α2-Macroglobulin Capture Allows Detection of Mast Cell Chymase in Serum and Creates a Reservoir of Angiotensin II-Generating Activity

Wilfred W. Raymond, Sharon Su, Anastasia Makarova, Todd M. Wilson, Melody C. Carter, Dean D. Metcalfe and George H. Caughey

*J Immunol* 2009; 182:5770-5777; doi: 10.4049/jimmunol.0900127

http://www.jimmunol.org/content/182/9/5770
α2-Macroglobulin Capture Allows Detection of Mast Cell Chymase in Serum and Creates a Reservoir of Angiotensin II-Generating Activity

Wilfred W. Raymond,2* Sharon Su,2‡ Anastasia Makarova,8 Todd M. Wilson,1 Melody C. Carter,1 Dean D. Metcalfe,1 and George H. Caughey3*‡§¶

Human chymase is a highly efficient angiotensin II-generating serine peptidase expressed by mast cells. When secreted from degranulating cells, it can interact with a variety of circulating antipeptidases, but is mostly captured by α2-macroglobulin, which sequesters peptidases in a cage-like structure that precludes interactions with large protein substrates and inhibitors, like serpins. The present work shows that α2-macroglobulin-bound chymase remains accessible to small substrates, including angiotensin I, with activity in serum that is stable with prolonged incubation. We used α2-macroglobulin capture to develop a sensitive, microtiter plate-based assay for serum chymase, assisted by a novel substrate synthesized based on results of combinatorial screening of peptide substrates. The substrate has low background hydrolysis in serum and is chymase-selective, with minimal cleavage by the chymotryptic peptidases cathepsin G and chymotrypsin. The assay detects activity in chymase-spiked serum with a threshold of ~1 pM (30 pg/ml), and reveals native chymase activity in serum of most subjects with systemic mastocytosis. α2-Macroglobulin-bound chymase generates angiotensin II in chymase-spiked serum, and it appears in native serum as chymostatin-inhibited activity, which can exceed activity of captopril-sensitive angiotensin-converting enzyme. These findings suggest that chymase bound to α2-macroglobulin is active, that the complex is an angiotensin-converting enzyme inhibitor-resistant reservoir of angiotensin II-generating activity, and that α2-macroglobulin capture may be exploited in assessing systemic release of secreted peptidases. The Journal of Immunology, 2009, 182: 5770–5777.

Received for publication January 14, 2009. Accepted for publication February 26, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by National Institutes of Health Grant HL024136.
2 W.W.R. and S.S. contributed equally to this work.
3 Address correspondence and reprint requests to Dr. George H. Caughey, Veterans Affairs Medical Center, San Francisco, CA 94121; E-mail address: george.caughey@ucsf.edu
4 Abbreviations used in this paper: ACE, angiotensin converting enzyme; α1ACT, α1-antichymotrypsin; α1M, α1-macroglobulin; AAPF-4NA, succinyl-L-Ala-Ala-Pro-Phe-4-nitroanilide; AEPF-4NA, succinyl-L-Ala-Glu-Pro-Phe-4-nitroanilide; VPP-4NA, succinyl-L-Val-Pro-Phe-4-nitroanilide; RETF-4NA, acetyl-L-Arg-Glu-Thr-Phe-4-nitroanilide; kcat, turnover number; Kcat, Michaelis constant; kcat/Km, specificity constant.
from other circulating antipeptidases in key ways. It is nonspecific with regard to peptidase class (serine, aspartyl, thiol, metallo) and attracts peptidases with a broad range of peptide target preferences (18). Although it attaches covalently to peptidases via a thiol ester that becomes reactive after cleavage of a target region in $\alpha_M$, this connection is made with a lysine on the surface of the peptidase and does not involve the canonical antipeptidase mechanism of occupying the substrate binding site (19). Instead, $\alpha_M$ traps the peptidase in a cage-like structure, which is inaccessible to protein targets of the peptidase but may allow access by small substrates to the trapped peptidase. The present findings suggest that human chymase circulates bound to $\alpha_M$, where it is active and can be assayed in serum using a selective, newly developed substrate. The findings further reveal that chymase captured by $\alpha_M$ generates angiotensin II. This suggests that chymase, after secretion by mast cells, remains active longer than once thought and may circulate bound to $\alpha_M$, in which form it can generate angiotensin II.

