predict cleavage sites within putative protein targets like the proform of interleukin-18, or to develop specific Pr3 peptide-derived inhibitors, because none is available for further studies on the physiopathological function of this protease.

Protease 3 (Pr3)$^3$ was initially described as an elastin-degrading protease whose structural and functional properties are similar to those of human neutrophil elastase (HNE) (1, 2). Pr3 is stored as an active enzyme within the primary granules of human neutrophils, together with HNE and cathepsin G, and is released from activated cells as a free or membrane-bound protease (3, 4). Its three-dimensional structure is very similar to that of HNE, with which its sequence is 57% identical (5). Pr3 and HNE also have extended interaction sites that greatly influence substrate binding and specificity (5). The active sites of the two proteases are also very similar, and both preferentially accommodate small aliphatic residues in their S1 subsite$^6$ (6, 7). This is why there was no substrate that discriminated between the two proteases until fluorescence resonance energy transfer (FRET) peptides became available. These can be used to study the specificity on both sides of the cleavage site (8, 9). However, kinetic and structural studies have shown that the substrate binding site in Pr3 is more polar, and some subsites are more restrictive than those in HNE (5, 10). This partly explains why Pr3, but not HNE, is not inhibited by the low molecular weight inhibitor SLPI present in the upper airways, even though both proteases are inhibited by the $\alpha$-1-protease inhibitor ($\alpha$-1-Pi) in lung secretions (6, 11).

Although the primary function of Pr3 and HNE is commonly thought to be the intralysosomal degradation of phagocytized microorganisms, both also act extracellularly to break down matrix proteins (6, 12, 13), release cytokines from their precursors, and cleave active cytokines (14). But Pr3 also has specific functions in acute and chronic myeloid leukemia, cell proliferation, and differentiation (15–18), endothelial cells apoptosis (19, 20), and Wegener granulomatosis (21–23). These make it a unique neutrophil protease, rather than a redundant enzyme, although the reasons for its specificity are not well understood.

We have compared the electrostatic surface potentials in the substrate binding sites of Pr3 and HNE. The findings have been used to produce new Pr3 substrates with extended binding sites that could help explain the specific cleavage of biological protein substrates and identify new protein targets of pathophysiological relevance.
Materials—Protease 3 (EC 3.4.21.76), human neutrophil elastase (EC 3.4.21.37), and α1-P, were obtained from Athens Research and Technology (Athens, GA). All other reagents were of analytical grade.

**Design and Synthesis of FRET Peptides**—All Fmoc-protected amino acids were of the L-configuration and were purchased from Advanced Chemtech (Louisville, KY), Applied Biosystems (Warrington, UK), and Neosystem (Strasbourg, France). Abz-peptidyl-EDDnp fluorogenic substrates were prepared by solid-phase synthesis with Fmoc methodology using an automated multiple peptide synthesizer (PSSM-8, Shimadzu Co., Kyoto, Japan). Substrates were purified by semipreparative reversed-phase chromatography using a 50-min linear (0–100%) gradient of acetonitrile in 0.1% trifluoroacetic acid and checked for homogeneity by analytical reversed-phase HPLC on a C18 column and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (ToFSpec-E, Micromass, Manchester, UK) or peptide sequencing (Applied Biosystems Procise Sequencer) with the chemicals and program recommended by the manufacturer. 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. Cys-containing substrate stock solutions were supplemented with 10 mM dithiothreitol.

**Enzyme Assays**—Free Pr3 and HNE were measured in 50 mM Hepes, pH 7.4, 750 mM NaCl, supplemented with 0.05% Igepal CA-630 (v/v) to take into account the great propensity of these proteases to stick to plastic and glass surfaces when in dilute solution. Free Pr3 and HNE were titrated with α1-PI, the titers of which had been determined using bovine trypsin titrated with p-nitrophenyl-p’-guanidinobenzoate (24). A 1:1 stoichiometry was used for the HNE/α1-PI complex, whereas that for the Pr3/α1-PI complex was 1:1.3, to allow for partial α1-PI degradation via the substrate pathway. Trypsin was prepared as a 2 × 10⁻⁴ M stock solution in 100 mM Tris/HCl buffer, pH 8.50, 500 mM CaCl₂, then used in the same buffer as HNE and Pr3. 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 spectrofluorometer. The system was standardized using Abz-FR-OH prepared by the total tryptic hydrolysis of an Abz-FR-pNA solution, and its concentration was determined from the absorbance at 410 nm, assuming F₄₁₀ nm = 8,800 M⁻¹ cm⁻¹ for p-nitroanilide. Concentrations of Abz-peptidyl-EDDnp substrate were determined by measuring the absorbance at 365 nm, using F₃₆₅ nm = 17,300 M⁻¹ cm⁻¹ for EDDnp.

