Substrate Conformation Modulates Aggrecanase (ADAMTS-4) Affinity and Sequence Specificity

SUGGESTION OF A COMMON TOPOLOGICAL SPECIFICITY FOR FUNCTIONALLY DIVERSE PROTEASES*3

Received for publication, May 31, 2006, and in revised form, November 2, 2006. Published, JBC Papers in Press, November 9, 2006, DOI 10.1074/jbc.M605236200

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Protease-substrate interactions are governed by a variety of structural features. Although the substrate sequence specificities of numerous proteases have been established, “topological specificities,” whereby proteases may be classified based on recognition of distinct three-dimensional structural motifs, have not. The aggrecanase members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family cleave a variety of proteins but do not seem to possess distinct sequence specificities. In the present study, the topological substrate specificity of ADAMTS-4 (aggrecanase-1) was examined using triple-helical or single-stranded poly(Pro) II helical peptides. Substrate topology modulated the affinity and sequence specificity of ADAMTS-4 with $K_m$ values indicating a preference for triple-helical structure. In turn, non-catalytic ADAMTS-4 domains were critical for hydrolysis of triple-helical and poly(Pro) II helical substrates. Comparison of ADAMTS-4 with MMP-1 (collagenase 1), MMP-13 (collagenase 3), trypsin, and thermolysin using triple-helical peptide (THP) and single-stranded peptide (SSP) substrates demonstrated that all five proteases possessed efficient “triple-helical peptidase” activity and fell into one of two categories: $(k_{cat}/K_m)_\text{SSP} > (k_{cat}/K_m)_\text{THP}$ (thermolysin, trypsin, and MMP-13) or $(k_{cat}/K_m)_\text{THP} \cong (k_{cat}/K_m)_\text{SSP}$ and $(K_m)_\text{SSP} > (K_m)_\text{THP}$ (MMP-1 and ADAMTS-4). Overall these results suggest that topological specificity may be a guiding principle for protease behavior and can be utilized to design specific substrates and inhibitors. The triple-helical and single-stranded poly(Pro) II helical peptides represent the first synthetic substrates successfully designed for aggrecanases.

Collagen catabolism is considered a committed physiological event in remodeling of tissues and embryonic development as well as a critical step in the pathology of numerous diseases (1–3). The triple-helical structure of collagen renders it resistant to most proteases. Several members of the metallopeptidase “clan” MA(M), specifically matrix metalloproteinases (MMPs), possess collagenolytic activity (4). For example, one or more of the interstitial collagens (types I–III) are hydrolyzed within their triple-helical domain by MMP-1, -2, -8, -13, -14, and -18 (5). Other members of the MMP family, such as MMP-3, share similar primary and tertiary structures and substrate sequence specificities with collagenolytic MMPs but do not cleave triple-helical structures (6–9). Triple-helical structure itself provides favorable interactions with several MMPs (i.e. MMP-1 and MMP-8 hydrolyze a triple-helical substrate more efficiently than an analogous single-stranded one), whereas the activities of other MMPs, such as MMP-3, are reduced when susceptible sequences are incorporated within a triple-helical structure (9–12). Considerable evidence has accumulated that non-catalytic domains or regions beyond the active site, termed exosites, play significant roles in MMP recognition and processing of natural protein substrates (8, 13–15). The apparent regulatory role played by the collagen triple helix, in concert with recognition by regions beyond the protease active site, suggests that topology of the substrate may be an element of proteolytic specificity.

In the present study we considered the topological specificity of the metallopeptidase family M12(B), which are members of the same MA(M) clan as MMPs but are not known to cleave triple-helical domains within collagens. More specifically, we examined the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family to evaluate the role of substrate topology on enzyme activity. Among the ADAMTS family members, several have been described to have proteolytic activity toward aggrecan (ADAMTS-1, -4, -5, -9, and -15),

* This work was supported by National Institutes of Health Grants AR 39189 (to H. N.) and CA 77402, CA 98799, and EB 000289 (to G. B. F.), the Wellcome Trust (Reference Number 057508 (to H. N.)), a Glenn/American Federation for Aging Research (AFAR) Scholarship (to J. L. L.-F.), and the Florida Atlantic University Center of Excellence in Biomedical and Marine Biotechnology. 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.

†The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S7.