Materials and Methods

Materials

Recombinant human prochymase was expressed in Trichoplax ni cells and purified as described (20). Mature human chymase was activated from recombinant prochymase and repurified as described (20, 21). Human cathepsin G and bovine $\alpha$-chymotrypsin were purchased from MP Biomedicals and Sigma-Aldrich, respectively. Peptidase substrate succinyl-$\varepsilon$-Ala-$\varepsilon$-Ala-$\varepsilon$-Pro-$\varepsilon$-Phe-$\varepsilon$-nitroanilide (AAPF-4NA) was from Sigma-Aldrich and succinyl-$\varepsilon$-Ala-$\varepsilon$-Glu-$\varepsilon$-Pro-$\varepsilon$-Phe-$\varepsilon$-nitroanilide (AEFP-4NA) was from Bachem; succinyl-$\varepsilon$-Val-$\varepsilon$-Pro-$\varepsilon$-Phe-$\varepsilon$-nitroanilide (VPF-4NA) was provided by Dr. John Burnier of Genentech. Angiotensin I was purchased from Peninsula Laboratories (now Bachem). Antipeptidases $\alpha_M$ and $\alpha_{ACT}$ were from EMD Biosciences/Calbiochem, and human serum used in assay development was from Sigma-Aldrich.

Design and synthesis of a colorimetric substrate of chymase

Screening of recombinant human chymase with a combinatorial library of tetrapeptide substrates (21) identified Arg-Glu-Thr-Tyr or Arg-Glu-Thr-Phe as being highly favored in the $P_4-P_1$ positions on the N-terminal side of the site of hydrolysis. A synthetic inhibitor, $N^\varepsilon$-benzoxycarbonyl-$\varepsilon$-Arg-$\varepsilon$-Glu-$\varepsilon$-Thr-$\varepsilon$-Phe-$\varepsilon$-phosphonate, which was synthesized based on these sequences, inhibited chymase selectively in comparison with cathepsin G (21), which encouraged us to design an assay substrate, acetyl-$\varepsilon$-Arg-$\varepsilon$-Glu-$\varepsilon$-Thr-$\varepsilon$-Phe-$\varepsilon$-nitroanilide (RETF-4NA), which was custom-synthesized by AnaSpec.

Assessment of concentration of active enzyme

Chymase activity was measured by addition of enzyme to buffer containing 1 mM AAPF-4NA, 0.45 mM Tris-HCl (pH 8.0), 1.8 M NaCl, and 10% DMSO. Cathepsin G activity was measured by addition of enzyme to assay buffer containing 1 mM VPF-4NA, 0.1 M HEPES (pH 7.5), 0.5 M NaCl, and 10% DMSO. Chymotrypsin activity was measured in buffer containing 1 mM AAPF-4NA, 0.1 M HEPES (pH 7.5), 0.5 M NaCl, and 10% DMSO. Change in $A_{410}$ nm was monitored at 25°C. The concentration of active enzyme in each preparation was determined by referencing observed activity under these conditions to reported specific activity, which is $2.1 \times 10^5$, $2.4 \times 10^5$, and $1.7 \times 10^5$ $A_{410}$ min/M for human cathepsin G (22), human chymase (6), and bovine chymotrypsin (23), respectively.

Kinetic comparisons of peptide-based colorimetric substrates

Hydrolysis of substrates was compared using recombinant human chymase, human cathepsin G, and bovine chymotrypsin in the presence and absence of $\alpha_M$. For experiments involving $\alpha_M$, each enzyme was incubated with 1000-fold molar excess of $\alpha_M$ in PBS (pH 7.4) at 25°C for 30 min, followed by incubation for 30 min with 2-fold molar excess of $\alpha_{ACT}$ to inactivate any residual free enzyme. To assess relative sensitivity and specificity for chymase free in solution and when bound to $\alpha_M$, we compared kinetic attributes of the novel substrate RETF-4NA with those of AAPF-4NA, AEFP-4NA, and VPF-4NA. Substrates were dissolved in PBS (pH 7.4) containing 0.05% DMSO and 0.01% Triton X-100. Reactions were initiated by addition of free or $\alpha_M$-bound enzyme. The reaction mixture was pipetted in triplicate in 180-μl aliquots into wells of a Costar 3320 flat bottom 96-well plate (Corning Life Sciences), which then was sealed with TempPlate RT optical film (USA Scientific) to minimize evaporation under these conditions to reported specific activity, which is 2.1 $A_{410}$ cuvette vs microtiter plate). Conditions were optimized for assay of native chymase activity in serum using the sealed, 96-well microtiter plate format described in the preceding paragraph. Briefly, 20 μl of serum was diluted 10-fold in 20 mM Tris-HCl (pH 7.9) containing 2 M NaCl, 0.05% DMSO, and 1% Triton X-100, and 10 μl of RETF-4NA was measured in 1 ml cuvettes in a Genesys 5 spectrophotometer (Thermo Fisher Scientific).

