Measurement of free and membrane-bound cathepsin G in human neutrophils using new sensitive fluorogenic substrates

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Activated human polymorphonuclear neutrophils at inflammatory sites release the chymotrypsin-like protease cathepsin G, together with elastase and proteinase 3 (myeloblastin), from their azurophil granules. The low activity of cathepsin G on synthetic substrates seriously impairs studies designed to clarify its role in tissue inflammation. We have solved this problem by producing new peptide substrates with intramolecularly quenched fluorescence. These substrates were deduced from the sequence of putative protein targets of cathepsin G, including the reactive loop sequence of serpin inhibitors and the N-terminal domain of the protease-activated receptor of thrombin, PAR-1. Two substrates were selected, Abz-TPFSGQ-EDDnp and Abz-EPFWEDQ-EDDnp, that are cleaved very efficiently by cathepsin G but not by neutrophil elastase or proteinase 3, with specificity constants (k_{cat}/K_m) in the $10^5\ M^{-1} \cdot s^{-1}$ range. They can be used to measure subnanomolar concentrations of free enzyme *in vitro* and at the surface of neutrophils purified from fresh human blood. Purified neutrophils express 0.02–0.7 pg of cathepsin G/cell ($n = 15$) at their surface. This means that about 10^4 purified cells may be enough to record cathepsin G activity within minutes. This may be most important for investigating the role of cathepsin G as an inflammatory agent, especially in bronchoalveolar lavage fluids from patients with pulmonary inflammatory disorders.

Key words: kinetics, serine protease, synthetic substrate.

INTRODUCTION

Cathepsin G is one of the three major serine proteinases of the chymotrypsin family present in the azurophil granules of human polymorphonuclear neutrophils (PMNs); the other two are human neutrophil elastase (HNE) and proteinase 3 (Pr3; myeloblastin). All three enzymes are released from activated neutrophils at the same time and in response to the same stimuli at inflammatory sites [1]. Because they are released simultaneously from primary granules and can all remain bound to neutrophil membranes as active enzymes [2–4], the functions of these three proteases during inflammation have not been yet clearly identified. All can participate, for example, in connective tissue degradation (reviewed in [5]), regulation of cytokine bioactivity [6] and inhibition of thrombin-induced cell activation [7], but the contribution of cathepsin G is uncertain. Cathepsin G also participates in a variety of other functions including platelet activation [8], proteolysis of blood coagulation factors [9], angiotensin II generation [10], chemotactic activity on monocytes [11], and anti-fungal [12] and bactericidal [13] activity. It also increases the susceptibility of monocytes/macrophages to acute HIV-1 infection [14] and activates progelatinase B *in vitro* more efficiently than HNE or Pr3 [15]. Cathepsin G specificity differs from those of HNE and Pr3, as it prefers a Phe or a Lys at P1 [16]. The chromogenic [*p*-nitroanilide (pNA)] and fluorogenic (7-amino-4-methyl-coumarin hydrochloride) substrates most commonly used to measure cathepsin G activity have a Pro-Phe pair at P2-P1 [17–19]. However, cathepsin G cleaves synthetic substrates much more slowly than related chymotrypsin-like proteinases [18,20]. This seriously hampers studies of its biological function and monitoring of its activity in biological fluids or in tissue extracts, where it is present at low concentrations. We recently designed a sensitive substrate derived from the α_1-antichymotrypsin (ACT) reactive-site loop that is more specific than those currently used [21]. This resulted from a study of optimal substrate occupation that included the prime side subsites (S′ sites) of the protease. But the first attempts to measure cathepsin G activity in the presence of other neutrophil-derived serine proteases showed that this substrate was also cleaved by these proteases. We have therefore developed specific, sensitive substrates of neutrophil cathepsin G to investigate the role of this protease during inflammation and possibly use it as a target for therapeutic drugs. These substrates were used to measure cathepsin G activity at the surfaces of native and activated PMNs.

The nomenclature used for the individual amino acid residues (P_1, P_2, …) of a substrate and corresponding residues of the enzyme subsites (S_1, S_2, ….) is that of Schechter and Berger [22].

EXPERIMENTAL

Chemicals

Human cathepsin G (EC 3.4.21.20) was from ICN Pharmaceuticals. HNE (EC 3.4.21.37), Pr3 (EC 3.4.21.76), α_1-proteinase inhibitor and ACT were from Athens Research and Technology

Abbreviations used: Abz, *o*-aminobenzoic acid; ACT, α_1-antichymotrypsin; CMK, chloromethyl ketone; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; HNE, human neutrophil elastase; PAR, protease-activated receptor; PMN, polymorphonuclear neutrophil; pNA, *p*-nitroanilide; Pr3, proteinase 3; SBzl, thiobenzyl ester; Z, benzyloxycarbonyl; Suc, succinyl; MeOSuc, methoxysuccinyl.