**Specificity constants** (kcat/Km) were determined under first-order conditions using substrate concentrations far below the Km (maximum, 1 μM). Final Pr3 and HNE enzyme concentrations were 1–100 nm. Under these conditions, the Michaelis-Menten equation is reduced to v = kobs·S, where kobs = Vₘ/Km. Integrating this equation over time gives ln(S) = −kobs·t + ln(S)o, with (S)o and (S) being the substrate concentrations at time 0 and time t, respectively. Because Vₘ = kcat·(E)t, where (E)t is the final enzyme concentration, dividing kobs by (E)t gives the kcat/Km ratio. The kobs for the first-order substrate hydrolysis was calculated by fitting experimental data to the first-order equation using Enzfitter software (Elsevier Science Publishers, Amsterdam, The Netherlands). Km values were determined by measuring hydrolysis rates at five substrate concentrations from 0.5–20 μM.

**Chromatography and Analysis of Peptide Products**—Fluorogenic substrates (5–8 μM final) were incubated with Pr3 or HNE (10–100 nM) in reaction buffer at 37 °C. The reaction was stopped by adding 4 volumes of absolute ethanol and incubating for 15 min on ice. Precipitated protein was removed by centrifugation at 13,000 × 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 reversed-phase chromatography on a C18 column (2.1 mm × 30 mm) using a P200 pump and a Spectrasystem UV3000 detector (TSP, Les Ulis, France). They were eluted at 0.3 ml/min with a linear (0–60%, v/v) gradient of acetonitrile in 0.01% trifluoroacetic acid for 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. All cleavage sites were identified either by mass spectrometry or by N-terminal sequencing using an Applied Biosystems (Courtaboeuf, France) Procise 494 protein sequencer attached to a model 140C Micro-gradient System and a 610A Data Analysis System with the chemicals and program recommended by the manufacturer.

**Electrostatic Potential Calculations**—The electrostatic potentials of Pr3 and HNE were calculated by the Poisson-Boltzmann method implemented in the Delphi module of Insight II (Accelrys Inc., San Diego, CA) using formal charges at pH 7.4 (arginine, lysine, N terminus, +1; glutamate, aspartate, and the C terminus, –1; and histidine, neutral), an ionic strength in the aqueous environment of 0.15 M, dielectric constants of 2 for the interior and 80 for the exterior of the protein, and a temperature of 300 K. The x-ray coordinates of HNE were extracted from the complex formed by HNE with the turkey ovomucoid inhibitor OMTKY-III (PDB ID 1PPF). The structure of Pr3 (PDB ID 1FUJ) was superimposed on that of HNE to visualize both molecules with the same orientation. The location of subunits S4 to S3 in the active site of each protease was inferred from the position of the side chains of the corresponding P4–P3 residues of OMT-III complexed with HNE. The solvent-accessible molecular surface was calculated and colored according to the electrostatic potential using Insight II (Accelrys Inc.).

**MD Simulations**—The initial set of coordinates used for MD simulations were taken from the x-ray structures of Pr3 (PDB ID 1FUJ, resolution 2.2 Å) and HNE complexed with OMTKY-III (PDB ID 1PPF, resolution 1.8 Å). The complexes between enzymes and peptides were built and simulated using molecular dynamics as reported previously (25).

All simulations were performed using version 2.5 of the NAMD program (26), with version 27 of the Charmm force field (27). Production runs were performed for 2000 ps once the systems had been heated up to 300 K and equilibrated for 100 ps.
The MD simulations were validated using a strategy similar to that used earlier (25). The interactions between the enzymes and ligands were analyzed using Charmm to detect hydrogen bonds and hydrophobic contacts.

RESULTS

Differences in the Charge Distributions of Pr3 and HNE—Pr3 and HNE are homologous proteases that have evolved by gene duplication from a common ancestor and so are quite distinct from the main branches of the chymotrypsinogen superfAMILY of serine proteases (28). Their sequences are 57% identical, and about half of the 61 residues that differ between the two proteases are charged residues, but only 10 are found at the same position in the whole sequences (Fig. 1). The solvent-accessible surfaces in Pr3 and HNE show that Asp-61, Lys-99, and Arg-143 in Pr3 and Arg-217 in HNE are in the vicinity of the substrate binding site that extends from at least substates S4 to S3’ (5) (Fig. 1, B and C). Both proteases also have a cluster of positively charged residues remote from the active site that could favor binding to negatively charged substrates (Fig. 1, B and C). However this positive cluster is disrupted by negatively charged residues in Pr3 and is therefore smaller than that of HNE. Molecular dynamic simulations based on the three-dimensional structure of Pr3 show that substates S1’ to S3’ in Pr3 are all in the vicinity of charged residues that are not present in HNE (Fig. 1). Asp-61 is close to the putative substates S1’ and S3’ (Fig. 1B). Those have the same orientation and could influence the specificities for both P1’ and P3’ residues (5). The residue at position 99, which is close to substates S2 and S4, is a Lys in Pr3 and a Leu in HNE, whereas position 217 is occupied by a positively charged Arg residue in HNE and a hydrophobic Ile in Pr3 (Fig. 1, B and C). We studied the contribution of charged residues to the extended substrate binding sites in Pr3 and the importance of these charged residues for the specificity of Pr3 so as to better understand the intimate interaction of this protease with its biological target substrates and thus its particular biological function. FRET substrates with extended sequences on both sides of the cleavage site and MD simulations were used for this purpose.

