Characterization of Two Azurophil Granule Proteases with Active-site Homology to Neutrophil Elastase*

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Much of the tissue damage associated with emphysema and other inflammatory diseases has been attributed to the proteolytic activity of neutrophil elastase, a major component of the azurophil granule. Recently, two additional azurophil granule proteins with NH2-terminal sequence homology to elastase were isolated (Gabay, J. E., Scott, R. W., Campanelli, D., Griffith, J., Wilde, C., Marra, M. N., Sooger, M., and Nathan, C. F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5610–5614) and designated azurophil granule protein 7 (AGP7) and azurocidin. Azurocidin and AGP7 represent significant protein components of the azurophil granule, together comprising approximately 15% of the acid-extractable protein as judged by reverse-phase high performance liquid chromatography analysis. AGP7 migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as four distinct glycoforms of molecular mass 28–34 kDa, whereas azurocidin exhibits three predominant bands with molecular mass of 28–30 kDa. Treatment of intact azurophil granules with [3H]diisopropyl fluorophosphate resulted in labeling of elastase, cathepsin G, and AGP7, whereas azurocidin was not labeled. Tryptic mapping resulted in labeling of elastase, cathepsin G, and AGP7, whereas azurocidin was not labeled. Tryptic mapping of [3H]-labeled AGP7 allowed us to identify and sequence the active-site polypeptide that has 70% identity to elastase over 20 residues. The active site peptide of AGP7 allowed us to identify and sequence the active-site polypeptide that has 70% identity to elastase over 20 residues. The active site peptide of AGP7 allowed us to identify and sequence the active-site polypeptide that has 70% identity to elastase over 20 residues. The active site peptide of AGP7 allowed us to identify and sequence the active-site polypeptide that has 70% identity to elastase over 20 residues. The active site peptide of AGP7 allowed us to identify and sequence the active-site polypeptide that has 70% identity to elastase. Surprisingly, the catalytic serine of azurocidin is replaced by glycine, explaining its inability to label with [3H]diisopropyl fluorophosphate. Thus, we have identified two azurophil proteins closely related to neutrophil elastase, one of which has apparently lost its proteolytic activity due to mutation of the catalytic serine.

Neutrophils bind to target organisms via Fc and complement receptors and internalize them in phagosomes. The phagosomes then fuse with primary (azurophil) granules to obtain a soluble fraction for chromatography. 

EXPERIMENTAL PROCEDURES

Materials—Diisopropyl fluorophosphate (DFP) was purchased from Sigma. [3H]DFP from Amersham Corp., polynvinylidene difluoride from Millipore, 4-vinylpyridine from Aldrich, and TPCK-treated trypsin from Worthington. 

Isolation and Subcellular Fractionation of Granulocytes—Granulocytes were isolated from buffy coats as described previously (10). Isolated granulocytes (2 x 10^7 cells/ml in phosphate-buffered saline, pH 7.4) were treated with 5 mM DFP for 15 min at 4 °C. The cells were washed, resuspended in lysis buffer (10 mM Pipes, pH 6.8, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2), and disrupted by nitrogen cavitation (Parr Instrument Co., Moline, IL). Granules were isolated from the postnuclear supernatant by Percoll density gradient centrifugation as described previously (11). The azurophil granule fraction was collected, and Percoll was removed from the granules by centrifugation (180,000 x g, 2 h). When isolated granules were labeled with [3H]DFP, treatment of whole cells with the inhibitor was omitted. Isolated granules from 2.3 x 10^9 cells were treated with 20 μCi [3H]DFP (180 mCi/mmol) for 1 h at room temperature before freezing at -70 °C for later extraction.

Preparation of Granule Extracts—Isolated granules were lysed by five freeze/thaw cycles on dry ice/ethanol and after addition of an equal volume of 100 mM glycine, pH 2.0, were extracted with vigorous agitation for 40 min at room temperature. The acid extract was centrifuged at 30,000 x g for 20 min and at 200,000 X g for 30 min to obtain a soluble fraction for chromatography.