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(Athens, GA, U.S.A.). Bovine chymotrypsin (EC 3.4.21.1) was from Roche Molecular Biochemicals (Meylan, France). Human recombinant chymase (EC 3.4.21.39) was provided by Professor Norman Schechter (Department of Dermatology, University of Pennsylvania, Philadelphia, PA, U.S.A.). Suc-AAPF-SBzl (where Suc is succinyl and SBzl is thiobenzyl ester) was from Bachem (Voisins-le-Bretonneux, France). Z-GLF-CMK (where Z is benzyloxycarbonyl and CMK is chloromethyl ketone) and MeOSuc-AAPA-CMK (where MeOSuc is methoxysuccinyl) were from Enzyme System Products (Livermore, CA, U.S.A.). *N*,*N*-Dimethylformamide and acetonitrile were from Merck (Darmstadt, Germany), C_{18} cartridges for reversed-phase chromatography were from Touzart et Matignon (Paris, France) and Interchim (Montluçon, France). Polymorphprep™ and Lymphoprep™ were from Nycomed Pharma (Oslo, Norway). Igepal CA-630 was from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Isolation and activation of blood PMNs

Human neutrophils were purified from 10 ml of peripheral blood of volunteers ($n = 15$) collected into EDTA-containing tubes. Aliquots (5 ml) of blood were layered over 5 ml of Polymorphprep and centrifuged at 2000 ***g*** for 20 min at 25 °C. The neutrophil-enriched bands (75 % PMNs and 25 % lymphocytes) were harvested, diluted with an equal volume of half-strength PBS and centrifuged at 500 ***g*** for 10 min. The resulting pellet was washed once with PBS, adjusted to 6 ml with PBS and layered over 3 ml of Lymphoprep. After centrifugation at 1100 ***g*** for 20 min at 20 °C, the neutrophil band at the bottom of the gradient was recovered and washed three times in PBS + 1 mg/ml glucose + 4 mM EGTA at 500 ***g*** for 5 min at 20 °C. Residual erythrocytes were removed by hypotonic lysis in 4 mM EGTA, pH 7.4. Purified PMNs were pelleted at 500 ***g*** for 5 min and contaminating erythrocyte membranes were removed. The PMNs were suspended in PBS + 1 mg/ml glucose + 4 mM EGTA to approx. 10^4 cells/μl, and their cathepsin G activity assayed. Differential counting of cytocentrifuge preparations (Cytospin II; Shandon) and Hemacolor (Merck) staining showed > 99 % PMNs, with the remainder of the cells being eosinophils. These preparations contained no monocytes, lymphocytes or platelets.

Design and synthesis of quenched fluorescent substrates

All quenched fluorogenic substrates were prepared by solid-phase synthesis with the Fmoc (fluoren-9-ylmethoxycarbonyl) methodology using a multiple automated peptide synthesizer (PSSM-8; Shimadzu Co.) [23–25]. Glutamine was the C-terminal residue in all peptides, due to a requirement of the synthesis strategy [23]. Substrate purity was checked by matrix-assisted laser-desorption ionization–time-of-flight MS (TofSpec-E; Micromass) and by reversed-phase chromatography on a C_{18} column eluted at 2 ml/min with a 10 min linear gradient of acetonitrile (0–60 %) in 0.075 % trifluoroacetic acid.

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

Cathepsin G, chymotrypsin and chymase were titrated as reported previously [21]. HNE and Pr3 were titrated with α_1-proteinase inhibitor, the titre of which had been determined using bovine trypsin titrated with *p*-nitropheny-*p*′-guanidinobenzoate [26]. Experimental conditions were optimized, taking into account the propensity of diluted solutions of neutrophil proteases to stick to plastic and glass surfaces. Assays were carried out at 37 °C in 300 μl of 50 mM Hepes buffer, pH 7.4, 100 mM NaCl and 0.01 % Igepal CA-630 (v/v) for cathepsin G and chymotrypsin, 0.1 M Tris/HCl, pH 8.0, and 1.8 M NaCl for chymase, and 50 mM Hepes, pH 7.4, 750 mM NaCl and 0.05 % Igepal CA-630 (v/v) for HNE and Pr3.

The hydrolysis of Abz-peptidyl-EDDnp [where Abz is *o*-aminobenzoic acid and EDDnp is *N*-(2,4-dinitrophenyl)-ethylenediamine] substrates was followed by measuring the fluorescence at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm in a Hitachi F-2000 spectrofluorometer. The system was standardized using Abz-FR-OH prepared from Abz-FR-pNA total tryptic hydrolysis, and its concentration was determined from the absorbance at 410 nm, assuming $\epsilon_{410} = 8800\ M^{-1} \cdot cm^{-1}$ for *p*-nitroaniline. Alternatively, Abz-peptidyl-EDDnp concentration was determined by the absorbance measured at 365 nm, with $\epsilon_{365} = 17300\ M^{-1} \cdot cm^{-1}$ for EDDnp.