Prime Specificity: Contribution of Asp-61 to the Specificity of Pr3—Abz-VADCADQ-EDDnp (Table 1, substrate 2), which is cleaved at the C–A bond (29), was used as the lead substrate. We introduced additional residues at P3’ in this substrate and measured the specificity constants $k_{cat}/K_m$. Positively charged, negatively charged, and neutral residues (Table 1, substrate 3–5) were all accepted at P3’ and improved the specificity constant significantly over to that of the lead substrate. This suggests that ionic interactions involving residue P3’ and Asp-61 are not essential for substrate binding but that elongation of the peptide chain helps to stabilize the substrate, thus improving catalytic efficiency. HNE did not significantly hydrolyze these elongated substrates (Table 1). Asp-61 also lies in the vicinity of the S1’ subsite and may be an important residue in the S1’ site of Pr3 (25). Because there is no negatively charged residue at that position in HNE, we checked the contribution of this residue to the Pr3 P1’ specificity. Both positively charged and negatively charged residues were accepted at P1’ (Table 1, substrates 6 and 7), but the $k_{cat}/K_m$ was about ten times greater for the substrate containing P1’-Arg. Because polar interactions can modify even distant recognition substates (25), we checked the influence of positively and negatively charged residues at P1’ on the specificity constants using a substrate that had no other charge from P3 to P3’ and that was cleaved at the same site by both Pr3 and HNE. The P1’-Met of the monocyte neutrophil elastase inhibitor-derived substrate Abz-GIATFCMLMPEQ-EDDnp was replaced by either an Arg or an Asp (Table 2). A positively charged residue remained the preferred residue at position P1’ for Pr3 hydrolysis, whereas HNE had no preference at that position (Table 2, substrates 9 and 10). This emphasizes the importance of the S1’ subsite for Pr3 specificity even though an electrostatic interaction at S1’ in the Pr3 active site is not a prerequisite for substrate binding. A positively charged residue at P2’ in the monocyte neutrophil elastase inhibitor-derived substrate resulted in a marked decrease in the $k_{cat}/K_m$ for Pr3 but not for HNE (Table 2, substrate 11), confirming previous indication of the importance of Pr3 Arg-143 for P2’ specificity (29).

The interactions between Pr3/HNE and the same substrates were derived from MD simulations (25); we identified and analyzed all hydrogen bonds (H-bonds) and hydrophobic interactions between enzymes and substrates during the simulations. The snapshots of the complexes at the end of the MD simulations (Fig. 2) highlight the partners of the substrates when they bind to the enzymes.

The lead substrate (Table 2, substrate 8, and Fig. 2, A and B) binds to Pr3 and HNE in a favorable way, similar to what we have described earlier (25). Pr3 interacted with the nonprime side of substrate 8 via strong H-bonds and hydrophobic interactions (between P5-Ile and Trp-218, between P3-Thr and Val-216 and between Lys-99 and both residues P4 and P2 of the substrate) (Fig. 2A). The durable H-bonds between P1-Cys and the backbone of both Gly-193 and Ser-195 stabilized the oxyanion hole in Pr3 and the prime side of substrate 8 binds Pr3 via strong H-bonds between P2’-Leu and Phe-41 and between the side chains of P5’-Glu and Arg-143. As shown in Fig. 2B, the interactions between substrate 8 and HNE were weaker though very similar to the ones described for Pr3; the oxyanion hole was stabilized via proper interactions at P1; H-bonds between P5-Ile and Arg-217 and between P3-Thr and Val-216 were observed at the nonprime side and between P2’-Leu and the backbone of Phe-41, P6’-Gln, and Asn-61 at the prime side.

MD simulations of Pr3 bound to substrates 9 and 10 were analyzed to determine the importance of the P1’ position. The resulting interaction networks of substrates 9 and 10 with Pr3 were quite similar to that between Pr3 and substrate 8 described above, except that the interaction between the prime side (in particular P5’) of the substrate and Arg-143 was less strong (Fig. 2, C and D). The main differences were caused by the nature of the residue at P1’. When P1’ was negatively charged (substrate 10) it formed a strong H-bond (lifetime >40%) with Arg-60 of Pr3. But when P1’ was an arginine (substrate 9), there were strong interactions (H-bonds lifetime >80%) with the polar charged side chain of Asp-61 in Pr3. Because these interactions involved residues of opposite charges, they probably helped to stabilize the enzyme-substrate complexes.
The network of hydrogen bonds between the residues of the catalytic triad is an indication of how well the active site was preserved in each of the complexes; the lifespans of hydrogen bonds crucial for the catalytic reaction during the MD simulations are reported in Table 3. When Pr3 was complexed with the three substrates 8–10, there were durable hydrogen bonds between the three residues of the catalytic triad, showing that the active site is not disrupted by the different residues at P1'.