Trypic Mapping—Granule extract was fractionated by chromatography on a Bio-Sil TSK-125 high performance size exclusion column (Bio-Rad) equilibrated and developed in 50 mM glycine, pH 2.0, 100 mM NaCl. Azurocidin and AGP7 were purified from the appropriate size exclusion fractions by reverse-phase HPLC. Samples were reconstituted in 200 mM Tris, pH 8.5, 5 mM guanidine hydrochloride, 20 mM EDTA, 0.1% 2-mercaptoethanol (30 min, 37 °C) and alkylated by the addition of 50 mM iodoacetamide.

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1The abbreviations used are: AGP7, azurophil granule protein 7; DFP, diisopropyl fluorophosphate; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPCK, 1-tosylamide-2-phenylethyl chloromethyl ketone; Pipes, piperazine-N,N'-bis[2-ethanesulfonic acid].
addition of 4-vinylpyridine to a final concentration of 1% (3 h, room temperature). Repurified samples of reduced and alkylated azurocidin (80 μg) and AGP7 (40 μg) were brought to 1.0 ml in 100 mM ammonium bicarbonate, pH 8.0, and digested for 4 h at 37 °C with TPCK-treated trypsin (1/40, w/w). Tryptic fragments were isolated from the digest by reverse-phase HPLC on an Aqapurp RP-300 column (2.1 × 30 mm; Brownlee) eluted with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The fragment of azurocidin having sequence homology to the neutrophil elastase active site was further purified by rechromatography on the same column eluted with a gradient from 15 to 35% acetonitrile.

Sequence Analysis of Reverse-phase Purified Peptides—Automated Edman degradation was carried out using an Applied Biosystems 477A pulsed-liquid phase sequenator. Phenylthiohydantoins were analyzed on-line using an Applied Biosystems model 120A liquid chromatograph.

Protein Sequence Homology Search—Computerized homology search of the Swiss Prot database (release 6; Feb. 1988) for protein sequences homologous to those of azurocidin and AGP7 was carried out using PC/Gene software (Intelligenetics, Mountain View, CA).

**RESULTS**

Acid extraction of intact granules and reverse-phase HPLC of extracted proteins revealed 10 major protein peaks (Fig. 1). Analysis of the peaks by NH2-terminus sequencing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a relatively simple complement of proteins, similar to that described previously for an azurophil granule membrane fraction (9) but with notable differences. One new peak (peak 4a) was identified and found to contain primarily eosinophil-derived neutrophil protein. Peak 5 consisted of lysozyme with little major basic protein detected. The predominant species in each peak is derived from neutrophils with the exception of peaks 3 and 4a. Only these peaks increased in size when granule preparations from eosinophil-rich granulocytes were analyzed (data not shown).

The predominant species comprising peak 6 (azurocidin) and peak 7 (AGP7) each exhibited NH2-terminal sequence homology to neutrophil elastase, cathepsin G, and other members of the trypsin superfamily (9). Table I shows a comparison of the first 20 residues of azurocidin and AGP7 to other granule-associated leukocyte proteases. AGP7 is identical to cathepsin G and the cytotoxic T cell proteases (granzymes) across a stretch of 8 residues from Pro9 to Ala16 and shows 70% homology to granzyme B. Azurocidin is less similar (35%) to the granzymes than AGP7, and of the granule proteases, it is most clearly related to elastase.

Analysis of peak 4 (cathepsin G), peak 6 (azurocidin), peak 7 (AGP7), and peak 8 (elastase) by SDS-PAGE is shown in Fig. 2. AGP7 migrated as four discrete bands in the M, range 28,000–34,000. The relative size and staining intensity were similar to those seen for glycoforms of elastase. For azurocidin, the three bands around M, 90,000, were somewhat diffuse, indicating a more heterogeneous range of glycoforms.