All buffers were supplemented with the thiodisulphide reagent 5,5′-dithio-bis(2-nitrobenzoic acid) ('DTNB'; 5 mM final) to follow the hydrolysis of Suc-AAPF-SBzl (2.5–5 μM) by cathepsin G and related proteases (2–5 nM final). This was followed at 405 nm and 37 °C with a Shimadzu UV-160A spectrophotometer.

Specificity constants (k_{cat}/K_m) were determined under first-order conditions, using a substrate concentration far below the estimated K_m, and a final cathepsin G concentration of 10–50 nM. Under these conditions, the Michaelis–Menten equation is reduced to $v = k_{obs} \cdot S$, where $k_{obs} = V_{max}/K_m$. Integrating this equation over time gives $\ln[S] = -k_{obs} \cdot t + \ln[S]_0$ with $[S]_0$ being the substrate concentrations at time 0 and [S] at time t. Since $V_{max} = k_{cat} \cdot [E]_t$, where $[E]_t$ is the final enzyme concentration, dividing k_{obs} by $[E]_t$ gives the k_{cat}/K_m ratio. The k_{obs} for the first-order substrate hydrolysis was calculated by fitting experimental data to the first-order law using Enzfitter software (Elsevier Science, Amsterdam, The Netherlands). The same experiments were performed using chymotrypsin (7×10^{-7} M final), chymase (20 nM final) and Abz-peptidyl-EDDnp substrates.

The observed K_m values [$K_{m(obs)}$] for the hydrolysis of Abz-peptidyl-EDDnp by cathepsin G were determined using 8–10 substrate concentrations (0.1–10 μM); depending on the substrate, the final concentration of cathepsin G was 10–100 nM. Experimental data were fitted to the hyperbolic Michaelis–Menten rate equation using Enzfitter. Direct measurements of the Michaelis constant were not possible when substrate concentrations exceeded 10 μM, probably due to intermolecular quenching. In that case, $K_{m(obs)}$ values were determined using two competing substrates whose hydrolysis products could be measured independently. Under these mixed alternative substrate conditions, each substrate acts as a competitive inhibitor of the other. $K_{m(obs)}$ values of intramolecularly quenched fluorogenic substrates were obtained by measuring the dissociation constant (K_i) towards a chromogenic pNA substrate. Assays were carried out by adding 5 nM cathepsin G to a mixture of 100–500 μM Suc-AAPF-pNA (whose K_m is 1.7 ± 0.2 mM [19]) and 0–50 μM fluorogenic Abz-peptidyl-EDDnp derivative. The hydrolysis of Suc-AAPF-pNA was monitored at 410 nm with less than 5 % of substrate hydrolysed. The velocity of the enzymic reaction is described by the following equation:

$$v_i/v_o = (K_m + S)/[K_m \cdot (1 + I/K_i) + S] \quad (1)$$

where v_i is the initial velocity at a given substrate concentration with the Abz-peptidyl-EDDnp competitor, v_o is the initial velocity at the same substrate concentration without competitor, K_m is the Michaelis constant of the substrate, S is the chromogenic substrate concentration and I is the Abz-peptidyl-EDDnp sub-

strate concentration. The K_i value corresponds to the observed Michaelis constant [$K_{m(obs)}$] of Abz-peptidyl-EDDnp substrate as a competitor.

Enzymic activity at the surface of PMNs

Purified PMNs (10^4–10^5) were incubated in 50 mM Hepes buffer, pH 7.4, 150 mM NaCl and 0.05 % Igepal CA-630 (v/v) with 5 μM Abz-peptidyl-EDDnp substrate in 200 μl of reaction medium in microplate wells at 37 °C. The fluorescence was recorded using a Spectra Max Gemini microplate fluorescence reader (Molecular Devices) that allows continuous stirring during the course of the reaction. The enzyme activity in supernatants obtained by washing PMNs in PBS was also assayed. CMK inhibitors Z-GLF-CMK and MeOSuc-AAPA-CMK were assayed at final concentrations of 10^{-4} M and incubated with PMNs for 2 h at 37 °C before measuring residual activity with Abz-TPFSGQ-EDDnp.

Chromatographic procedures and analysis of peptide products

Once the enzyme reaction was complete, the reaction medium was incubated with 4 vol. of absolute ethanol for 15 min on ice and centrifuged at 13000 ***g*** for 10 min. The supernatant containing the hydrolysis products was recovered, air-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 C_{18} column (2.1 mm × 30 mm, Brownlee; or 2 mm × 33 mm, Uptisphere), using a Thermo Separation Products P200 pump and a Spectrasystem UV3000 detector (Thermo Separation Products), at a flow rate of 0.3 ml/min, with a linear (0–60 %, v/v) gradient 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 online model 120A analyser.