Stability of chymase and cathepsin G in serum

Activity was compared in PBS (containing 0.05% DMSO and 0.01% Triton X-100) and in enzyme-spiked serum during 8 h of incubation at 37°C. Aliquots were withdrawn at intervals to measure residual chymase and cathepsin G activity using AAPF-4NA and VPF-4NA, respectively, in 1-ml cuvettes. In additional experiments, stability to five cycles of freezing and thawing was examined in serum spiked with 10 ng/ml active chymase.

Size exclusion chromatography and immunoblotting of chymase-spiked serum

Normal human serum (100 μl) spiked with 340 ng of active human chymase or prochymase was chromatographed using an AKTA purifier system (GE Healthcare) on a Superose 6 GL 10/300 size exclusion column equilibrated with PBS. Outflow was monitored for absorbance at 280 nm. Aliquots of column fractions were assayed for amidolytic activity with RETF-4NA in a 96-well format as noted for chymase-spiked serum. Aliquots from each fraction were divided into six pools covering distinct molecular mass regions. Portions of each pool were electrophoresed, electrobotted to a polyvinylidene difluoride membrane, and probed with anti-human $\alpha_M$ mAb 2D9 (Abcam) and anti-human chymase (CC-1; Abcam). The column was calibrated with thyroglobulin (669 kDa), apoferritin (460 kDa), and BSA (66 kDa). Human chymase was also chromatographed in PBS to establish elution behavior in the absence of $\alpha_M$ and other serum proteins.

Recruitment and pathological stratification of subjects with mastocytosis

Study participants were evaluated at the National Institutes of Health (Bethesda, MD) as part of Institutional Review Board-approved research protocols exploring the pathogenesis of mastocytosis. Twenty-five patients who met World Health Organization criteria for mastocytosis between 2003 and 2008 were included (24). The 15 adult subjects were classified as follows: 13 with indolent systemic mastocytosis and 2 with aggressive systemic mastocytosis. Of the 10 pediatric subjects, 7 were classified as indolent systemic mastocytosis and 3 as cutaneous mastocytosis.

Measurement of immunoreactive tryptase in subjects with mastocytosis

As part of establishing World Health Organization diagnostic criteria for systemic mastocytosis, a total tryptase level was obtained for all participants. Serum was collected at the National Institutes of Health, frozen to -20°C, and then shipped to the Mayo Medical Laboratories, where serum total tryptase was measured via fluorescence enzyme immunoassay with normal level of <11.5 ng/ml, according to the laboratory. Serum for the chymase experiments was handled and mailed to the San Francisco Veterans Affairs Medical Center in a similar manner.
Measurement of chymase activity in subjects with mastocytosis

Serum from subjects with mastocytosis was assayed in duplicate for RETF-4NA-hydrolyzing activity in sealed microtiter plates as described for assays of chymase-spiked serum. RETF-4NA-hydrolyzing activity was measured in duplicate in separate aliquots of the same serum samples after preincubation with 100 μM chymostatin, a chymase inhibitor. Activity observed in the presence of chymostatin was considered background. The difference in ΔA410 nm measured with and without chymostatin was considered to be chymase-like activity. Concentration of active chymase in native samples was determined by extrapolation from standard curves generated using serum spiked with known concentrations of recombinant active chymase.

Assessment of angiotensin II-generating activity in chymase bound to inhibitors

Active chymase (1 pmol) was incubated in 7 μl of PBS at 37°C for 15 min with 5 pmol of human α2M or 5 pmol of human α1ACT. To verify the reaction of chymase with α2M, 1 pmol of chymase was first incubated in PBS at 37°C for 15 min with 5 pmol of α2M and then for 15 min with 5 pmol of α1ACT. Following these incubations, 1 μl of the resulting mixtures (containing 170 fmol of chymase) was incubated with 1 nmol of angiotensin I in 50 μl of PBS for 30 min at 37°C. Reactions were terminated by addition of 1 μl of 12 N HCl, diluted with 60 μl of an aqueous solution of 10% acetonitrile/0.1% trifluoroacetic acid, and injected onto a 2.1 × 250-mm BioBasic C-18 column (Thermo Scientific) equilibrated in 10% acetonitrile/0.1% trifluoroacetic acid on the AKTA purifier system (GE Healthcare). Angiotensin I and cleavage products were eluted with a linear gradient of 10–40% acetonitrile over 2.7 ml (three column volumes). Outflow was monitored for A280 nm. Chromatograms were analyzed using Unicorn 5.0 software (GE Healthcare).