We then modeled the complex between Pr3-HNE and substrate 11 to investigate the consequences of having a positively charged residue (Lys) at P2'. Fig. 2 (E and F) shows a snapshot of the complexes with Pr3 and HNE. There was a strong repulsion between P2'-Lys and Arg-143 of Pr3. This repulsion was probably the reason why the tail of the substrate became exposed to solvent. There was no interaction of its negatively charged residue at P5 with Arg-143. In addition, there were new H-bonds and hydrophobic contacts between the essential histidine of the active site of Pr3 and the P2, P1, and P1' residues of the substrate. For example, the H-bond between P1-Cys and the N\textsuperscript{92} of His-57 was present for 98% of the simulation time. These interactions competed with the hydrogen bond network of the catalytic triad, which was consequently greatly disrupted; the calculated lifespans of the His-Ser and Ser-Asp hydrogen bonds (Table 3) during the simulations were 7% and 0%, respectively. In contrast, the interaction network between substrate 11 and HNE (Fig. 2F) was very similar to that between HNE and substrate 8, described above (Fig. 2B). The only differences were a loss of the H-bond between Asn-61 and P6', a reduction in the hydrophobic contacts between Phe-41 and both P1' and P2', and unexpected hydrophobic contacts between P1' and Gly-193 and between P4' and Gly-39. The H-bond network in the catalytic triad of HNE was not disrupted by the presence of substrate 11 (Table 3).

Nonprime Pr3 Specificity: Influence of the Lys-99/Leu and Ile-217/Arg Substitutions in Pr3 and HNE—Another critical difference between the three residues of the catalytic triad, showing that the active site is not disrupted by the different residues at P1'.

We replaced the P2-Asp residue by an Arg residue in the lead Pr3 substrate Abz-VA\textsuperscript{R}CRDRQ-EDDnp (substrate 6). The specificity constant measured using Abz-VA\textsuperscript{R}CRDRQ-EDDnp (Table 4, substrate 12), was 60-times lower than that with Abz-VA\textsuperscript{R}CRDRQ-EDDnp. The network of hydrodynamic bonds preserved in each of the complexed; the lifespans of hydrogen bonds crucial for the catalytic reaction during the MD simulations are reported in Table 3. When Pr3 was complexed with the three substrates 8–10, there were durable hydrogen bonds between the three residues of the catalytic triad, showing that the active site is not disrupted by the different residues at P1'.
VADCRDQR-EDDnp. This decrease in $k_{cat}/K_m$ depended mainly on a decrease in $k_{cat}$ but not in $K_m$ that remained in the micromolar range. This indicates that charge distribution at P2 does not impair substrate binding but helps optimize substrate orientation within the active site. There was also slight but significant hydrolysis of this P2-Arg containing substrate by HNE, whereas the P2-Asp substrate was completely resistant (Table 4, substrates 6 and 12). We obtained the same result with an Asp at P2 in the polyvalent substrate derived from the monocytic neutrophil elastase inhibitor reactive loop (substrate 13). This was an excellent substrate for Pr3 and was cleaved slowly by HNE (Table 4). MD simulations also showed favorable interactions in the complex between substrate 13 and Pr3, including strong H-bonds between residues with opposite charges: P2-Lys-99, P1-Asp-61, P2'-Arg-143, and P3'-Asp-61. However, none of the charged residues of the substrate (at P2, P1', P2', P3', or P5') found a partner in HNE when the substrate 13 was bound to HNE. Predictions of subsite positions within the Pr3 active site also showed that Lys-99 was close to the hydrophobic S4 subsite, which is smaller in Pr3 than in HNE (5). The two proteases differ in this area in that HNE has an Arg at position 217, whereas Pr3 has an Ile. Ile/Arg-217 together with Trp-218 probably contribute to the P4/P5 specificity. The importance of a charged residue at position 217 in HNE is also illustrated by the discovery that an Arg-217/Gln mutation is responsible for a severe form of neutropenia (OMIM 130130), although no relationship has been established so far between the disease and the protease catalytic activity (30). We first compared the hydrolysis of Abz-GIATFCMLMPEQ-EDDnp (substrate 8), that is hydrolyzed by both HNE and Pr3, with that of Abz-GATFCMLMPEQ-EDDnp (substrate 14), which has an Arg at P5. The Arg/Ile substitution at position 217 did not significantly modify the $k_{cat}/K_m$ of either protease, suggesting that P5 is not important for substrate specificity (Table 4). We set up the MD simulations of substrate 14 bound to Pr3 and HNE and calculated the interaction networks between this substrate and the two proteases (Fig. 2, G and H). The partners found by the substrate in Pr3 and HNE were very similar to those described for substrate 8 (Fig. 2, A and B). The introduction of an Arg at P5 induced repulsion with the Lys-99 of Pr3, which lead to the loss of interactions between Lys-99 and residues at position P4 and P2 of the peptide. However, this was the only noticeable change in enzyme-substrate interactions, both the important oxyanion hole at P1 and the favorable S'-P' interactions were maintained. The findings for HNE were similar; with the exception of the repulsion between P5-Arg and Arg-217, the partners in HNE found by substrate 14 were similar to those found by substrate 8.