RESULTS AND DISCUSSION

It is difficult to investigate the biological function of cathepsin G because of its poor capacity to cleave synthetic peptide substrates as compared with related chymase-like proteases such as chymotrypsin and chymase [18,20,21]. There is little doubt that cathepsin G is intimately involved in the metabolism of PMNs and particularly the function of azurophil granules [1]. But the roles of the azurophil granule proteases in biological fluids and tissues can only be determined when sensitive, specific substrates are available for each protease. We have already produced a fluorogenic cathepsin G substrate of great sensitivity, its specificity constant (k_{cat}/K_m) being greater than 10^5 $M^{-1} \cdot s^{-1}$ [21]. This chimaeric substrate was derived from the sequence of the reactive-site loop of ACT, a fast-acting serpin inhibitor of cathepsin G, by placing a Pro-Phe pair at P2-P1 in the fluorogenic substrate Abz-TPFSALQ-EDDnp. As cathepsin G is released from activated neutrophils at the same time as HNE and Pr3 at inflammatory sites, we first used Abz-TPFSALQ-EDDnp as a substrate for these two proteases and found that it was hydrolysed at a low rate by both of them (Table 1).

Designing a new, sensitive substrate from Abz-TPFSALQ-EDDnp

The cleavage sites in Abz-TPFSALQ-EDDnp by all three primary granule serine proteases were identified by reversed-phase HPLC and N-terminal sequencing of the EDDnp-containing fragments. This showed that cathepsin G hydrolysed the substrate at the F–S bond, whereas HNE cleaved at the L–Q bond and Pr3 at the S–A bond (Figure 1). Although the k_{cat}/K_m for HNE and Pr3 were lower than that for cathepsin G (Table 1) and the cleavage sites differed for the three proteases, Abz-TPFSALQ-EDDnp is not ideally suited for measuring cathepsin G specifically in biological samples where the other PMN serine proteases may be present. We therefore produced a new substrate lacking the HNE and Pr3 cleavage sites by replacing the A–L pair with a G residue, which is generally not accomodated by the S1 subsite of these proteases. The resulting substrate (Abz-TPFSGQ-EDDnp) was hydrolysed by cathepsin G with a k_{cat}/K_m in the 10^5 $M^{-1} \cdot s^{-1}$ range, similar to that of the former substrate, but was resistant to HNE and Pr3 hydrolysis (Table 1). The $K_{m(obs)}$ was 38.7 μM, which is in the same range as that reported for Abz-TPFSALQ-EDDnp [21]. This value was determined by competition experiments with the chromogenic pNA substrate Suc-AAPF-pNA, because intermolecular quenching might occur at Abz-peptidyl-EDDnp concentrations above 10 μM. Chymotrypsin and chymase, two related enzymes that differ from cathepsin G by their potent activity on synthetic substrates, also cleave Abz-TPFSGQ-EDDnp but less efficiently than Abz-TPFSALQ-EDDnp (Table 1). The same is true for the currently used substrate Suc-AAPF-SBzl, which is cleaved more rapidly by chymase and chymotrypsin than by cathepsin G and is also a substrate for HNE (Table 1).

Abz-EPFWEDQ-EDDnp, a sensitive protease-activated receptor (PAR)-1-derived cathepsin G substrate resistant to HNE and Pr3

In addition to serpins, whose reactive-site loop is cleaved in a substrate-like manner by cathepsin G, some other natural protein

Table 1 Specificity constants (k_{cat}/K_m) for the hydrolysis of fluorogenic substrates by the three serine proteases of PMNs, and chymase and chymotrypsin

The k_{cat}/K_m values for the hydrolysis of Suc-AAPF-SBzl are given for comparison. Values are means ± S.D. ($n = 3$).

	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)				
Substrate	Cathepsin G	Pr3	Elastase	Chymase	Chymotrypsin
Abz-TPFSALQ-EDDnp	153.8 ± 4.9*	11.5 ± 1.7	7.11 ± 0.04	1870.0 ± 27.0*	231.3 ± 6.4*
Abz-TPFSGQ-EDDnp	161.9 ± 5.4	< 1	< 1	1018.7 ± 20.1	117.9 ± 1.9
Abz-EPFWEDQ-EDDnp	242.7 ± 2.9	No hydrolysis	< 1	459.3 ± 12.0	No hydrolysis
Suc-AAPF-SBzl	2096 ± 358	No hydrolysis	69.7 ± 5.6	17033 ± 888	11025 ± 248

* Values from Réhault et al. [21].