Angiotensin generation by chymase in native human serum

One microliter of native serum or chymase-spiked serum was incubated with 20 nmol of angiotensin I in PBS for 16 h at 37°C with or without 2 mM captopril, 0.4 mM chymostatin, or both inhibitors. Products were extracted on PepClean C-18 spin columns (Thermo Scientific/Pierce), eluted with 50% acetonitrile/0.1% trifluoroacetic acid, and dried by vacuum centrifugation. Pellets were resuspended in 110 μl of 10% acetonitrile/0.1% trifluoroacetic acid and subjected to reverse-phase HPLC as described for angiotensin hydrolyzed in the presence of purified inhibitors.

Results

RETF-4NA is a sensitive and selective substrate for chymase when free or bound to α2M

As revealed in Fig. 1 and Table I, the kinetic performances of the colorimetric substrates compared in this study differ markedly. For chymotrypsin, the best substrate in terms of maximum hydrolytic rate is AAPF-4NA, which is much less rapidly hydrolyzed by cathepsin G and chymase, although this commercially available substrate has been used by investigators to assay all three peptidases. For cathepsin G, the best substrate by far was VPF-4NA, although this peptidase is weak overall compared with chymotrypsin and chymase (as revealed by kcat/Km specificity constants in Table I). Consequently, VPF-4NA is more efficiently hydrolyzed by chymotrypsin and chymase than by cathepsin G, and it has comparatively little ability to discriminate among these enzymes. For chymase, VPF-4NA and RETF-4NA are the best of the substrates examined and yield similar specificity constants. However, as revealed in Fig. 1B and as hypothesized from results of combinatorial screening, our novel substrate RETF-4NA is substantially more selective than the other substrates for chymase in comparison with cathepsin G and chymotrypsin. When kcat/Km is compared for enzymes incubated in PBS, the ratios for chymase, chymotrypsin, and cathepsin G are 15:8.5:1 for AEPF-4NA and a much more selective 55:8.0:1 for RETF-4NA. A selectivity advantage is also noted for AEPF-4NA incubated with α2M, as shown in Table I. Based on these sensitivity and selectivity profiles, AEPF-4NA and RETF-4NA were tested as candidate substrates with which to construct a serum-based, chymase-selective assay.

Chymase measured with high selectivity and sensitivity in chymase-spiked serum

In pilot experiments (not shown), background activity in serum was higher when using AEPF-4NA than when using RETF-4NA. We tested AEPF-4NA because it is commercially available and because our laboratory previously identified a preference by chymase for peptidic substrates with Glu in the P3 position, that is, three residues on the N-terminal side of the site of hydrolysis (21). Indeed, as shown in Fig. 1 and Table I, AEPF-4NA is more readily hydrolyzed by chymase than by the other peptidases. However, AEPF-4NA is not as selective as custom-synthesized RETF-4NA, which is fully optimized based on preferences identified by combinatorial substrate screening. To test RETF-4NA selectivity in serum, 10 pM active chymase, chymotrypsin, and cathepsin G were added separately to aliquots of low-background serum containing 1.4 mM RETF-4NA. At this concentration, which is well above the predicted Ks of chymase and chymotrypsin but likely well below that of cathepsin G, chymotrypsin activity was 10% that of chymase, and cathepsin G activity was 0. This was as predicted by screening of enzyme-substrate combinations in PBS and α2M (Table I), which revealed that hydrolytic rates at substrate concentrations well above Ks are much higher for chymase than
likely reflecting the large molar excess of cathepsin G preincubated with aldosterone. This is evidence that the assay has a wide dynamic range, with the ability to measure activity over a large range of enzyme concentrations. More detailed kinetic evaluation of RETF-4NA hydrolysis by chymase in spiked serum revealed a rate constant of 9.6 ± 0.3 s⁻¹ and a Michaelis-Menten constant of 0.48 ± 0.03 mM (yielding nominal kcat/Km of 20 s⁻¹M⁻¹). The kcat and kcat/Km estimates in this case are minimum values because they assume that all chymase added to serum remains active, which is likely not to be the case. The net effect is that chymase added to serum behaves similarly to the same concentration of chymase in serum (0.03 mM) and observed rates of substrate hydrolysis, as reflected by change in milliabsorbance (ΔmA410 nm) per minute. Standard curves based on this relationship allow determination of levels of chymase-like activity in native serum, as in Fig. 4.

**FIGURE 2.** RETF-4NA-hydrolyzing activity in chymase-spiked serum. Human serum was spiked with human chymase over the range of concentrations of enzyme indicated by the x-axis. There is a strong linear correlation between concentrations of chymase in spiked serum and observed rates of substrate hydrolysis, as reflected by change in milliabsorbance (ΔmA410 nm) per minute. Standard curves based on this relationship allow determination of levels of chymase-like activity in native serum, as in Fig. 4.