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§The abbreviations used are: MMP, matrix metalloproteinase; ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; Dnp, 2,4-dinitrophenyl; Fmoc, 9-fluorenylmethoxy carbonyl; fTHP, fluorogenic triple-helical peptide; Hyp, 4-hydroxy-L-proline; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; Mca, (7-methoxycoumarin-4-yl)acetyl; SSP, single-stranded peptide; TIMP, tissue inhibitor of metalloproteinases; THP, triple-helical peptide; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bis-Tris, 2-[bis(hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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whereas others can remove the N-propeptide region from native interstitial procollagens (ADAMTS-2, -3, and -14) or cleave von Willebrand factor (ADAMTS-13) (16–18). Interestingly the processing of the collagen N-propeptide region by ADAMTS-2 appears to require retention of substrate superecondary (triple-helical) structure (19, 20). In a similar fashion, N-terminal truncations in the interglobular domain of aggregan can reduce ADAMTS-4 activity toward the aggregcanase cleavage site (21) possibly due to alterations in the proteoglycan three-dimensional structure. Consistent with these results, short or random coil peptides modeled after aggregcan cleavage sites are not hydrolyzed efficiently by ADAMTS-1, ADAMTS-4, or ADAMTS-5 (21–24). These results suggest that members of the ADAMTS family require a distinct topology for efficient substrate hydrolysis and that some family members (such as ADAMTS-2, -3, and -14) may interact with a three-stranded motif. Furthermore ADAMTS-1 processes collagen-associated proteins (25, 26). Thus, triple-helical topology may be generally recognized by ADAMTS family members.

In the present study, we (a) designed potential substrates for ADAMTS-4 based on triple-helical or poly(Pro) II helical topologies, (b) utilized deletion mutants of ADAMTS-4 to evaluate the enzyme domains critical for processing of designed substrates, and (c) examined the collagenolytic activity and “topological specificity” of ADAMTS-4 in comparison with collagenolytic MMPs and proteases that cleave a wide variety of substrates (trypsin and thermolysin). Ultimately we were able to develop unique substrates for ADAMTS-4 and propose a role for the triple helix as a topology generally recognized by proteases.

**EXPERIMENTAL PROCEDURES**

**Materials**—All standard chemicals were peptide synthesis or molecular biology grade and purchased from Fisher Scientific. Guinea pig skin type I collagen and gelatin were obtained as described previously (15), while human type IV collagen was purchased from Fisher Scientific. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, 1-hydroxybenzotriazole, and Fmoc-amino acid derivatives (including Fmoc-Lys(Mca) and Fmoc-Lys(Dnp)) were obtained from Novabiochem. Amino acids are of the L-configuration. Hexanoic acid (CH$_3$-(CH$_2$)$_4$-CO$_2$H, designated C$_6$) and decanoic acid (CH$_3$-(CH$_2$)$_9$-CO$_2$H, designated C$_{10}$) were purchased from Fisher Scientific. THPs and SSPs were synthesized by Fmoc solid-phase methodology and purified in our laboratory by methods described previously (12, 27, 28). The syntheses and characterizations of fTHP-4, fSSP-3, and (α1(IV)1263–1277) THP have been described previously (12, 28–30). All peptides were synthesized as C-terminal amides to prevent diketopiperazine formation (31).

**Peptide Analyses**—Analytical reversed-phase high performance liquid chromatography was performed on a Hewlett-Packard 1100 liquid chromatograph equipped with a Vydac 218TP5415 C$_18$ reversed-phase column (5-μm particle size, 300-Å pore size, 150×4.6 mm). Eluants were 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B). The elution gradient was 0–100% B in 20 min with a flow rate of 1.0 ml/min. Detection was at λ = 220, 324, and 363 nm. MALDI-TOF-MS was performed on a Hewlett-Packard G2025A LD-TOF or ABD DE-STR Voyager mass spectrometer using either α-cyano-4-hydroxycinnamic acid or a 2,5-dihydroxybenzoic acid/2-hydroxy-5-methoxybenzoic acid (9:1, v/v) matrix (32, 33). Single chain mass values were as follows: (α1(IV)1263–1277) SSP, [M + H]$^+$ 1463.1 Da (theoretical mass, 1463.6 Da); fTHPa, [M + H]$^+$ 1464.1 Da (theoretical mass, 1464.0 Da); and fSSPa, [M + H]$^+$ 1465.1 Da (theoretical mass, 1464.2 Da). All mass values were within 0.07% of theoretical mass.

**Circular Dichroism Spectroscopy**—CD spectra were recorded over the range λ = 190–250 nm on a Jasco J-600 or J-810 spectropolarimeter using a 1.0-cm-path length quartz cell. Thermal transition curves were obtained by recording the molar ellipticity ([θ]) at λ = 222 nm while the temperature was continuously increased in the range of 5–95 °C at a rate of 0.2 °C/min. Temperature was controlled using a Jasco PTC-348W temperature control unit. For samples exhibiting sigmoidal melting curves, the inflection point in the transition region (first derivative) is defined as the melting temperature ($T_m$).

