Compared action of neutrophil proteinase 3 and elastase on model substrates. Favorable effect of S’-P’ interactions on proteinase 3 catalysis

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Running title: Neutrophil proteinase 3 catalytic activity
FOOTNOTES

1Abbreviations: Pr3: human neutrophil proteinase 3, NE: human neutrophil elastase, Suc: succinyl, MeOSuc: methoxysuccinyl, Ac: acetyl, Boc: tert-butyloxycarbonyl, pNA: p-nitroanilide, L2p: Lysyl-(2-picolinoyl), Mca: (7-methoxycoumarin-4-yl) acetyl, Dpa: N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl, TFA: trifluoroacetic acid, the S and S’ subsites of the substrate binding site and the corresponding P and P’ residues of the substrate are labeled according to the nomenclature of Schechter and Berger [1].
ABSTRACT

Neutrophil proteinase 3 (Pr3) and elastase (NE) may cause lung tissue destruction in emphysema and cystic fibrosis. These serine proteinases have similar P₁ specificities. We have compared their catalytic activity using acyl-tetrapeptide-\( p \)-nitroanilides which occupy the S₅ – S₁ subsites of their substrate binding site and intramolecularly-quenched fluorogenic heptapeptides which theoretically bind at S₅ – S₄. Most \( p \)-nitroanilide substrates are turned over slowly by Pr3 as compared to NE. These differences disappear with the fluorogenic heptapeptides some of which are hydrolyzed even faster by Pr3 than by NE: elongation of substrates strongly increases the catalytic efficiency of Pr3 whereas it has little effect on NE catalysis. These different sensitivities to S’- P’ interactions show that Pr3 and NE are not interchangeable enzymes despite their similar P₁ specificity.
INTRODUCTION

The azurophilic granules of polymorphonuclear neutrophils contain three serine proteinases, elastase (NE)\(^1\) cathepsin G and proteinase 3 (Pr3), which participate in lysosomal bacterial digestion and neutrophil migration through the extracellular matrix at sites of inflammation. These enzymes are ca. 30 kDa glycoproteins which belong to the chymotrypsin family of serine proteinases. Pr3 is the most recently discovered, the most difficult to isolate and, hence, the less well studied proteinase of the three. It is identical to three independently-discovered proteins: (i) myeloblastin which regulates the growth and differentiation of leukemic cells, (ii) p29b which has microbicidal activity, (iii) the target antigen of antineutrophil cytoplasmic autoantibodies detected in patients with Wegner’s granulomatosis [2]. Pr3 cleaves extracellular matrix proteins including elastin, type IV collagen, fibronectin, laminin and vitronectin [3, 4]. It is able to produce lung emphysema in hamsters [3] and may thus, in concert with NE and cathepsin G, be responsible for lung tissue destruction occurring in emphysema and cystic fibrosis.

While many model substrates have been used to map the active site of NE [5-8], literature on the substrate specificity and the catalytic activity of Pr3 is poorly documented. Fuginaga et al. [9] have shown that the two enzymes have similar substrate-binding sites. This explains why both proteinases cleave the oxidized insulin A and B chains at peptide bonds involving small aliphatic amino acid residues [4]. The specificity of Pr3 for non bulky residues has been confirmed with a limited number of model substrates [4, 10-12]. As a rule, these substrates were turned over at a much lower rate by Pr3 than by NE, an unexplained observation. We have undertaken the present work in order to understand this puzzling finding.
MATERIALS AND METHODS

Materials

NE was isolated and active site titrated as described previously [13]. Pr3 came from ART, Athens GA, USA and was titrated with α1-proteinase inhibitor [4]. This enzyme preparation was electrophoretically pure. The presence of NE was tested by reacting 0.14 µM Pr3 with increasing amounts of secretory leukoprotease inhibitor (1 - 8 µM, final concentration) which inhibits NE but does not inhibit Pr3 [3]. The buffer was the same as that used for enzyme kinetics. After 5 min at 25°C, 2.1 mM MeOSuc-Ala2-Pro-Val-pNA, a very sensitive NE substrate [5], was added to the mixtures and the enzymic rates were measured at 410 nm and 25°C. The lack of enzyme activity indicated that this commercial preparation of Pr3 was free of contaminating NE. Most p-nitroanilide substrates were purchased from Bachem, Bubendorf, Switzerland. Suc-Ala2-Asp-Val-pNA, Suc-Ala2-Glu-Val-pNA, Suc-Leu-Val-Glu-Ala-pNA, Suc-Ala4-pNA, MeOSuc-L2p-Tyr-Asp-Ala-pNA, and MeOSuc-L2p-Tyr-Asp-Val-pNA were synthesized by ESP, Livermore, CA, USA. The intramolecularly quenched fluorogenic substrates Mca-peptide-Dpa-NH2 were synthesized using the same methodology as earlier [14]. The compounds were purified by reverse-phase chromatography and their identity was confirmed by mass spectrometry. All substrates were dissolved in dimethylformamide. Their concentration was checked spectrophotometrically using ε315nm = 14,600 M⁻¹ cm⁻¹ and ε410nm = 7,500 M⁻¹ cm⁻¹

for p-nitroanilides and fluorescent substrates, respectively.