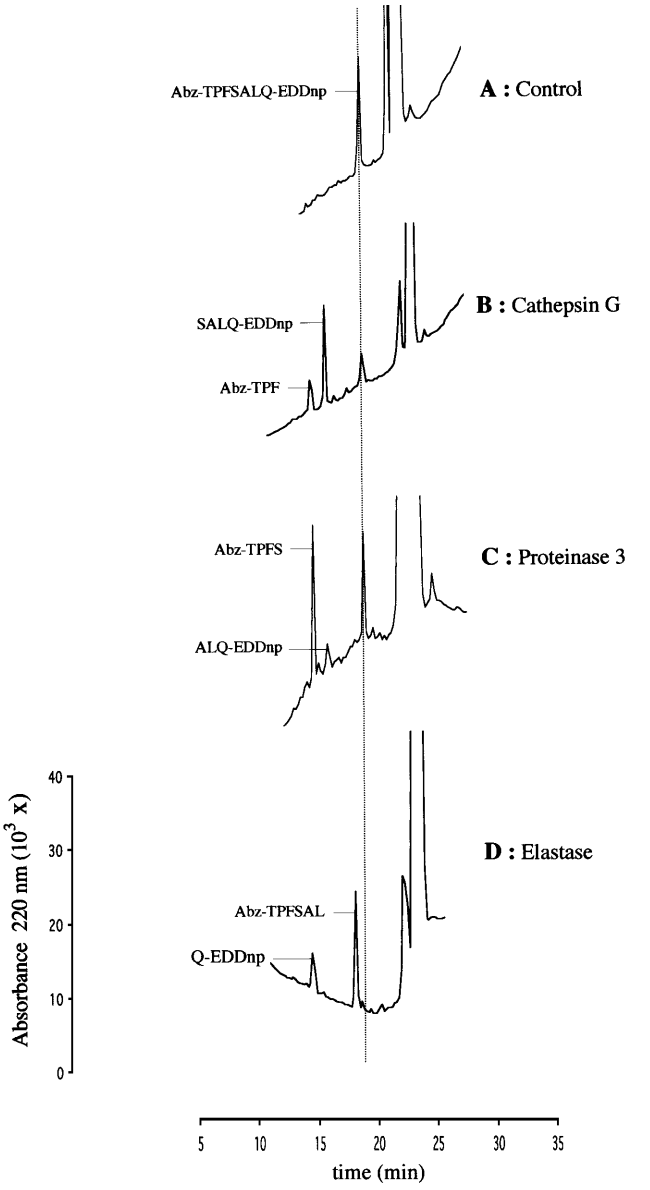

Figure 1 Identification of the cleavage sites within Abz-TPFSALQ-EDDnp after hydrolysis by azurophil granule serine proteases from human PMNs

Reversed-phase HPLC chromatograms of the substrate after hydrolysis by cathepsin G, elastase and Pr3. (**A**) 10 μM Abz-TPFSALQ-EDDnp, no enzyme. (**B**) 10 μM Substrate + 10^{-8} M cathepsin G. (**C**) 10 μM Substrate + 5×10^{-8} M Pr3. (**D**) 10 μM Substrate + 5×10^{-8} M elastase. The peaks eluting at 23 min correspond to Igepal CA-630. The cleavage sites were identified by N-terminal sequencing of the EDDnp-containing fragments having an absorbance peak at 360 nm. For the sake of clarity, absorbances at 320 nm (Abz-) and 360 nm (-EDDnp) are not reported.

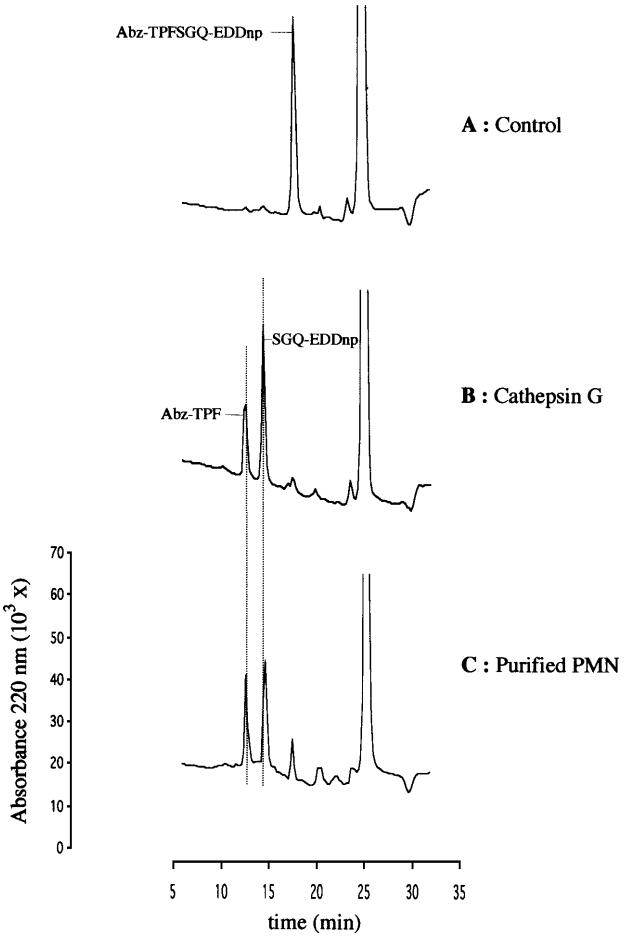

Figure 2 Identification of the cleavage site within Abz-TPFSGQ-EDDnp after hydrolysis by free cathepsin G and purified PMNs