### Table 1. Kinetic comparisons of peptidyl nitroanilide hydrolysis by chymotryptic peptidases

<table>
<thead>
<tr>
<th>Chymase</th>
<th>AAPF-4NA</th>
<th>AEPF-4NA</th>
<th>RETF-4NA</th>
<th>VPF-4NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>kcat</td>
<td>9.3 ± 0.8</td>
<td>8.8 ± 0.2</td>
<td>19.7 ± 0.1</td>
<td>17.2 ± 0.4</td>
</tr>
<tr>
<td>Km</td>
<td>0.31 ± 0.07</td>
<td>0.15 ± 0.01</td>
<td>0.64 ± 0.05</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>kcat/Km</td>
<td>30</td>
<td>59</td>
<td>31</td>
<td>59</td>
</tr>
<tr>
<td>Chymase + α2M</td>
<td>49 ± 2</td>
<td>51 ± 1</td>
<td>87 ± 1</td>
<td>8.9 ± 0.6</td>
</tr>
<tr>
<td>kcat</td>
<td>0.28 ± 0.02</td>
<td>0.08 ± 0.08</td>
<td>0.32 ± 0.01</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>kcat/Km</td>
<td>170</td>
<td>640</td>
<td>270</td>
<td>55</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>1.4 ± 0.1</td>
<td>3.7 ± 0.8</td>
<td>7.3 ± 11.9</td>
<td>13.8 ± 0.4</td>
</tr>
<tr>
<td>kcat</td>
<td>0.97 ± 0.06</td>
<td>0.93 ± 0.08</td>
<td>13 ± 36</td>
<td>0.69 ± 0.07</td>
</tr>
<tr>
<td>kcat/Km</td>
<td>1.4</td>
<td>3.9</td>
<td>0.56</td>
<td>20</td>
</tr>
<tr>
<td>Cathepsin G + α2M</td>
<td>0 ± 0</td>
<td>0.71 ± 0.26</td>
<td>0 ± 0</td>
<td>2.4 ± 2.1</td>
</tr>
<tr>
<td>kcat</td>
<td>0.12 ± 0.15</td>
<td>5.9</td>
<td>0.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>kcat/Km</td>
<td>9</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>68 ± 8</td>
<td>12.8 ± 0.5</td>
<td>5.0 ± 0.4</td>
<td>11.8 ± 0.4</td>
</tr>
<tr>
<td>kcat</td>
<td>0.52 ± 0.14</td>
<td>0.39 ± 0.04</td>
<td>1.1 ± 0.2</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>kcat/Km</td>
<td>130</td>
<td>33</td>
<td>4.5</td>
<td>170</td>
</tr>
<tr>
<td>Chymotrypsin + α2M</td>
<td>48 ± 1</td>
<td>31 ± 1</td>
<td>3.9 ± 0.4</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>kcat</td>
<td>0.035 ± 0.003</td>
<td>0.17 ± 0.02</td>
<td>0.057 ± 0.026</td>
<td>0.037 ± 0.007</td>
</tr>
<tr>
<td>kcat/Km</td>
<td>1400</td>
<td>180</td>
<td>68</td>
<td>59</td>
</tr>
</tbody>
</table>

*Units of kcat, Km, and kcat/Km are s⁻¹, mM, and s⁻¹M⁻¹, respectively.*

Serum stabilizes chymase and cathepsin G activity

As shown in Fig. 3, chymase activity in serum is remarkably stable to assay over time in serum, as compared with PBS. This stability contributes to the enhancement of assay sensitivity achieved by prolonged incubation.

**FIGURE 3.** Stability of chymase and cathepsin G activity in serum. Left panel, Examples of repeated measurements of ΔmA410 nm in single wells of a 96-well plate in a kinetic spectrophotometer at 37°C. Individual wells contained RETF-4NA substrate (0.5 mM) and spiked chymase concentrations as follows: ○, 2.1 ng/ml; □, 0.68 ng/ml; ■, 0.23 ng/ml; and □, 0.08 ng/ml. Right panel, Results of long-term incubations of human cathepsin G and chymase in serum (○, cathepsin G; ■, chymase) and PBS (□, cathepsin G; □, chymase). Enzyme-spiked serum and PBS were incubated as stocks for 8 h. Aliquots were withdrawn at the indicated intervals and subjected to cuvette-based spectrophotometric assay of chymotryptic activity using AAPF-4NA for chymase and VPF-4NA for cathepsin G. Results are expressed as percentage of activity relative to activity measured at the start of incubation.
Chymase activity in serum co-elutes with $\alpha_2$M

As shown in Fig. 4, the vast majority of activity of chymase, when the enzyme is spiked into serum and size-fractionated by gel chromatography, elutes at an apparent $M_r$ consistent with capture by $\alpha_2$M. Furthermore, $\alpha_2$M immunoreactivity of $\alpha_2$M in column fractions coelutes with the peak of chymase activity, providing further evidence that chymase, when mixed with the complex mixture of peptidase inhibitors and other proteins in serum, binds mainly to $\alpha_2$M, which preserves its activity.