Kinetics of substrate hydrolysis

All kinetic measurements were done at 25°C and pH 7.4 (50 mM Hepes, 150 mM
The enzymatic reactions were initiated by adding a small volume of enzyme solution to the buffered substrate solution contained in a spectrophotometer or a fluorometer cuvette. The final concentration of dimethylformamide was 5% (v/v) throughout.

The initial rate of p-nitroanilide hydrolysis was measured at 410 nm using variable concentrations of substrate and constant concentrations of enzyme. For the most sensitive substrates, the final Pr3 and NE concentrations were 140 nM and 60 nM, respectively. For the less sensitive substrates 10 to 50-fold higher enzyme concentrations were used. The kinetic parameters \( k_{cat} \) and \( K_m \) were calculated from nonlinear least square fits to the Michaelis-Menten equation (Enzfitter software). The Pr3-catalyzed hydrolysis of Suc-Ala4-pNA was so slow that \( k_{cat} \) and \( K_m \) could not be determined separately. We therefore recorded the full hydrolysis of 10 – 30 µM Suc-Ala4-pNA by 3.3 µM Pr3. The \( k_{cat}/K_m \) ratio for this enzyme-substrate pair was calculated from the progress curve as outlined below for the fluorogenic substrates.

The cleavage of the fluorogenic substrates was monitored at \( \lambda_{ex} = 328 \text{ nm} \) and \( \lambda_{em} = 393 \text{ nm} \) [14] using substrate concentrations below 10 µM to avoid absorptive fluorescence quenching of the hydrolysis product. The absorptive quenching also precluded the separate measurement of \( k_{cat} \) and \( K_m \). The hydrolysis of the fluorogenic substrates was therefore followed to completion. At low substrate concentrations ([S]₀ < \( K_m \)) the Michaelis-Menten equation simplifies to \( v = k_{cat}/K_m[E]_0[S]_0 \) so that the release of product P with time is a first-order reaction described by \( d[P]/dt = k_{obs} [S] \) where \( k_{obs} = k_{cat}/K_m[E]_0 \). The pseudo-first-order rate constant \( k_{obs} \) was calculated by nonlinear regression analysis of progress curves recorded using 0.5 – 5 µM substrate concentrations and 0.1 – 1 µM enzyme.
concentrations. The progress curves were all first order and $k_{\text{obs}}$ did not significantly change with $[S]_0$. This indicates that $[S]_0$ was indeed lower than $K_m$.

**Site of substrate cleavage**

The $p$-nitroanilide substrates (2µM) dissolved in the above buffer + 5% dimethylformamide were reacted with 0.3 – 15 µM enzyme in a total volume of 1 ml. After about 20% substrate hydrolysis, the reaction was stopped with 10 µl TFA, the medium was diluted 200-fold with buffer A (buffer + 0.1% TFA) and 40 µl of this dilution was applied to a 3.9 x 100 mm C18 Novo-pack Column equilibrated with buffer A. A linear gradient formed with buffer A and buffer B (0.1% TFA in acetonitrile) was used to separate the reaction products. Elution was followed at 214 nm at a flow rate of 0.8 ml min$^{-1}$.

The fluorogenic substrates (5 µM) dissolved in the buffer + 5% dimethylformamide were reacted with 30 nM to 1.6 µM enzyme in a total volume of one ml. After about 50% hydrolysis, the reaction was stopped with 30 µl glacial acetic acid. The digests were absorbed on C18 reverse phase cartridges, washed with 5% acetonitrile to remove the salts and then the peptide fragments eluted with 60% acetonitrile. The eluant mixtures were rotary evaporated to dryness and taken up in 0.1 ml of 60% acetonitrile. The fragments were identified by electrospray ionization mass spectrometry. The fragment signals were 10-100 times that of the background.