Both azurocidin and AGP7 represent major components of the azurophil granule extract, comprising 11.0 ± 0.4% and 4.3 ± 0.5%, respectively of extracted protein as determined by computer integration of reverse-phase HPLC peaks (triplicate analyses). Although yields of granules and extracted proteins were somewhat variable among preparations, recoveries of azurocidin and AGP7 were typically around 200 and 100 ng/106 cells, respectively, compared with approximately 150 ng/106 cells for elastase. Our detection of these proteins as major components may be attributable to extraction and purification under acidic conditions. We have observed a pronounced tendency of both azurocidin and AGP7 to precipitate when an acid extract is adjusted toward neutral pH or when the salt concentration of the extract is raised (data not shown).

To determine whether AGP7 and azurocidin are active as packaged in granules, we treated isolated azurophil granules with [3H]DFP, a membrane-permeant reagent that covalently labels the active-site serine of serine proteases. Proteins were extracted from labeled granules and fractionated by size exclusion and reverse-phase HPLC. Analysis of reverse-phase column fractions identified cathepsin G, AGP7, and elastase but not azurocidin as labeled peaks (Fig. 3). The extent of labeling of AGP7 was comparable to that of elastase and cathepsin G.

Specific labeling of AGP7 with [3H]DFP was demonstrated directly by SDS-PAGE of reverse-phase-purified AGP7 followed by electroblotting onto polyvinylidene difluoride membranes. Identical blotted lanes were prepared; one was sliced and counted for the presence of [3H]DFP, and the other was Coomassie stained and subsequently utilized for sequence analysis. The major peak of radioactivity was associated with an approximately 30-kDa band that yielded the NH2 terminal sequence expected for AGP7 (Fig. 4).

To determine the active site sequence of AGP7, [3H]DFP labeled material was reduced, alkylated, and subjected to digestion with trypsin. A single H-labeled tryptic peptide with the sequence shown in Table II was isolated by reverse-phase HPLC. Comparison of this sequence with the active-site region of neutrophil elastase revealed extensive homology, including the expected alignment of the catalytic serine at position 173 (Table III).

Although peak 6 was not labeled by [3H]DFP, we were able to identify a putative active site peptide by sequence analysis of the major tryptic fragments. This peptide has strong homology with the active site of elastase and AGP7 so that alignment of the fragment was unequivocal (Table III). The residue that aligns with the catalytic Ser175 of elastase is Gly8 of this peptide. The absence of the active-site serine in azurocidin likely accounts for the failure of peak 6 to label with [3H]DFP and identifies azurocidin as a proteolytically inactive member of the trypsin superfamily.

The insolubility of AGP7 near neutral pH noted above
**TABLE I**

Sequence comparison of neutrophil proteases to the granzymes

|    | AGP7 | Azurocidin | Elastase | Cathepsin G⁺ | Granzyme A⁻ | Granzyme B | Granzyme C⁻ | Granzyme D⁻ | Granzyme E⁻ |
|----|------|------------|----------|--------------|-------------|------------|-------------|-------------|-------------|
| 1  | Ile  | Val        | Gly       | His          | Gly         | Ala        | Gin         | Pro         | Ser         |
| 2  | Val  | Gly        | Arg       | Lys          | Ala         | Arg        | Pro         | Pro         | Phe         |
| 3  | Gly  | Arg        | Arg       | Pro          | His         | Ala        | Trp         | Pro         | Phe         |
| 4  | Arg  | Grz A      | Grz A     | Grz A        | Grz A       | Grz A      | Grz A       | Grz A       | Grz A       |
| 5  | Ile  | Ile        | Ile       | Ile          | Ile         | Ile        | Ile         | Ile         | Ile         |
| 6  | Val  | Val        | Val       | Val          | Val         | Val        | Val         | Val         | Val         |
| 7  | Pro  | Pro        | Pro       | Pro          | Pro         | Pro        | Pro         | Pro         | Pro         |
| 8  | Gly  | Gly        | Gly       | Gly          | Gly         | Gly        | Gly         | Gly         | Gly         |
| 9  | His  | His        | His       | His          | His         | His        | His         | His         | His         |
| 10 | Ser  | Ser        | Ser       | Ser          | Ser         | Ser        | Ser         | Ser         | Ser         |
| 11 | Leu  | Leu        | Leu       | Leu          | Leu         | Leu        | Leu         | Leu         | Leu         |
| 12 | Glu  | Glu        | Glu       | Glu          | Glu         | Glu        | Glu         | Glu         | Glu         |
| 13 | Ala  | Ala        | Ala       | Ala          | Ala         | Ala        | Ala         | Ala         | Ala         |

*Ref. 16.
⁺Ref. 17.
⁻Ref. 18.