**Enzymes**—Pro-MMP-1 was expressed in Escherichia coli and folded from the inclusion bodies as described previously (8). Pro-MMP-1 was activated by reaction with 1 mM 4-aminophenylmercuric acetate and a 1/10 molal amount of MMP-3 at 37 °C for 6 h. After activation, MMP-3 was completely removed from MMP-1 by affinity chromatography using an anti-MMP-3 IgG Affi-Gel 10 column. Pro-MMP-13 was a generous gift from Dr. Maureen Horrocks, AstraZeneca Pharmaceuticals, and was activated with 1 mM 4-aminophenylmercuric acetate. The amounts of active MMP-1 and MMP-13 were determined by titration with recombinant tissue inhibitor of metalloproteinases-1 (TIMP-1) (34) over a concentration range of 0.1–3 μg/ml. Pro-MMP-1 with the C-terminal hemopexin-like domain deleted (residues 243–450; designated pro-MMP-1(Δ243–450)) was expressed in E. coli using the expression vector pET3a (Novagen), folded from inclusion bodies, and purified as described previously (35). The zymogen was activated as described above for the full-length pro-MMP-1.

ADAMTS-4 and C-terminal deletion mutants were expressed in 293-EBNA cells and purified as described previously (36). The concentrations of active recombinant ADAMTS-4 and variants were determined by titration with N-TIMP-3 (36). Trypsin and thermolysin were obtained from Promega and Sigma, respectively.

**Assays**—Two different peptidase assay methods were utilized, the first for discontinuous fluorometric analyses and the second for continuous fluorometric analyses. For the discontinuous assay method, (α1(IV)1263–1277) THP and (α1(IV)1263–1277) SSP were prepared as stock solutions in “fluorometric” assay buffer (50 mM Tricine, pH 7.5, 50 mM NaCl, 10 mM CaCl$_2$, 0.05% Brij-35). Enzyme (MMP-1, MMP-13, trypsin, and thermolysin) assays were carried out in assay buffer by incubating a range of substrate concentrations (20–200 μM) with 5–40 nM enzyme at 30 °C. Enzymatic activity was terminated by the addition of 20 μl of the enzyme/substrate solution to 30 μl of o-phenanthroline (20 μM) or 50 μl of fluorescein at appropriate times. Rates of hydrolysis were monitored by the addition of 200 μl of fluorescein solution. Fluorescein
solution was prepared by first dissolving fluorogenic substrate in acetone at a concentration of 40 mM and then diluting the fluorogenic concentration to 5 mM with assay buffer minus Brij-35. Fluorescine reacts with free amino groups, resulting in a fluorophore with $\lambda_{\text{excitation}} = 387$ nm and $\lambda_{\text{emission}} = 480$ nm. Fluorescence was measured on a Molecular Devices SPECTRAmax Gemini dual scanning microplate fluorometer. Rates of hydrolysis were obtained from plots of fluorescence versus time using only data points corresponding to less than 40% full hydrolysis. The slope from these plots was divided by the fluorescence change corresponding to complete hydrolysis. The slope from these plots was divided by the fluorescence change corresponding to complete hydrolysis and then multiplied by the substrate concentration to obtain hydrolysis rates in units of $\mu$M/s.

For the continuous assay method, substrates fTHP, fSSP, fTHP-4, and fSSP-3 were prepared as stock solutions in fluorometric assay buffer. Enzyme (MMP-1, MMP-13, ADAMTS-4, trypsin, and thermolysin) assays were carried out in assay buffer by incubating a range of substrate concentrations (1–64 $\mu$M) with 5–50 nM enzyme at 30 °C. Fluorescence was measured using $\lambda_{\text{excitation}} = 325$ nm and $\lambda_{\text{emission}} = 393$ nm. Hydrolysis rates were obtained from plots of fluorescence versus time using only data points corresponding to less than 40% full hydrolysis. The slope from these plots was divided by the fluorescence change corresponding to complete hydrolysis and then multiplied by the substrate concentration to obtain initial velocity in units of $\mu$M/s. The fluorescence change corresponding to complete hydrolysis differed for fTHPs and fSSPs and as a function of temperature, and thus trends in rates of hydrolysis (where fluorescence is not corrected) occasionally differ from trends in $k_{\text{cat}}/K_m$. To determine activation energies ($E_a$), kinetic parameters were determined at 25, 30, 35, and 40 °C, and an Arrhenius plot of $\log k_{\text{cat}}$ versus 1/temperature (K) was constructed where the slope $= -E_a/2.3R$ ($R$ is the molar gas constant). For all $E_a$ value determinations, the highest temperature utilized was $\sim 10$ °C below the $T_m$ of the triple-helical substrate.