Reversed-phase HPLC chromatograms of: (**A**) 5 μM Abz-TPFSGQ-EDDnp and no enzyme; (**B**) 5 μM substrate + 10^{-8} M cathepsin G; and (**C**) 5 μM substrate + 4×10^4 purified PMNs. The peaks eluting at 25 min correspond to Igepal CA-630. The cleavage sites were identified by N-terminal sequencing of the EDDnp-containing fragments having an absorbance peak at 360 nm. An identical cleavage site was also found in Abz-EPFWEDQ-EDDnp with purified cathepsin G and neutrophils (not shown). For clarity, absorbances at 320 nm (Abz-) and 360 nm (-EDDnp) are not reported.

targets of this protease, including members of the PAR family, have been identified [7,8,27,28]. PAR-1 and PAR-3 are inactivated by cathepsin G and by other neutrophil proteases, thus blocking the cell activation induced by thrombin [7,27], whereas PAR-4 is activated by cathepsin G in human platelets [8]. A cathepsin G-sensitive sequence in the exodomain of the thrombin receptor PAR-1 has a Pro-Phe pair at P2-P1 and is specifically cleaved by cathepsin G [7]. We have produced an intramolecularly quenched fluorescent peptide based on this sequence on both sides of the cathepsin G-cleavage site (Phe-55–Trp-56). This substrate (Abz-EPFWEDQ-EDDnp) is rapidly cleaved by cathepsin G with a cleavage site at the F–W bond and a k_{cat}/K_m in the same range as that reported for Abz-TPFSGQ-EDDnp (Table 1). We found however that the $K_{m(obs)}$ was significantly lower than that for Abz-TPFSGQ-EDDnp so that it could be determined either directly by recording reaction rates at different substrate concentrations or by competition with Suc-AAPF-pNA (Table 1). The reason why the $K_{m(obs)}$ value towards Abz-EPFWEDQ-EDDnp is lower than that of Abz-TPFSGQ-EDDnp remains a matter for conjecture. It could be that the presence of three negatively charged residues at P3, P2′ and P3′ in the former favours substrate binding in a non-productive mode at the active site of cathepsin G. Such binding results in a decrease in both K_m and k_{cat} without altering the value of the specificity constant k_{cat}/K_m [29].

Abz-EPFWEDQ-EDDnp is completely resistant to hydrolysis by HNE and Pr3 so that it can be used to follow cathepsin G activity in biological fluids or at the surface of cells when all three proteases are present. Abz-EPFWEDQ-EDDnp is also com-

Table 2 Michaelis constants (K_m) for the hydrolysis of fluorogenic substrates by cathepsin G

Values are means ± S.D. ($n = 2$).

Substrate	$K_{m(obs)}$ (μM)
Abz-TPFSALQ-EDDnp	10.7 ± 1.8
Abz-TPFSGQ-EDDnp	38.7 ± 2.5
Abz-EPFWEDQ-EDDnp	1.1 ± 0.1

pletely resistant to chymotrypsin and is cleaved by mast cell chymase far less efficiently than several other substrates of this strongly reactive protease [21].

Cathepsin G activity at the surface of PMNs

Because of their great sensitivity and selectivity, Abz-EPFWEDQ-EDDnp and Abz-TPFSGQ-EDDnp were used to measure cathepsin G activity at the surface of purified neutrophils. SBzl substrates could not be used for this purpose since, in addition to their lack of specificity, kinetic measurements using this kind of substrate require an additional thiodisulphide reagent that interferes with any free thiol groups present in reaction mixtures containing cells or unfractionated biological fluids.

Suspensions of purified blood neutrophils containing as few as 10^4 cells/200 μl cleaved both substrates. This activity remained after the cells were washed with PBS, centrifuged and suspended in the reaction buffer, demonstrating that the activity was on the cell membranes. HPLC analysis of the soluble fraction after incubation of cells with fluorogenic substrates showed that both substrates were cleaved at a single site, identical with that of cathepsin G (Figure 2). The membrane-bound protease hydrolysed Abz-EPFWEDQ-EDDnp less rapidly than Abz-TPFSGQ-EDDnp, unlike free cathepsin G. It is unlikely that there is another protease which cleaves Abz-TPFSGQ-EDDnp but not, or to a lesser extent, Abz-EPFWEDQ-EDDnp at the surface of neutrophils because each substrate is hydrolysed at a single and identical site by free and membrane-bound cathepsin G (Figure 2) and activity is completely inhibited by the irreversible low-M_r cathepsin G inhibitor Z-GLF-CMK but not by the elastase-like inhibitor MeOSuc-AAPA-CMK (not shown). More probably, the three negative charges on the peptidyl moiety of Abz-EPFWEDQ-EDDnp impair access to the negatively charged membrane surface. We checked this by measuring the rate of hydrolysis of increasing concentrations (1–10 μM) of Abz-EPFWEDQ-EDDnp by free and membrane-bound cathepsin G. The $K_{m(obs)}$ for the membrane-bound enzyme (6 ± 1 μM) was significantly higher than that for free cathepsin G (Table 2), which explains the slower hydrolysis of Abz-EPFWEDQ-EDDnp by membrane-bound cathepsin G. It also suggests that free and membrane-bound cathepsin G cleave physiological target substrates differently, as already observed for angiotensin II generation [10]. As membrane-bound proteases from the azurophil granules of PMNs are also remarkably resistant to naturally occurring serpin proteinase inhibitors [3,4], their role during inflammation could differ significantly depending on whether they are on the surface of cells or released into extracellular medium.