**RESULTS**

**Compared action of Pr3 and NE on acyl-tetrapeptide p-nitroanilides**

All substrates listed in table I are hydrolyzed more or less efficiently by NE but many of them are more resistant to Pr3 than to NE. The proteolytic coefficient $k_{\text{cat}}/K_m$ may be up
to 380-fold lower for Pr3 than for NE (see substrate 1). For compound 8, the best NE substrate [5], the difference is 100-fold. With a few exceptions, the differences in reactivity are due to differences in $k_{\text{cat}}$. Very poor NE substrates are not hydrolyzed at all by Pr3 (see nb 10, 11, 13). Also, none of the substrates Suc-Ala-Ala-Pro-X-pNA ($X = \text{Asp, Glu, Lys, and Orn}$) is hydrolyzed by either NE or Pr3. The poor catalytic power of Pr3 is not due to nonproductive enzyme-substrate binding since HPLC analyses showed that the only reaction products resulting from partial hydrolysis by Pr3 (or NE) were acyl-tetrapeptides + $p$-nitroaniline, which indicates that binding involves subsites $S_5$ to $S'_1$ of the active center.

A number of compounds are cleaved by Pr3 and NE with comparable proteolytic coefficients. These are either poor (nb 12, 14, 15) or good NE substrates (nb 18-20). Compound 19 is the only $p$-nitroanilide whose $k_{\text{cat}}$ for Pr3 is significantly higher than that for NE.

**Compared action of Pr3 and NE on fluorogenic peptides**

We have synthesized a number of heptapeptides to cover both S and S’ subsites of the active center of Pr3 and NE. To follow their hydrolysis using fluorescence emission, we have included the fluorescent label Mca and the fluorescence quencher Dpa [14] at their N-and C-terminus, respectively. For reasons given in the experimental section, $k_{\text{cat}}$ and $K_m$ could not be determined separately. Their ratio is, however, meaningful since (i) it represents the second-order acylation rate constant whether acylation is rate-limiting or not and whether substrate binding is productive or not [15], (ii) it is a good measure of specificity [16].

The cleavage sites of the fluorogenic substrates were determined by electrospray ionization mass spectrometry. The masses (Da) of the N-terminal peptides generated by Pr3 and NE were 609.5 and 609.3 for substrate 21 (theoretical mass of Mca-Ala-Ala-Pro-Leu =
609.7), 547.1 and 545 for substrate 22 (Mca-Ala₄ = 541.5), 839.7 and 839.4 for substrate 24 (Mca-L₂p-Tyr-Asp-Ala = 839.8), 867.7 and 865.5 for substrate 25 (Mca-L₂p-Tyr-Asp-Val = 867.9), 880.8 and 879.5 for substrate 26 (Mca-L₂p-Tyr-Asp-Ile = 881.9), 789.3 and 788.2 for substrate 27 (Mca-L₂p-Val-Glu-Ala = 789.8). The N-terminal peptide generated by the action of NE on substrate 23 had a mass of 595.6 (Mca-Ala₂-Pro-Val = 596.6). The Pr₃ + substrate 23 mixture did not give a clear-cut mass profile due to paucity of material.

Table II shows that Pr₃ hydrolyses most fluorogenic substrates very rapidly. It is even more effective than NE on compounds 24 to 27. Table II also compares the proteolytic coefficients of Pr₃ and NE on six heptapeptidic and tetrapeptidic substrates encompassing identical N-terminal tetrapeptidic sequences. It can be seen that the catalytic activity of Pr₃ dramatically increases with peptide chain elongation while that of NE does not significantly vary with substrate length.

DISCUSSION

Pr₃ and NE belong to the chymotrypsin-like family of serine proteinases whose active site is composed of a substrate-binding site responsible for specificity and of a catalytic site responsible for substrate hydrolysis. The later is formed of a highly conserved Asp-His-Ser triad whose hydrogen bonding transforms the serine $O_\gamma$ into a powerful nucleophile that attacks the scissile peptide bond of the substrate. The efficacy of catalysis is due in part to a precise binding of substrate with resultant proper orientation of the scissile bond with respect to $O_\gamma$ of the catalytic serine [17]. Fuginaga et al. [9] have shown that Pr₃ and NE have similar substrate-binding sites which accounts for their preference for small aliphatic residues at $P_1$ [4]. However, the use of some model substrates and inhibitors indicated that Pr₃ is a much poorer catalyst than NE [4, 10-12]. We thought this might be due to the fact that Pr₃ (but not
NE) binds these compounds in a predominantly non-productive way. We therefore used a series of acyl-tetrapeptide-p-nitroanilides which all bind productively at subsites S5 to S'1. This series included substrates with P2 = Asp or Glu to explore the possibility of electrostatic S2-P2 interactions [9]. Since the catalytic differences persisted for most of these compounds, we hypothesized that occupancy of subsites S5 to S'1 of Pr3 may not be sufficient to align the substrate in a position favorable for efficient catalysis whereas it may be sufficient in the case of NE. The use of fluorogenic heptapeptides confirmed our hypothesis: elongation of the substrates strongly increased the catalytic efficiency of Pr3 whereas it had much less effect on NE catalysis. Thus, the poor catalytic activity of Pr3 on tetrapeptidic substrates is no longer observed with heptapeptidic substrates some of which are turned over even more rapidly by Pr3 than by NE. It is interesting to note that the rate-enhancing effect due to full occupancy of the S’ region of Pr3 is more pronounced for poor p-nitroanilide substrates (nb 1, 7, 11 with P4 = Ala) than for good ones (nb 19, 20 with P4 = L2p). This suggests that S4-P4 and S’-P’ interactions play a complementary role in binding substrates nb 19 and 20 in a position favorable for catalysis.