We investigated the influence of a positive charge from P4 to P6 on peptide cleavage by Pr3 and HNE using a series of peptides derived from Abz-GIATFCMLMPEQ-EDDnp (substrate 8) that lacked first the Abz group (+GIATFCMLMPEQ-EDDnp), then one (+ATFCMLMPEQ-EDDnp) and two (+ATFCMLMPEQ-EDDnp) residues, to produce peptides bearing an NH$_3^+$ at P6, P5, and P4, respectively. Their hydrolysis was investigated by reversed-phase HPLC on a C18 cartridge working with constant amounts of substrate and protease, because they lacked a fluorescence group. Both proteases hydrolyzed the peptides with N-terminal charges from P4 to P6 at rates that did not significantly differ from those for the lead substrate (Abz-GIATFCMLMPEQ-EDDnp) (not shown). We conclude that a positive charge from P4 to P6 does not significantly affect hydrolysis by Pr3 or HNE. Further, the presence of residues beyond P4 does not seem to be important for hydrolysis. MD simulations confirmed these results, showing that a positive charge at P5 affected only the S4 subsite as a result of the repulsion with Lys-99, without any other significant change (not shown).

Unprimed Specificity: Replacing Cys at P1 to Obtain Oxido-resistant Pr3 Substrates—Most of the Pr3 FRET substrates we have developed so far contain an oxidizable Cys residue at P1 (8, 29). This does not impair measurements of purified Pr3 activity that can be made in a reducing agent-containing buffer but could be a drawback when measuring Pr3 activity in a strongly oxidative environment such as inflammatory, neutrophil-containing, biological fluids. Preliminary experiments designed to measure Pr3 activity at the neutrophil surface showed that Cys-containing substrates were not fully hydrolyzed, thus impairing kinetic measurements. We exposed a gently stirred 10 $\mu$m solution of Abz-VADCAQ-EDDnp to ambient air for 2 h and separated the products by reversed-phase HPLC on a C18 cartridge. The air-exposed peptide was eluted with a longer retention time than the native substrate and was completely resistant to cleavage by Pr3. This was fully reversible by incubation with dithiothreitol, which strongly suggests that an inactive substrate dimer had formed due to oxidation of the P1 cysteine
Substrate Specificity of Human Neutrophil Protease 3

Protease 3

Abz-GIATFCMLMPEQ-EDDnp

Elastase

Abz-GIATFCMLMPEQ-EDDnp

P1'

C

Abz-GIATFCRLMPEQ-EDDnp

D

P2'

E

Abz-GIATFCMKEPQ-EDDnp

F

P5

G

Abz-GRATFCMLMPEQ-EDDnp

H
TABLE 3
Catalytic triade hydrogen bond networks
The crucial hydrogen bonds were monitored during MD simulations, and their lifespans are given as a percentage of the whole simulation time. Simulations of HNE were performed only with substrates 8, 11, 13, and 14.

| Atom name | Substrate number | Pr3 | HNE |
|-----------|------------------|-----|-----|
| N6-His-57 | O2-Oter-195      | 8   | 99  |
| 9         | 97               |     |     |
| 10        | 96               | 95  | 94  |
| 11        | 7                | 7   |     |
| 13        | 92               | 92  |     |
| 14        | 99               | 99  |     |
| O31-Asp-102 | N3 | 8   | 63  |
| 9         | 91               |     |     |
| 10        | 78               |     |     |
| 11        | 0                | 0   |     |
| 13        | 47               | 47  |     |
| 14        | 83               | 83  |     |

TABLE 4
Influence of positively charged residues at P2 and P5 on the specificity of Pr3 and HNE as deduced from specificity constants $k_{cat}/K_m$ values are the means of $n \geq 4$ experiments. The error for $k_{cat}/K_m$ is $<15%$. The arrow indicates protease 3 cleavage sites.