Based on the assumption that Abz-TPFSGQ-EDDnp is cleaved similarly by free and membrane-bound cathepsin G, it was used to quantify cathepsin G at the surface of purified neutrophils. We used a linear standard curve from 5×10^{-10} M to 5×10^{-8} M cathepsin G and assayed 10^4–10^5 cells in a final volume of 200 μl. The number of cells was adjusted to obtain a final cathepsin G molar concentration in the range 0.5–1.5 nM. In these conditions, purified neutrophils expressed 0.02–0.7 pg cathepsin G/cell ($n = 15$) at their surface (median value 0.35 pg/cell). This corresponds to about 10–20 % of the cellular content of this neutral protease and agrees with the data of Owen et al. [30].

Thus, the substrates described above are suitable for directly measuring subnanomolar concentrations of cathepsin G at the surface of purified human blood neutrophils. It is not clear at present whether blood neutrophils bear cathepsin G and other azurophil granule proteases on their surface constitutively, or whether the cell-surface activity is due to partial degranulation during purification. Nevertheless, the fluorogenic substrates described here may be most useful for investigating the role of cathepsin G in the cellular and soluble fraction of bronchoalveolar lavage fluids from patients suffering from pulmonary inflammatory disorders where PMNs have been recruited and activated.

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REFERENCES

1 Owen, C. A. and Campbell, E. J. (1999) The cell biology of leukocyte-mediated proteolysis. J. Leukoc. Biol. **65**, 137–150
2 Bangalore, N. and Travis, J. (1994) Comparison of properties of membrane bound versus soluble forms of human leukocytic elastase and cathepsin G. Biol. Chem. Hoppe-Seyler **375**, 659–666
3 Campbell, E. J., Campbell, M. A. and Owen, C. A. (2000) Bioactive proteinase 3 on the cell surface of human neutrophils: quantification, catalytic activity, and susceptibility to inhibition. J. Immunol. **165**, 3366–3374
4 Owen, C. A., Campbell, M. A., Sannes, P. L., Boukedes, S. S. and Campbell, E. J. (1995) Cell surface-bound elastase and cathepsin G on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. J. Cell Biol. **131**, 775–789
5 Caughey, G. H. (1994) Serine proteinases of mast cell and leukocyte granules. A league of their own. Am. J. Respir. Crit. Care Med. **150**, S138–S142
6 Bank, U. and Ansorge, S. (2001) More than destructive: neutrophil-derived serine proteases in cytokine bioactivity control. J. Leukoc. Biol. **69**, 197–206
7 Renesto, P., Si-Tahar, M., Moniatte, M., Balloy, V., Van Dorsselaer, A., Pidard, D. and Chignard, M. (1997) Specific inhibition of thrombin-induced cell activation by the neutrophil proteinases elastase, cathepsin G, and proteinase 3: evidence for distinct cleavage sites within the aminoterminal domain of the thrombin receptor. Blood **89**, 1944–1953
8 Sambrano, G. R., Huang, W., Faruqi, T., Mahrus, S., Craik, C. and Coughlin, S. R. (2000) Cathepsin G activates protease-activated receptor-4 in human platelets. J. Biol. Chem. **275**, 6819–6823
9 Turkington, P. T. (1992) Cathepsin G, a regulator of human vitamin K, dependent clotting factors and inhibitors. Thromb. Res. **67**, 147–155
10 Owen, C. A. and Campbell, E. J. (1998) Angiotensin II generation at the cell surface of activated neutrophils: novel cathepsin G-mediated catalytic activity that is resistant to inhibition. J. Immunol. **160**, 1436–1443
11 Chertov, O., Ueda, H., Xu, L. L., Tani, K., Murphy, W. J., Wang, J. M., Howard, O., Sayers, T. J. and Oppenheim, J. J. (1997) Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. J. Exp. Med. **186**, 739–747
12 Newman, S. L., Gootee, L., Gabay, J. E. and Selsted, M. E. (2000) Identification of constituents of human neutrophil azurophil granules that mediate fungistasis against Histoplasma capsulatum. Infect. Immun. **68**, 5668–5672
13 Shafer, W. M., Hubalek, F., Huang, M. and Pohl, J. (1996) Bactericidal activity of a synthetic peptide (CG 117–136) of human lysosomal cathepsin G is dependent on arginine content. Infect. Immun. **64**, 4842–4845
14 Moriuchi, H., Moriuchi, M. and Fauci, A. S. (2000) Cathepsin G, a neutrophil-derived serine protease, increases susceptibility of macrophages to acute human immunodeficiency virus type 1 infection. J. Virol. **74**, 6849–6855