Whereas many studies have probed the S region of the substrate binding site of serine proteinases, literature is poorly documented on the S binding region [19]. Stein and Strimpler [7] have shown that the favorable effect of S’-P’ interaction on NE catalysis [18] is mainly due to occupancy of subsite S’1 since there are no important binding interactions available past S’1. This is in agreement with our data showing that elongation of the NE substrates beyond S’1 has no significant effect on catalysis.

The present work is the first report on S’-P’ interactions in Pr3. It explains why
p-nitroanilides [4, 12] and thiobenzyl esters [10, 11] were found to be poor substrates of Pr3. It also allows concluding that Pr3 and NE cannot be considered two interchangeable enzymes despite their common specificity for small aliphatic amino acid residues. Indeed, the divergent effects of S’-P’ binding may lead to different specificities on biological protein substrates. In this context, it is noteworthy that Pr3 and NE show differences in the digestion pattern of fibronectin, lamanin and collagen [4].

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Table I
Kinetics of the hydrolysis of acyl-tetrapeptide p-nitroanilides by Pr3 and NE at pH 7.4 and 25°C. For the sake of clarity the kinetic constants are rounded off and the errors attached to them are not given. The errors on \(k_{\text{cat}}\) and \(K_m\) were equal or lower than 14% and 19% respectively. n.d. = not determined.

| S  | U  | B  | S  | T  | R  | A  | T  | E  | Structure | Enzyme | \(k_{\text{cat}}\) (s\(^{-1}\)) | \(K_m\) (mM) | \(k_{\text{cat}}/K_m\) (M\(^{-1}\) s\(^{-1}\)) |
|----|----|----|----|----|----|----|----|----|------------|--------|--------------------------|-------------|---------------------------------|
| nb | P5 | P4 | P3 | P2 | P1 | P'1|     |    |            |         |                          |             |                                  |
|   | Peptide                   | digest | NE  | NE  |   |
|---|---------------------------|--------|-----|-----|---|
| 1 | Suc-Ala-Ala-Ala-Ala-pNA    | Pr3    | 0.45| 0.13| 3460 |
| 2 | Ac-Ala-Ala-Pro-Ala-pNA     | Pr3    | 0.22| 2.7 | 80  |
| 3 | Suc-Ala-Ala-Pro-Ala-pNA    | Pr3    | 0.23| 1.9 | 120 |
| 4 | Boc-Ala-Ala-Pro-Ala-pNA    | Pr3    | 0.5 | 2.00| 250 |
| 5 | Suc-Ala-Ala-Pro-Abu-pNA    | Pr3    | 0.3 | 0.38| 790 |
| 6 | Ac-Ala-Ala-Pro-Val-pNA     | Pr3    | 1.0 | 2.8 | 360 |
| 7 | Suc-Ala-Ala-Pro-Val-pNA    | Pr3    | 0.2 | 0.6 | 360 |
| 8 | MeOSuc-Ala-Ala-Pro-Val-pNA | Pr3    | 0.5 | 0.47| 1060 |
| 9 | Suc-Ala-Ala-Pro-Nva-pNA    | Pr3    | 0.09| 0.5 | 180 |
| 10| Suc-Ala-Ala-Pro-Met-pNA    | Pr3    | not | hyd| 130 |
| 11| Suc-Ala-Ala-Pro-Leu-pNA    | Pr3    | not | hyd| 190 |
| 12| Suc-Ala-Ala-Pro-Ile-pNA    | Pr3    | 0.27| 0.64| 420 |