Sites of substrate hydrolysis by ADAMTS-4, thermolysin, and trypsin were established by MALDI-TOF-MS and/or Edman degradation sequence analysis using an Applied Biosystems 477A protein sequencer/120A analyzer as described previously (33). Sites of fTHP-4 and fSSP-3 hydrolysis by MMP-1 and MMP-13 have been evaluated previously (12).

Type I collagen, type IV collagen, and type I gelatin hydrolysis was analyzed as described previously (15, 37). Protein concentrations were 60–50 nM for MMP-1, MMP-13 (243–450), and MMP-13; 525 nM for ADAMTS-4-3; 15 nM for thermolysin; 20 nM for trypsin; 1.8 mg/ml for type I collagen and gelatin; and 3.0 mg/ml for type IV collagen. Five milliliters of enzyme were used for each assay, followed by fluorescence and MALDI-MS analyses indistinguishable from glucosylated transferrin, fibromodulin, decorin, and versican (36, 39) and that the thrombospondin type I domain of ADAMTS-4 was critical for proteolytic activity (36). If ADAMTS-4 possesses topological specificity, the use of ADAMTS-4 deletion mutants may identify the domain responsible for this specificity.

Treatment of fTHPs with full-length ADAMTS-4 (ADAMTS-4-1) and four ADAMTS-4 deletion mutants at 25 °C followed by fluorescence and MALDI-MS analyses indistinguishable from glucosylated transferrin, fibromodulin, decorin, and versican (36, 39) and that the thrombospondin type I domain of ADAMTS-4 was critical for proteolytic activity (36). If ADAMTS-4 possesses topological specificity, the use of ADAMTS-4 deletion mutants may identify the domain responsible for this specificity.

The use of C- and C10-modified peptides results in triple helices that are stabilized by the alkyl chains (28, 29) in a fashion similar to covalent crosslinking of the three chains (33). This greatly reduces concentration-dependent effects on triple helix stability (86), although, even without addition of the alkyl chains, such effects are minimal based on the range of substrate concentrations examined here. Additionally, differences in substrate activities are not complicated by substrate aggregation. THPs containing N-terminal, C14 branched alkyl chains can aggregate to form micelles (87), and C10 and C14 alkyl chains induce small aggregates of 18 and 22 triple helices, respectively (88). However, in these prior cases, the triple-helical peptide concentration was 0.5–1.5 mM. The present study used C5- and C10-triple-helical peptides at considerably lower concentrations (1–20 $\mu$M). Aggregates at these concentrations were not observed previously by dynamic light scattering (89). This result is consistent with the high critical micellar concentrations of the potassium salts of octadecanoic acid (C18$, K_m$), cis-$\Delta9$-octadecenoic acid (C18:1$, K_m$), and 12-hydroxy-cis-$\Delta9$-octadecenoic acid (C18:1,OH$, K_m$) at 0.5, 1.5, and 3.6 mM, respectively (90).

**RESULTS**

**ADAMTS-4 Substrate Design**—To investigate topological requirements of a typical aggregcanase (ADAMTS-4, aggregcanase 1), we used chemically synthesized fluorogenic substrates in which (7-methoxycoumarin-4-yl)acetyl (Mca) is the fluorophore and 2,4-dinitrophenyl (Dnp) is the quencher (9, 12, 30). Substrate sequences were based on known aggregcanase cleavage sites in human aggregcan (38). An ADAMTS-4 substrate was designed that utilized the aggregcan sequences (1545° cis-Gly1545° cis-Gly1545° cis-cleavage site within a triple-helical structure. Residues 1540–1552 from human aggregcan (Thr-Ala-Ser-Glu-Leu-Glu-Leu-Glu-Gly-Arg-Gly-Thr-Lys(Dnp) Gln-Ile-Ser) (Gly-Pro-Hyp-Hyp-Gly)45° cis-Gly-Pro-Hyp-Hyp-Gly (Gly-Pro-Hyp-Gly-Thr-Lys(Mca)-Gly-Glu-Leu-Glu-Gly-Arg-Gly-Thr-Lys(Dnp) Gln-Ile-Ser) (Gly-Pro-Hyp-Hyp-Gly)45° cis-Gly-Pro-Hyp-Hyp-NH$_2$).