| No. | Substrate | $k_{cat}/K_m$ |
|-----|-----------|---------------|
|     |           | Pr3 | Elastase |
| 6   | Abz-VADCDRQ-EDDnp | 6500 | NSH* |
| 12  | Abz-VARCRDQ-EDDnp | 105  | 8.1  |
| 13  | Abz-GIATDCRDPRQ-EDDnp | 2000 | 5.1  |
| 14  | Abz-GRAFCHLMPEQ-EDDnp | 834  | 1520 |

**NSH**, no significant hydrolysis.

residues. We inserted the closest oxidizable structural homologue of cysteine, norvaline, at P1 in selected Pr3 substrates as to retain the advantage of the efficient accommodation of Cys at P1 in Pr3 substrates (Table 5). The specificity constants compared well with those obtained with the Cys-containing substrates, and the substrates were not cleaved by HNE (Table 5). Abz-VADnVADYQ-EDDnp (Table 5, substrate 16) appeared to be the most sensitive Pr3 substrate ever reported ($k_{cat}/K_m$ = 7,000 mom⁻¹ s⁻¹).

Specific Pr3 Cleavage of Protein Substrates—Several putative biological functions of Pr3 have been deduced from the identification of specific cleavage sites within target proteases like NFkB (19), p21 (18), and pro-TNFα (31, 32). We have prepared FRET peptides based on the sequences cleaved in these target proteases, and have measured the specificity constants with Pr3 and HNE. All FRET peptides contained 4 residues on both their nonprime and their prime sides to take into account the results reported above. NFkB- and p21-derived peptides were cleaved at the expected site by Pr3 but not by HNE as in the parent molecule, whereas the pro-TNFα-derived peptide was also cleaved by HNE at the same site but at a lower rate. Specificity constants for NFkB- and p21-derived peptides compare well with those derived from the lead sequence used to prepare the specific Pr3 substrates (Table 6) (8, 29). However, Cys was preferred to Ala at P1 as judged from the values obtained with substrates 6 and 20 (Table 6). Their sequence was also very similar to that of the substrates deduced from the structural and kinetic analysis, so that a consensus sequence can be deduced that is preferentially accommodated within the Pr3 active site. This bears 4 residues on each side of the sensitive bond, including small aliphatic residues such as Ala and Val or Cys at P1, a positively charged residue at P1’, and negatively charged residues at P2’ and P2.

**DISCUSSION**

Although serine proteases from the neutrophil primary granule, Pr3, HNE, and cathepsin G all hydrolyze extracellular proteins at inflammatory sites, each of them has other unrelated, specific functions (33, 34). The simultaneous presence of these three proteases in neutrophils, where they are stored within the granule, or in the extracellular space when cells are activated and degranulate, greatly complicates investigation of their individual function. Nevertheless, it has been shown that Pr3 has specific properties (see Ref. 34 for a recent review), although the molecular reasons that make this protease unique...
are not well understood. Developing molecular tools that specifically interact with Pr3 may therefore help elucidate its biological function. However, no specific Pr3 substrate was available until recently due to the importance of the S' subsites for substrate binding (8, 9, 29). S' subsites cannot be investigated using conventional chromogenic or fluorogenic substrates but require FRET peptides whose sequences extend on both sides of the cleavage site. We and others prepared such substrates for neutrophil serine proteases and demonstrated the importance of the S2' subsite for discriminating between the activities of Pr3 and HNE (8, 9, 29).

Inspection of the cell surface potential of Pr3 and HNE active sites based on the Pr3 three-dimensional structure and molecular modeling studies revealed that they differ not only at S2' but also at S4, S2, S1', and S3 subsites (25), but it is surprising that Pr3 and HNE, whose primary sequences are very similar, differ mainly by their charged surface residues. The presence of Asp-61 at the interface between the S1' and S3' subsites in Pr3 suggests that Pr3 preferentially accommodates a positively charged residue at P1' and P3'. However, kinetic and MD studies indicate that this is not a prerequisite at neither S1' nor S3'. The S3' subsite of Pr3 accommodates any type of residue without any significant change in the specificity constant, but a C-terminal extension of the peptide chain beyond the S3' subsite significantly improves the specificity constant. This participates in the discrimination between Pr3 and HNE, because the favorable effects of the S'-P' interaction in the latter is limited to the S1' subsite (35). Although positively charged residues are preferred at S1' in Pr3, probably because they form ionic interactions with Asp-61 (25), a negatively charged residue can be also accommodated at that position and even better than a small hydrophobic residue (29), which suggests that the S1' subsite is exposed to solvent. This agrees with the data from MD simulations showing that water molecules can come close to, and interact with, the residue in the S1' subsite. We deduced from these results that the sequence positive/negative/positive or neutral residues at P1', P2', and P3' is the consensus sequence to optimize interactions with the S' Pr3 subsites, in agreement with molecular dynamic simulations (18, 25).