15 Kolkenbrock, H., Zimmermann, J., Burmester, G. R. and Ulbrich, N. (2000) Activation of progelatinase B by membranes of human polymorphonuclear granulocytes. Biol. Chem. **381**, 49–55
16 Hof, P., Mayr, I., Huber, R., Korzus, E., Potempa, J., Travis, J., Powers, J. C. and Bode, W. (1996) The 1.8 Å crystal structure of human cathepsin G in complex with Suc-Val-Pro-PheP-$(OPh)_2$: a Janus-faced proteinase with two opposite specificities. EMBO J. **15**, 5481–5491
17 Nakajima, K., Powers, J. C., Ashe, B. M. and Zimmerman, M. (1979) Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. Studies with peptide substrates related to the alpha 1-protease inhibitor reactive site. J. Biol. Chem. **254**, 4027–4032
18 Tanaka, T., Minematsu, Y., Reilly, C. F., Travis, J. and Powers, J. C. (1985) Human leukocyte cathepsin G. Subsite mapping with 4-nitroanilides, chemical modification, and effect of possible cofactors. Biochemistry **24**, 2040–2047
19 Ermolieff, J., Duranton, J., Petitou, M. and Bieth, J. G. (1998) Heparin accelerates the inhibition of cathepsin G by mucus proteinase inhibitor: potent effect of O-butyrylated heparin. Biochem. J. **330**, 1369–1374
20 Powers, J. C., Tanaka, T., Harper, J. W., Minematsu, Y., Barker, L., Lincoln, D., Crumley, K. V., Fraki, J. E., Schechter, N. M., Lazarus, G. G. et al. (1985) Mammalian chymotrypsin-like enzymes. Comparative reactivities of rat mast cell proteases, human and dog skin chymases, and human cathepsin G with peptide 4-nitroanilide substrates and with peptide chloromethyl ketone and sulfonyl fluoride inhibitors. Biochemistry **24**, 2048–2058
21 Réhault, S., Brillard-Bourdet, M., Juliano, M. A., Juliano, L., Gauthier, F. and Moreau, T. (1999) New, sensitive fluorogenic substrates for human cathepsin G based on the sequence of serpin-reactive site loops. J. Biol. Chem. **274**, 13810–13817
22 Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. **27**, 157–162
23 Hirata, I. Y., Cezari, M. H. S., Nakaie, C. R., Boschcov, P., Ito, A. S. and Juliano, M. A. (1994) Internally quenched fluorogenic protease substrates: solid phase synthesis and fluorescence spectroscopy of peptides containing *ortho*-aminobenzoyl/dinitrophenyl groups as donor-acceptor pairs. Lett. Pept. Sci. **1**, 299–308
24 Chagas, J. R., Juliano, L. and Prado, E. S. (1991) Intramolecularly quenched fluorogenic tetrapeptide substrates for tissue and plasma kallikreins. Anal. Biochem. **192**, 419–425
25 Oliveira, M. C., Hirata, I. Y., Chagas, J. R., Boschcov, P., Gomes, R. A., Figueiredo, A. F. and Juliano, L. (1992) Intramolecularly quenched fluorogenic peptide substrates for human renin. Anal. Biochem. **203**, 39–46
26 Serveau, C., Moreau, T., Zhou, G. X., El Moujahed, A., Chao, J. and Gauthier, F. (1992) Inhibition of rat tissue kallikrein gene family members by rat kallikrein-binding protein and alpha 1-proteinase inhibitor. FEBS Lett. **309**, 405–408
27 Cumashi, A., Ansuini, H., Celli, N., De Blasi, A., O'Brien, P. J., Brass, L. F. and Molino, M. (2001) Neutrophil proteases can inactivate human PAR3 and abolish the co-receptor function of PAR3 on murine platelets. Thromb. Haemost. **85**, 533–538
28 Loew, D., Perrault, C., Morales, M., Moog, S., Ravanat, C., Schuhler, S., Arcone, R., Pietropaolo, C., Cazenave, J. P., van Dorsselaer, A. and Lanza, F. (2000) Proteolysis of the exodomain of recombinant protease-activated receptors: prediction of receptor activation or inactivation by MALDI mass spectrometry. Biochemistry **39**, 10812–10822
29 Fersht, A. (1985) Enzyme Structure and Mechanism, pp. 109–111, W.H. Freeman & Co, New York
30 Owen, C. A., Campbell, M. A., Boukedes, S. S. and Campbell, E. J. (1995) Inducible binding of bioactive cathepsin G to the cell surface of neutrophils. A novel mechanism for mediating extracellular catalytic activity of cathepsin G. J. Immunol. **155**, 5803–5810

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