Fig. 2. SDS-PAGE analysis of serine proteases of the azurophil granule. Peaks 4, 6, 7, and 8 (lanes 1, 2, 3, and 4) from Fig. 1 were analyzed by SDS-PAGE under reducing conditions in 12.5% acrylamide gels (13). Proteins were visualized by silver staining (14).

Fig. 3. Labeling of azurophil granule proteins in situ with [³HJDFP. Isolated azurophil granules were labeled with [³HJDFP as described under “Experimental Procedures.” Granule proteins were extracted with glycine and subjected to gel filtration and reverse-phase HPLC on a Baker cyanopropyl column (RP-7107-00). Radioactive DFP was determined in aliquots of each fraction by liquid scintillation counting. The positions of absorbance peaks corresponding to cathepsin G, azurocidin, AGP7, and elastase are indicated.

complicated efforts to determine substrate specificity. Because serine proteases are generally resistant to denaturing treatments, we tested reverse-phase-purified AGP7 and elastase for amidolytic and esterolytic activity against a panel of chromogenic substrates (Table IV). Only the p-nitrophenyl ester of N-benzyloxycarbonylvaline was cleaved by AGP7, and no amidolytic activity was identified. Elastase retained activity against its characteristic substrates.

**TABLE II**

Analysis of [³H]DFP labeling of AGP7 active site

| Cycle no. | Residue | Yield | pmol | cpm  |
|-----------|---------|-------|------|------|
| 1         | Ala     | 40.9  | 75   |
| 2         | Gly     | 54.0  | 32   |
| 3         | Ile     | 42.0  | 41   |
| 4         | Cys     | 65    |      |
| 5         | Phe     | 43.4  | 96   |
| 6         | Gly     | 42.5  | 58   |
| 7         | Asp     | 32.0  | 144  |
| 8         | Gly     | 26.1  | 181  |
| 9         | Gly     | 29.6  | 144  |
| 10        | Pro     | 18.8  | 91   |
| 11        | Leu     | 17.5  | 42   |
| 12        | Ile     | 13.4  | 75   |

**DISCUSSION**

Azurocidin and AGP7 are primary granule proteins that show extensive NH₂-terminal sequence similarity to proteases of the trypsin superfamily. Azurocidin appears to be most closely related to elastase, whereas AGP7 is 70% identical to granzyme B, a serine protease found in the granules of cytotoxic T lymphocytes.

Labeling of AGP7 with [³H]DFP demonstrates that it is an
active serine protease. The extent of AGP7 labeling with this
reagent was comparable to that of elastase and cathepsin G.
We were able to take advantage of the covalent modification
of AGP7 labeling with this procedure to precipitate the proteinase 3
degrades elastin with an efficiency equivalent to that of neu-
triphil elastase (8). Proteinase 3, like AGP7, was found in
multiple isoforms in the molecular weight range around 30,000
and was extracted from granules under strongly acidic con-
ditions. Initial purification attempts using procedures de-
scribed by these workers resulted in complete loss of AGP7.
The relationship of proteinase 3 to AGP7 is under investi-
gation.

Azurocidin shows antimicrobial activity in vitro and prob-
ably plays a role in bacterial killing within the phagolysosome.
AGP7 is only slightly microbicidal, however, suggesting that
this protein has a different function. One interesting pos-
sibility is that AGP7 contributes to the proteolytic degradation
at inflammatory sites that has previously been attributed
solely to elastase and cathepsin G.

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