**FIGURE 1. Sequences of triple-helical (fTHP) and single-stranded (fSSP) fluorogenic substrates.** fTHP and fSSP incorporate models of the 1540–1552 sequence from human aggregcan. –, cleavage site.
cated that fTHPa was readily hydrolyzed by all proteases (Fig. 4, top) except ADAMTS-4-1 (data not shown). The lack of hydrolysis by ADAMTS-4-1 is not surprising as full-length ADAMTS-4 has a different aggrecan sequence specificity profile than the ADAMTS-4 deletion mutants (36). Deletion of the Cys-rich region of ADAMTS-4 (ADAMTS-4-2 → ADAMTS-4-3) resulted in a slight increase in the rate of substrate hydrolysis (Fig. 4, top). Further deletion of the thrombospondin type I domain (ADAMTS-4-3 → ADAMTS-4-4) and disintegrin-like domain (ADAMTS-4-4 → ADAMTS-4-5) was detrimental for activity (Fig. 4, top). Thus, the thrombospondin type I and disintegrin-like domains were critical for favored recognition and processing of triple-helical structure. This trend is identical to that reported for ADAMTS-4 general proteinase activity detected with S-carboxymethylated transferrin (36). However, hydrolysis of fTHPa at 37 °C or fSSPa at either 25 or 37 °C by ADAMTS-4 deletion mutants revealed a different trend as activity decreased only slightly upon deletion of the thrombospondin type I domain (ADAMTS-4-3 → ADAMTS-4-4) (Fig. 4). Instead activity toward fSSPa (at either 25 or 37 °C) was most affected by deletion of the Cys-rich and disintegrin-like domains, whereas activity toward fTHPa at 37 °C was substantially decreased by deletion of the disintegrin-like domain.

Individual kinetic parameters were then determined for ADAMTS-4-2 hydrolysis of fTHPa and fSSPa at 25 and 37 °C (Table 1). To calculate kinetic parameters, it was assumed that hydrolysis occurred rapidly through all three strands of fTHPa and that multiple cleavages had little effect on control fluorescence values. Hydrolysis curves were consistent with these assumptions as discussed previously (33). At 25 °C, fSSPa was hydrolyzed with a 6-fold worse $K_m$ than fTHPa and approximately half the overall $k_{cat}/K_m$ value (Table 1). When assays...

**FIGURE 2.** Thermal transition curve for purified THPa in 0.25% (v/v) fluorometric assay buffer at a substrate concentration of 2 μM. Molar ellipticities ([θ]) were recorded at λ = 225 nm while the temperature was increased from 5 to 85 °C. The inset is the first derivative of the transition curve where the $T_m$ value was determined from the trough.

**FIGURE 3.** Domain structures of ADAMTS-4 and deletion mutants. Dis, disintegrin-like domain; TS, thrombospondin type I domain.

**FIGURE 4.** Rates of fTHPa (top) and fSSPa (bottom) hydrolysis by ADAMTS-4 variants. Hydrolysis was examined using a 100 nM concentration of each ADAMTS-4 and 10 μM substrate where the black bar is 25 °C and the white bar is 37 °C. Rates were determined as described under “Experimental Procedures.” RFU, relative fluorescence units.

**TABLE 1**

Kinetic parameters for hydrolysis of single-stranded and triple-helical substrates by ADAMTS-4-2

| Substrate | $k_{cat}$ (μM s$^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ (μM$^{-1}$ s$^{-1}$) | $T$ (°C) |
|-----------|------------------------|------------|-------------------------------|---------|
| fTHPa     | 0.0120                 | 47.1       | 415.0                         | 25      |
| fSSPa     | 0.0720                 | 279.0      | 258.0                         | 25      |
| fTHPa 37°C| 0.181                  | 657.0      | 276.0                         | 37      |
| fSSPa 37°C| 0.433                  | 761.0      | 569.0                         | 37      |
were performed at 37 °C, which is above the \( T_m \) of fTHPa (33 °C), the \( K_m \) values for fTHPa and fSSPa hydrolysis by ADAMTS-4-2 were very similar, and the overall \( k_{cat}/K_m \) for fSSPa hydrolysis was twice that of fTHPa hydrolysis (Table 1). Virtually identical trends were observed with ADAMTS-4-3 (data not shown). Thus, ADAMTS-4 binding to and hydrolysis of substrate was sensitive to constraint in a triple-helical context.

The sequence specificity of ADAMTS-4 was also altered based on triple-helical structure. For example, after 18 h the Glu-Leu, Leu-Glu, Glu-Gly, and Arg-Gly bonds were hydrolyzed in fSSPa, but only Glu-Leu and Glu-Gly bonds were hydrolyzed in fTHPa (supplemental data, Figs. S1–S3). Based on mass spectrometric analyses, hydrolysis of these peptide bonds was due to endopeptidase activity.