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Table I (continued)

|    | peptide                     | Pr3  | hydrolyzed |     |     |
|----|-----------------------------|------|------------|-----|-----|
| 13 | Suc-Ala-Ala-Pro-Nle-pNA     | Pr3  | 0.3        | 90  | 400 |
|    |                             | NE   | 0.65       |     |     |
| 14 | Suc-Leu-Val-Glu-Ala-pNA     | Pr3  | 0.37       |     |     |
|    |                             | NE   | 0.62       | 75  |     |
| 15 | Suc-Ala-Ala-Asp-Val-pNA     | Pr3  | 1.4        |     |     |
|    |                             | NE   | 0.83       |     |     |
| 16 | Suc-Ala-Ala-Glu-Val-pNA     | Pr3  | 0.9        | 97  | 1200|
|    |                             | NE   | 0.83       |     |     |
| 17 | Suc-Ala-Tyr-Leu-Val-pNA     | Pr3  | 1.0        |     |     |
|    |                             | NE   | 0.83       |     |     |
| 18 | MeOSuc-L2p-Ala-Pro-Val-pNA  | Pr3  | 0.6        |     |     |
|    |                             | NE   | 0.14       |     |     |
| 19 | MeOSuc-L2p-Tyr-Asp-Ala-pNA  | Pr3  | 2.7        |     |     |
|    |                             | NE   | 0.03       |     |     |
| 20 | MeOSuc-L2p-Tyr-Asp-Val-pNA  | Pr3  | 2.3        |     |     |
|    |                             | NE   | 0.14       |     |     |

\(^a\)k\(_{cat}\) = 0.49 s\(^{-1}\), K\(_m\) = 0.06 mM, k\(_{cat}/K_m\) = 30 600 M\(^{-1}\) s\(^{-1}\) in 50 mM phosphate buffer pH 7.0 containing 0.05% Triton X-100 and 0.5% dimethylsulfoxide [12]
Table II

Compared catalytic activity of Pr3 and NE on intramolecularly-quenched fluorogenic substrates at pH 7.4 and 25°C. The error on $k_{\text{cat}}/K_m$ is ± 20%. The table also compares fluorogenic and $p$-nitroanilide substrates (from table I) encompassing identical N-terminal tetrapeptidic sequences. Fluo/pNA means $k_{\text{cat}}/K_m$ of fluorogenic substrate/$k_{\text{cat}}/K_m$ of pNA substrates). The arrow indicates the cleavage points. n.h. = no hydrolysis

| nb | Structures | Substrates | Pr3 | NE |
|----|------------|------------|-----|----|
|    |            |            | $k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$) | $k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$) |
|    |            |            | Fluo / pNA | Fluo / pNA |
|    |            |            |        |      |
|   | Peptide (sequence)                           | Series | pNa | Series | Series | Series |
|---|---------------------------------------------|--------|-----|--------|--------|--------|
| 11| Mca-Ala-Ala-Pro-Leu-Lys-Gly-Asp-Dpa          | 115    | -   | 1200   | 6.3    |
|   | NH2                                         |        |     |        |        |
| 22| Suc-Ala-Ala-Pro-Leu-pNa                      | 2600   | 290 | 5000   | 1.4    |
| 7 | Mca-Ala-Ala-Ala-Lys-Gly-Asp-Dpa              | 9      | 3   | 3500   |        |
|   | NH2                                         |        |     |        |        |
| 23| Suc-Ala-Ala-Ala-Ala-pNA                      | 62000  | 172 | 179000 | 2.3    |
| 7 | Mca-Ala-Ala-Pro-Val-Lys-Gly-Asp-Dpa          | 360    | 7   | 77000  |        |
|   | NH2                                         |        |     |        |        |
| 24| Suc-Ala-Ala-Pro-Val-pNa                      | 1100000| 34  | 2300   | 0.34   |
| 19| Mca-Ala-Ala-Pro-Val-Lys-Gly-Asp-Dpa          | 32000  | 6   | 6800   |        |
|   | NH2                                         |        |     |        |        |
| 25| Mca-L2p-Tyr-Asp-Ala-Lys-Gly-Asp-Dpa-NH2      | 4600000| 25  | 88000  | 1.04   |
| 20|                                            | 184600 | 84  | 6000   |        |
| 26| MeOSuc-L2p-Tyr-Asp-Ala-pNA                   | 910000 | 95  | 5000   |        |
| 27| Mca-L2p-Tyr-Asp-Val-Lys-Gly-Asp-Dpa-NH2      | 610000 | 55  |        |        |
|   | MeOSuc-L2p-Tyr-Asp-Val-pNA                   |        |     |        |        |
|   | Mca-L2p-Tyr-Asp-Ile-Lys-Gly-Asp-Dpa-NH2      |        |     |        |        |
|   | Mca-L2p-Val-Glu-Ala-Lys-Gly-Asp-Dpa-NH2      |        |     |        |        |
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