Inspection of the charge-surface potential of Pr3 and HNE active sites also shows that the Pr3 S2 subsite is positively charged, due to the presence of a Lys residue at position 99, instead of a Leu in HNE. Accordingly, a negatively charged residue at P2 is preferred by Pr3 substrates, although a positive charge can be accommodated in keeping with previous results (8). MD simulations predicted that the aliphatic part of the Lys-99 side chain also participates in the Pr3 S4 specificity, together with Phe-215 and Ile-217 (25). These two residues, plus Trp-216, define a hydrophobic environment that is not found in HNE due to the presence of an Arg at position 217 (Fig. 1, B and C). This position is even more important in HNE, because the Arg-217/Gln mutation in HNE is responsible of severe neutropenia in humans (36, 37). However, a positively charged residue at P4 and P5, which are thought to interact with residue 217 in both Pr3 and HNE, does not significantly alter peptide cleavage, suggesting that position P4/P5 is not essential for the specificities of Pr3 and HNE. This also suggests that the Arg-217/Gln mutation in HNE will not alter its catalytic efficiency and its interaction with inhibitors of the serpin family, in agreement with previous results (30). Nevertheless, the length of the peptide chain is important for the catalytic activity of HNE (38). Optimized substrates contain at least 4 residues on each side of the sensitive peptide bond.

Specific substrates of Pr3 must also have physicochemical properties that enable them to be used in an oxidative environment, such as may occur in vivo or ex vivo in a complex biological fluid. This is especially true when Pr3 activity is measured at the surface of activated neutrophils that release reactive oxygen species (39). We showed here a spontaneous oxidation of the P1-Cys-containing substrates when exposed to air, leading to substrate dimerization via disulfide bond formation and further impairment of their hydrolysis by Pr3. Experiments must therefore be carried out in the presence of reducing agents that may interfere or modulate the activity of other components in the reaction mixture. We have avoided this problem by using the unnatural Cys homologue norvaline at P1, which gives similar or even better results than a P1-Cys, but Val residues can also be used (9, 25). Nevertheless, some of the protein substrates of Pr3 are cleaved at Cys residues (19). We have synthesized three FRET peptides based on the sequences of known protein targets of Pr3 but not of HNE. They contained 4 amino acid residues on each side of their putative sensitive peptide bond. Two of them that were derived from p21 and NFκB were cleaved by Pr3 but not by HNE with specificity constants that are in the same range as those of the optimized FRET substrates developed in this study. These peptides are therefore very reliable tools that mimic the cleavage of physiological target proteins of neutrophil serine proteases. Furthermore, they contained the consensus sequence deduced from the kinetic analysis and molecular modeling. This consensus sequence may be used to predict cleavage sites within so far unidentified Pr3 targets or in Pr3 targets whose cleavage site has not been yet identified, because it extends some distance on both sides of the cleavage site and involves residues at several positions. For example, the pro-inflammatory cytokine IL-18 is processed by caspase 1, caspase 3, and Pr3, but only the sites cleaved by the two former proteases have been identified (40). Inspection of the primary sequence of pro-IL-18 revealed the presence of a putative Pr3 target sequence close to the caspase 3 cleavage sites but remote from the predicted Pr3 cleavage sites (41). This putative Pr3 cleavage site in the prosequence of IL-18 is located at the C–R bond in the sequence, -MDT73SDCRD76NA-, which fulfills most of requirements for Pr3 cleavage, i.e. a negatively charged residue at P2 and P2', a Cys at P1, and an Arg at P1', and contains the two processing sites by caspase 3 at D–S and D–N bonds. The preference of Pr3 for Asp residues at P2 and P2' explains why Pr3 behaves as a caspase 3-like protease (34). Caspase 3 preferentially cleaves after Asp residues within sequences that have another Asp residue at position P4 (42, 43). Thus, the two Asp residues at P1 and P4 in caspase 3 substrates can be superimposed on those at P2 and P2' in Pr3 substrates, explaining why Pr3 and caspase 3 may cleave the same target proteins, although at neighboring sites. This holds true also for NFκB that is cleaved by Pr3 two amino acids upstream of the reported caspase 3 site (19).
There is now growing evidence that human neutrophil Pr3 has roles other than that of a redundant protease that mimics neutrophil elastase in breaking down extracellular proteins, or caspases in activating the cytokine network. More has to be done to understand its biological function, especially in the regulation of immune function. This will require drugs that specifically interact with this protease. Optimized sequences that allow close interaction with the extended active site of Pr3 will now be used to develop peptide or pseudopeptide inhibitors that do not interfere with Pr3-related proteases, especially HNE.