**Analysis of Collagenolytic Activity of ADAMTS-4**—The triple-helical peptidase activity of ADAMTS-4 led us to consider the collagenolytic potential of this protease. Activity was examined toward a continuous triple helix (type I collagen) and an interrupted triple helix (type IV collagen). For comparison, collagenolytic MMPs (MMP-1 and MMP-13) and general proteases of different chemical mechanisms (thermolysin and trypsin) were included. Type I collagen was hydrolyzed into \( \frac{3}{4} \) and \( \frac{1}{4} \) fragments by MMP-1 and MMP-13 (Fig. 5A, lanes 3 and 5). In turn, type I collagen was not hydrolyzed by truncated MMP-1, which did not contain the C-terminal hemopexin-like domain (MMP-1(D243–450)) (Fig. 5A, lane 4). ADAMTS-4-3 was not active against type I collagen, whereas thermolysin and trypsin exhibited low levels of collagen hydrolysis (Fig. 5A, lanes 6–8). ADAMTS-4-1 (full-length protease) also did not hydrolyze type I collagen (data not shown). These collagenolytic activities are consistent with those reported previously for MMP-1, MMP-1(D243–450), MMP-13, and trypsin (5, 15, 40).

The same series of enzymes was compared for activity against type IV collagen. Only thermolysin showed significant type IV collagenolytic activity (Fig. 5B, lane 7). Prior studies had shown that MMP-1 and MMP-13 do not cleave type IV collagen (5). Finally, hydrolysis of denatured type I collagen (gelatin) was compared for all enzymes. MMP-1 and MMP-1(D243–450) showed modest gelatinolytic activities, whereas MMP-13 readily hydrolyzed the substrate (Fig. 5C, lanes 2–4). ADAMTS-4-3 had a low level of activity toward gelatin, whereas thermolysin and trypsin rapidly cleaved it (Fig. 5C, lanes 5–7). The relative activities of MMP-1 and MMP-13 toward gelatin had been observed previously (41), whereas an aggreganase related to ADAMTS-4 (ADAMTS-1) has recently been reported to cleave gelatin (25).

**Examination of Triple Helix Hydrolysis by Functionally Diverse Proteases**—ADAMTS-4 exhibited a very interesting, and rather unique, substrate profile in that it cleaved a short triple-helical structure (fTHPa) but not a long one (type I or type IV collagen). This result led us to consider a more widespread “triple-helical topological specificity” among proteases, i.e. the triple helix one of several structural elements that may serve as protease recognition elements. To explore this possibility, we considered a triple-helical structure that possesses potential cleavage sites for several general proteases but not MMPs. As shown previously, some MMP cleavage sites are susceptible to more general proteolysis presumably due to a “relaxed” substrate conformation (42–44). By using a triple-helical substrate not cleaved by MMPs, such potential biases are minimized. The type IV collagen al1(IV)1263–1277 gene-derived sequence, Gly-Val-Lys-Gly-Asp-Lys-Gly-Asn-Pro-Gly-

**FIGURE 5. Hydrolysis of type I collagen (A), type IV collagen (B), and type I gelatin (C) by MMP-1, MMP-13, ADAMTS-4, thermolysin, and trypsin.** For A, lanes 1 and 9 are molecular mass markers (in kDa), lane 2 is type I collagen, lane 3 is MMP-1 + collagen, lane 4 is MMP-1(D243–450) + collagen, lane 5 is MMP-13 + collagen, lane 6 is ADAMTS-4-3 + collagen, lane 7 is thermolysin + collagen, and lane 8 is trypsin + collagen. For B, lanes 1 and 9 are molecular mass markers (in kDa), lane 2 is type IV collagen, lane 3 is MMP-1 + collagen, lane 4 is MMP-1(D243–450) + collagen, lane 5 is MMP-13 + collagen, lane 6 is ADAMTS-4-3 + collagen, lane 7 is thermolysin + collagen, and lane 8 is trypsin + collagen. Type IV collagen hydrolysis was repeated using a higher ADAMTS-4-3 concentration (450 nM) or ADAMTS-4-1 (10 nM), but no hydrolysis was observed (data not shown). For C, lanes 1 and 9 are molecular mass markers (in kDa), lane 2 is type I gelatin, lane 3 is MMP-1 + gelatin, lane 4 is MMP-1(D243–450) + gelatin, lane 5 is MMP-13 + gelatin, lane 6 is ADAMTS-4-3 + gelatin, lane 7 is thermolysin + gelatin, and lane 8 is trypsin + gelatin.
Trp-Pro-Gly-Ala-Pro, has no obvious MMP cleavage sites but does contain potential trypsin and thermolysin cleavage sites. Synthetic miniproteins incorporating this sequence have been prepared and stabilized by addition of alkyl chains to the N terminus (28). Structural characterization by CD and one- and two-dimensional NMR spectroscopic methods demonstrated a continuous and properly aligned triple helix (28, 29). (α1(IV)1263–1277) THP and (α1(IV)1263–1277) SSP (Fig. 6) were utilized to examine the triple-helical peptidase potential of trypsin and thermolysin.