Detection of chymase activity in serum of subjects with mastocytosis

As shown in Fig. 5, most subjects with systemic mastocytosis have detectable serum chymase-like activity, which correlates weakly with serum levels of immunoreactive total tryptase. Levels of active chymase (in ng/ml) are always lower than those of immunoreactive tryptase in paired samples. The highest chymase activity is in a subject with aggressive systemic mastocytosis. Nearly all subjects with indolent systemic mastocytosis have readily detectable chymase-like activity. However, all three of the subjects with cutaneous mastocytosis have low levels at or near the threshold for detection.

Chymase captured and protected from serpins by $\alpha_2$M generates angiotensin II

As revealed in the chromatograms in Fig. 6, chymase generates bioactive angiotensin II from angiotensin I when preincubated with $\alpha_2$M, but it has no detectable activity when preincubated with the serpin $\alpha_1$ACT. However, when preincubated with $\alpha_2$M and $\alpha_1$ACT together, chymase’s angiotensin II-generating capacity is preserved, consistent with chymase reacting more slowly with $\alpha_1$ACT than with $\alpha_2$M, and gaining protection within the $\alpha_2$M “cage” from inhibition by the serpin. This finding also reveals that the size of the cage in chymase-bound $\alpha_2$M is sufficiently large to admit the decapetide angiotensin I, which is more than twice the length of the tri- and tetrapeptide nitroanilide substrates used in this work to develop the serum chymase assay. The results shown in Fig. 6 also suggest that rates of hydrolysis by $\alpha_2$M-bound chymase are, if anything, greater than for chymase free in solution.

Chymase generates angiotensin II in serum

As shown in Fig. 7, the combination of chymase and captopril almost fully ablates angiotensin-generating capacity of native and 

FIGURE 4. Coelution of chymase activity with $\alpha_2$M in serum. The chromatogram represented by the solid line shows absorbance of Superose 6 fractions generated by serum subjected to gel filtration chromatography in PBS. Downward arrows indicate elution positions of standard proteins of known size applied separately to the column in PBS. The asterisk indicates chymase (~30 kDa) applied to the column in PBS. The dashed line indicates levels of chymase-like (RETF-4NA-hydrolyzing) activity assessed in individual fractions of eluate generated by serum premixed with active chymase. Fractions were also collected in six larger pools, as indicated, then concentrated and subjected to reducing SDS-PAGE and immunoblotting using Abs recognizing $\alpha_2$M. Results, as revealed by the immunoblot, reveal strongest $\alpha_2$M immunoreactivity in pool 2, which also contains most of the chymase activity.

FIGURE 5. Serum chymase activity in mastocytosis. Chymase activity and immunoreactive tryptase was measured in serum from subjects with various types of mastocytosis. Chymase activity was measured using the RETF-4NA microtiter plate assay. Total immunoreactive $\alpha$ plus $\beta$ serum trypptase (protryptase plus mature trypptase) was measured by ELISA. Each symbol represents data from a single subject and sample. The dashed line indicates the active chymase detection threshold (~0.03 ng/ml) of the assay. Mastocytosis abbreviations are as follows: ASM, aggressive systemic mastocytosis; ISM, indolent systemic mastocytosis; CM, cutaneous mastocytosis.

FIGURE 6. Generation of angiotensin II by $\alpha_2$M-captured chymase. These HPLC chromatograms reveal products resulting from incubation of angiotensin I with chymase alone, chymase plus $\alpha_1$ACT, chymase plus $\alpha_2$M, or chymase plus the combination of $\alpha_1$ACT and $\alpha_2$M. Absorbance of the eluate was monitored continuously at 260 nm. Incubation time and chymase concentration were the same in each reaction and were selected so that digestion would allow visualization of parent as well as product peptides. Elution positions of angiotensin I and the bioactive product angiotensin II are as noted.
chymase-spiked serum alike. The sample of serum used in the studies in Fig. 7 was chosen for its low baseline chymase-like (i.e., chymostatin-sensitive) activity to allow exploration of concentration-responsiveness to chymase in spiking experiments. Addition of chymase to serum increases chymostatin-sensitive angiotensin II-generating activity in proportion to the concentration of added chymase. These findings establish that chymase can generate angiotensin II in serum. In the sample of serum used in the Fig. 7 studies, native ACE-like (captopril-sensitive) activity is at least ~4-fold greater than that of native chymase-like (chymostatin-sensitive) activity, as reflected by relative angiotensin II-generating capacity revealed in the first four bars of the graph. In other samples (not shown), native chymase-like angiotensin II-generating activity exceeds ACE-like activity. These findings suggest that chymase can generate angiotensin II in native serum and that its contribution can be similar to or even greater than that of soluble ACE.