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REFERENCES
1. Kao, R. C., Wehner, N. G., Skubitz, K. M., Gray, B. H., and Hoidal, J. R. (1988) J. Clin. Invest. 82, 1963–1973
2. Rao, N. V., Marshall, B. C., Gray, B. H., and Hoidal, J. R. (1993) Am. J. Respir. Cell Mol. Biol. 8, 612–616
3. Owen, C. A., and Campbell, E. J. (1999) J. Leukoc Biol. 65, 137–150
4. Campbell, E. J., Campbell, M. A., and Owen, C. A. (2000) J. Immunol. 165, 3366–3374
5. Fujinaga, M., Chernaia, M. M., Halenbeck, R., Koths, K., and James, M. N. (1991) J. Biol. Chem. 266, 267–278
6. Rao, N. V., Wehner, N. G., Marshall, B. C., Gray, W. R., Gray, B. H., and Hoidal, J. R. (1991) J. Biol. Chem. 266, 9540–9548
7. Brubaker, M. J., Groutas, W. C., Hoidal, J. R., and Rao, N. V. (1992) Biochem. Biophys. Res. Commun. 188, 1318–1324
8. Korkmaz, B., Attucci, S., Hazouard, E., Ferrandiere, M., Jourdan, M. L., Brillard-Bourdet, M., Juliano, L., and Gauthier, F. (2002) J. Biol. Chem. 277, 39074–39081
9. Koehl, C., Knight, C. G., and Bieth, J. G. (2003) J. Biol. Chem. 278, 12609–12612
10. Kam, C. M., Kerrigan, J. E., Dolman, K. M., Goldschmeding, R., Von dem Borne, A. E., and Powers, J. C. (1992) FEBS Lett. 297, 119–123
11. Korkmaz, B., Poutrain, P., Hazouard, E., de Monte, M., Attucci, S., and Gauthier, F. L. (2005) Am. J. Respir. Cell Mol. Biol. 33, 2840–2849
12. Presto, G. A., Zarella, C. S., Pendergraft, W. F., 3rd, Rudolph, E. H., Yang, J. J., Sekura, S. B., Jennette, J. C., and Falk, R. J. (2002) J. Am. Soc. Nephrol. 13, 2840–2849
13. Pendergraft, W. F., 3rd, Rudolph, E. H., Falk, R. J., Jahn, J. E., Grimmler, M., Hengst, L., Jennette, J. C., and Preston, G. A. (2004) Kidney Int. 65, 75–84
14. van der Woude, F. J. (1985) Lancet. 2, 48
15. Gauthier, F. L. (2005) J. Biol. Chem. 280, 30242–30253
16. Dublet, B., Ruello, A., Pederzoli, M., Hajjar, E., Courbebaisse, M., Cante- loup, S., Reuter, N., and Witko-Sarsat, V. (2005) J. Biol. Chem. 280, 30242–30253
17. Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P., and Halb-wachs-Mecarelli, L. (2000) Lab. Invest. 80, 617–653
18. Akita, K., Ohsu, T., Nakada, Y., Tamimoto, T., Namba, M., Okura, T., Takakura-Yamamoto, R., Torigoe, K., Gu, Y., Su, M. S., Fujii, M., Satoh-Itoh, M., Yamamoto, K., Kohno, K., Ikeda, M., and Kurimoto, M. (1997) J. Biol. Chem. 272, 26595–26603
19. Fantuzzi, G., Dinarello, C. A. (1999) J. Clin. Immunol. 19, 1–11
20. Steinnicke, H. R., Renatus, M., Meldal, M., and Salvesen, G. S. (2000) Biochem. J. 350, 563–568
21. Wei, Y., Fox, T., Chambers, S. P., Sintchak, J., Coll, T. J., Golec, J. M., Swenson, L., Wilson, K. P., and Charifson, P. S. (2000) Chem. Biol. 7, 423–432
22. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162
23. Hengst, L., Jennette, J. C., and Preston, G. A. (2004) Kidney Int. 65, 75–84
24. van der Woude, F. J. (1985) Lancet. 2, 48
25. Golschmeding, R., van der Schoot, C. E., ten Bokkel Huinink, D., Hack, C. E., van den Ende, M. E., Kallenberg, C. G., and von dem Borne, A. E. (1989) J. Clin. Invest. 84, 1577–1587
26. Rarok, A. A., Limburg, P. C., and Kallenberg, C. G. (2003) J. Leukoc. Biol. 74, 297–311
27. Takakura-Yamamoto, R., Torigoe, K., Gu, Y., Su, M. S., Fujii, M., Satoh-Itoh, M., Yamamoto, K., Kohno, K., Ikeda, M., and Kurimoto, M. (1997) J. Biol. Chem. 272, 26595–26603
28. Fantuzzi, G., Dinarello, C. A. (1999) J. Clin. Immunol. 19, 1–11
29. Steinnicke, H. R., Renatus, M., Meldal, M., and Salvesen, G. S. (2000) Biochem. J. 350, 563–568
30. Wei, Y., Fox, T., Chambers, S. P., Sintchak, J., Coll, T. J., Golec, J. M., Swenson, L., Wilson, K. P., and Charifson, P. S. (2000) Chem. Biol. 7, 423–432
31. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162