Treatment of (α1(IV)1263–1277) THP with ADAMTS-4, MMP-1, MMP-13, trypsin, and thermolysin followed by Edman degradation sequence and MALDI-MS analyses indicated that this miniprotein was not cleaved by ADAMTS-4, MMP-1, and MMP-13, but hydrolysis by trypsin and thermolysin did occur. (α1(IV)1263–1277) THP was hydrolyzed by thermolysin at the Gly-Val bond and by trypsin at the Lys-Gly bonds (supplemental data, Figs. S4 and S6). Individual kinetic parameters for hydrolysis of the triple-helical substrate, along with an analogous single-stranded sequence, were evaluated by a fluorescamine-based assay (10). The kinetic parameters were similar for trypsin and thermolysin triple-helical substrate proteolysis (Table 2). Much faster degradation of (α1(IV)1263–1277) SSP (data not shown) did not permit evaluation of kinetic parameters but did differ considerably from the rate at which the triple-helical substrate was hydrolyzed. Based on prior biochemical and physical studies (9, 10, 29), the apparent triple-helical peptidase activity of trypsin and thermolysin was not due to hydrolysis of a small subset of single-stranded substrate.

The triple-helical peptidase activities of trypsin and thermolysin were further evaluated and compared with MMPs using a quenched fluorogenic triple-helical substrate, designated fTHP-4, and the single-stranded analog fSSP-3 (Fig. 6). The substrate was modeled after a consensus sequence derived from the collagenolytic MMP cleavage sites in human types I–III collagen and contains potential sites of hydrolysis for trypsin, thermolysin, MMP-1, and MMP-13. fTHP-4 was hydrolyzed by MMP-1 and MMP-13 at the Gly-Leu bond, thermolysin at the Gly-Leu and Gly-Val bonds, and trypsin at the Arg-Gly bond (supplemental data, Figs. S5 and S7). ADAMTS-4-3 did not cleave fTHP-4. Individual kinetic parameters and activation energies were determined for hydrolysis of fTHP-4 by trypsin, thermolysin, MMP-1, and MMP-13 (Table 2). The kcat values for MMP-1, MMP-13, trypsin, and thermolysin hydrolysis of fTHP-4 were dissimilar, but Km values and activation energies were similar. The activation energies reflect roughly equivalent difficulties in reaching the transition state (30) and thus similar triple-helical peptidase abilities. It appears that these enzymes possess a common topological specificity. The recognition of triple-helical structure is not as pronounced for trypsin, thermolysin, and MMP-13 as it is for MMP-1 based on comparison of Km values for hydrolysis of fTHP-4 with an analogous single-stranded substrate (fSSP-3; Table 2). This is consistent with prior studies demonstrating MMP-13 to be much more efficient at hydrolyzing denatured triple helices (gelatin) than MMP-1 (41).

**DISCUSSION**

The present study initially examined the substrate features that direct aggrecanase (ADAMTS-4) activity. Aggrecanase members of the ADAMTS family, either collectively or individually, cleave several proteoglycans, such as aggrecan, versican, brevican, biglycan, and decorin, as well as gelatin, cartilage oligomeric matrix protein, tissue factor pathway inhibitor-2, transferrin, fibromodulin, nidogen-1 and -2, Mac-2-binding glycoprotein, and desmocollin 3 (16–18, 25, 26, 36, 45–47). There is no distinct sequence specificity directing hydrolysis of these substrates. It was initially reported that aggrecanases had a preference for Glu- X bonds, where X was Ala, Ser, Gly, or Leu.