Discussion

This work reveals that an active form of human chymase can be captured by α2M, in which form it can cleave small peptide substrates, including angiotensin I, and is protected from irreversible inactivation by serpins and other antiproteases in biological fluids. We exploited α2M binding to develop a sensitive and specific assay for chymase activity in the serum of subjects with mastocytosis. These studies reveal that chymase, after secretion by mast cells and capture by α2M, can cleave small peptides for longer than once thought possible. Extravascular chymase captured and protected by α2M may be an important source of non-ACE-generated angiotensin II near tissue sites of mast cell degranulation. The portion of α2M-caged chymase making its way to the bloodstream provides the basis of our serum assay and may be a mobile source of angiotensin II-generating chymase in blood and tissues remote from original sites of mast cell degranulation. The half-life of peptidase-bound α2M in blood in vivo is 9–12 min, with activity of labeled complex peaking at 30–40 min in liver (24), which appears to be a major site of uptake and destruction of the complex. Thus, chymase activity in blood will reflect a balance between rates of production and removal of the chymase–α2M complex. The half-life of peptidase-α2M complexes before entering the bloodstream is not known. Nonetheless, the duration of chymase activity following capture by α2M is profoundly longer compared with the fate of other secreted immune peptidases. For example, neutrophil elastase and chymase’s closest relative, cathepsin G, are inactivated in plasma by serpin-class inhibitors in a small fraction of 1 s (half life of ~0.4 ms (25)). The half-life of the chymase–α2M in blood, if typical of other serine peptidase-α2M complexes, is >10⁶-fold longer than that of neutrophil elastase.

Under the optimized conditions of our assay, the activity of chymase bound to α2M in serum ex vivo is remarkably stable, especially compared with stability of pure chymase in PBS or of chymase combined with serpins. We exploited this stability, which allows prolonged incubations with chymase substrates, to increase the sensitivity of the serum assay. The assay we report in this work is an alternative to the development and application of immunoassay-based assays. Although Abs raised against human chymase work well in immunohistochemical applications and in blotting of purified chymase, they are less successful as components of immunoassays for detecting chymase in complex biological fluids, including serum (26). This may be because most chymase released from mast cells becomes covalently linked to (and caged by) α2M, in which form its major Ab-binding epitopes may be shielded from interacting productively with Abs. To our knowledge, the only reported successful use of an immunoassay to detect chymase in human serum was in postmortem specimens in cases of anaphylaxis (27). However, in the vast majority of cases the level of chymase determined by immunoassay is below the level of detection (26, 27). Unlike activity-based assays, immunoassays have the potential to detect prochymase, denatured chymase, and other proteolytically inactive forms, which are not expected to be captured by α2M because they are unable to cleave the bait region.

Investigations in mice suggest that little if any prochymase is stored by mast cells, except in the case of animals lacking the intracellular chymase-activating enzyme dipeptidylpeptidase I (28). In humans, it is not known whether there is constitutive release of prochymase from mast cells in tissues. However, if a major portion of chymase were released in the proenzyme form, one would expect greater ease in developing immunoassays, since prochymase is not captured by α2M. In contrast, the great majority of circulating immunoreactive β tryptases, which are produced by most human mast cells, is thought to be immature, inactive proenzyme (29, 30). This is also true of β tryptases in humans who possess α genes (31). However, levels of mature β tryptases can rise substantially in some subjects shortly after anaphylaxis (32), presumably because the active tryptase tetramer, which is much larger than monomeric chymase, is too big to be engulfed by the α2M cage (33). The weak correlation of active chymase levels with tryptase levels in our mastocytosis samples, as well as the major difference between tryptase and chymase in the range of measured concentrations, may relate to major disparity between tryptases and chymase in the extent to which mast cells release the two peptidase types as proenzymes. If human chymase, unlike tryptases, is released mainly from granules via a regulated pathway, then there is the potential that chymases are released acutely in larger amounts in settings of anaphylaxis, which is a possibility that we are exploring. Nonetheless, it seems likely that chymase activity in serum is influenced by total body burden of mast cells, in that in vitro assays of mast cells suggest that chymase “leaks” from mast cell granules in the absence of specific stimulation at a low but steady rate (34). This hypothesis is consistent with our assay results in subjects with mastocytosis, in that chymase levels are much higher in systemic mastocytosis than in more localized cutaneous mastocytosis. Nonetheless, a few subjects with the indolent subtype of systemic mastocytosis had levels at or below the

FIGURE 7. Generation of angiotensin II by serum chymase. The graph shows results of measurement of angiotensin II-generating capacity of native serum and of the same sample spiked with recombinant human chymase (50 or 100 pM). Some samples were preincubated with an ACE inhibitor (captopril), a chymase inhibitor (chymostatin), or with both inhibitors, as indicated.

Note: The above text is a natural representation of the document content. It has been formatted for readability and clarity, ensuring that all relevant information is preserved.
level of detection, which may reflect low mast cell burden or possibly variations in peptidase phenotype, because mastocytosis cells can vary in relative expression of tryptases and chymase (35).

The present studies underscore the value of combinatorial peptidase substrate screening in developing selective substrates for specific peptidases. Our custom substrate RETF-4NA was synthesized for the present studies based on results of a screen of ~160,000 potential peptide substrates varying in amino acid composition at positions P1 through P4 in relation to the site of hydrolysis (21). In the serum chymase assay, RETF-4NA was clearly superior to standard, available substrates of chymotryptic serine peptidases. Although the $K_m$ of RETF-4NA is similar to that of several other peptidyl nitroanilide substrates cleaved by chymase, RETF-4NA is substantially more chymase-selective than other substrates. This undoubtedly contributes to the low levels of nonspecific RETF-4NA cleavage in serum, which in turn reduces the signal-to-noise ratio in the assay and provides an important boost to sensitivity. Intriguingly, RETF-4NA and certain of the other substrates are more avidly cleaved by chymase in its $\alpha_2$M-bound form than in its free, unbound form. Prior work with chymase and peptidyl nitroanilide substrates showed that the amido lytic activity of human chymase is sensitive to salt and solvent effects. Perhaps the environment in the $\alpha_2$M cage favors these types of interactions. Tight quarters may favor interactions between substrate and cage walls, which may enhance substrate binding. However, the kinetic data summarized in Table I suggest that the effect on binding (as reflected by lowering of $K_m$) is not as great as the effect on substrate turnover (as reflected by increases in $V_{max}$).

Due to the limited size of the $\alpha_2$M cage, it seems unlikely that $\alpha_2$M can engulf chymase complexed with macromolecular heparin proteoglycan (with which much or most chymase is exocytosed from secretory granules). Rather, we speculate that entrapment occurs as chymase dissociates from the noncovalent complex, or that $\alpha_2$M entraps the portion of the chymase pool that is bound to smaller fragments of heparin. Indeed, Walker et al. (7) showed that heparin glycans are covalently linked to the chymase, although it slowly association rates, affects neither the formation of a covalent complex between chymase and $\alpha_2$M nor the stoichiometry of inhibition.

The observed serum chymase activity in this study is likely to originate from mast cells in extravascular sites, since mast and mastocytosis cells in mature form circulate in small numbers or not at all. The significance of angiotensin II generated by chymase in blood per se is unclear. Although we identified samples of serum in which chymase-like activity makes a greater contribution than ACE-like activity, most angiotensin II generation by ACE in vivo is thought to be contributed by membrane-bound ACE attached to the luminal surface of endothelial cells, rather than by ACE shed into solution. Perhaps the greatest significance of activity in serum is as a marker of chymase released in extravascular tissues. Local angiotensin II-generating capacity can be assumed to be much greater at sites of mast cell degranulation, before dilution in plasma. Indeed, in tissues such as heart, angiotensin II-generating machinery appears to be compartmentalized, with ACE and chymase-like peptidases being responsible for intra- and extravascular (interstitial) production, respectively (36). In the absence of specific mast cell stimulation, baseline leak of chymase from resident mast cells, combined with $\alpha_2$M capture, could provide background production of angiotensin II, which could be responsible for proposed tonic effects on smooth muscle and stromal cells (16, 37, 38) contributing to remodeling, including arteriopathy and fibrosis. In settings of acute mast cell degranulation, chymase-generated angiotensin II can be expected to spike, producing short-term effects, such as changes in caliber of skeletal muscle resistance vessels (8).

Disclosures

The authors have no financial conflicts of interest.

References

1. Reilly, C. F., D. A. Tewksbury, N. B. Schechter, and J. Travis. 1982. Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell pro-

References