### Table 2

Kinetic parameters and activation energies for hydrolysis of single-stranded and triple-helical-substrates by MMP-1, MMP-13, trypsin, and thermolysin

All assays were performed at 30 °C (except where noted). NC, not cleaved; ND, not determined.

| Enzyme | Substrate | kcat | Km | kcat/Km | Ea |
|--------|-----------|------|----|---------|----|
| MMP-1  | (α1(IV)1263–1277) THP | NC   | NC | NC      | NC |
| MMP-1  | fSSP-3    | 0.25* | 171.0* | 1,462* | 3.4* |
| MMP-1  | fTHP-4    | 0.07* | 11.0* | 6,400* | 11.9* |
| MMP-13 | (α1(IV)1263–1277) THP | NC   | NC | NC      | NC |
| MMP-13 | fSSP-3    | 9.61 | 21.1 | 455,400 | 3.7 |
| MMP-13 | fTHP-4    | 4.57 | 49.4 | 92,400  | 10.5 |
| Trypsin| (α1(IV)1263–1277) THP | 0.90 | 134.5 | 6,656 | ND |
| Trypsin| fSSP-3    | 1.40 | 39.7 | 35,260 | <1.6 |
| Trypsin| fTHP-4    | 0.50 | 20.1 | 24,880 | 12.7 |
| Thermolysin | (α1(IV)1263–1277) THP | 1.13 | 111.3 | 10,190 | ND |
| Thermolysin | fSSP-3    | 28.0 | 51.4 | 545,000 | <1.6 |
| Thermolysin | fTHP-4    | 3.01 | 18.8 | 159,600 | 10.2 |

* Reported previously (12, 30, 89).
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Based on processing of aggrecan, versican, and brevican (18, 38, 48–50). However, ADAMTS-4 cleavage of carboxymethylated transferrin occurs at Met-Tyr, Gly-Tyr, Ser-Leu, Leu-Phe, and several other bonds (36). ADAMTS-4 also cleaves aggrecan at an Asn-Phe bond (51), biglycan at an Asn-Cys bond (47), and α₂-macroglobulin at a Met-Gly bond (52). To further complicate matters, small linear peptides that model aggrecan cleavage sites are often not hydrolyzed by aggrecanases (21–24), and deglycosylation of aggrecan results in different cleavage patterns than for the native protein (36). Finally the aggrecanase specificity toward native proteins can be altered by C-terminal processing of the enzyme (17, 36, 39, 53). These results suggest that specific features of the substrate, such as conformation and/or posttranslational modification, play a significant role in aggrecanase specificity. In turn, processed forms of aggrecanases may be affected to different degrees by substrate specificity determinants.

We found that substrate conformation has a significant role in ADAMTS-4 specificity. Alteration of substrate conformation from a triple helix to a poly(Pro) II helix (fTHPa versus fSSPa) resulted in (a) a 6-fold decrease in $K_m$ and (b) a change in sequence specificity. The change in sequence specificity is particularly notable and suggests that changes in aggrecanase specificity toward aggrecan upon substrate deglycosylation (36) may be due to alterations in substrate conformation. Aggrecanase activity toward aggrecan is also dependent upon the number of amino acids N-terminal to the aggrecanase cleavage site (21). It was hypothesized that the aggrecan N terminus formed a loop followed by a β-strand and then a loop at the site of aggrecanase cleavage (21). The N-terminal loop would serve as an important recognition element for an aggrecanase exosite. In the present study, the aggrecanase substrate has an N-terminal triple helix (fTHPa) to poly(Pro) II helix (fSSPa) that could direct hydrolysis toward the cleavage site in a similar fashion.

As shown here, the thrombospondin type I and disintegrin-like domains were important for recognition of triple-helical topology. Prior studies have shown or suggested that the thrombospondin type I and/or disintegrin-like domains from family M12(B) members ADAM, ADAMTS, and snake venom metalloproteinase may possess exosites that direct substrate recognition (36, 54–57). Recognition of triple-helical structure has also been reported within this family. Crotalus atrox snake venom metalloproteinase Ht-d and ADAM10 are type IV collagens, whereas C. atrox snake venom metalloproteinase catrocollastatin binds directly to type I collagen (54, 58, 59). The ADAMTS-4 deletion results for fTHPa hydrolysis contrast with those for the fSSPa in that deletion of the thrombospondin type I domain resulted in only slightly diminished activity toward fSSPa (Fig. 4). This suggests that there are specific interactions between the triple helix and the thrombospondin type I domain that are not present with the poly(Pro) II substrate. Future studies will be directed at identifying ADAMTS-4 features required for substrate recognition.

The results with ADAMTS-4 led us to examine whether the triple helix serves as a common recognition element for a variety of proteases. Triple-helical peptidase activity appears to fall into one of three categories: (a) a single-stranded peptide (SSP) is cleaved, whereas the analogous triple-helical peptide (THP) is not cleaved (i.e. MMP-3) (9, 10); (b) $k_{cat}/K_m$ for SSP > $k_{cat}/K_m$ for THP (thermolysin, trypsin, and MMP-13); or (c) $k_{cat}/K_m$ for THP ≤ $k_{cat}/K_m$ for SSP and $K_m$ for THP > $K_m$ for MMP-1 and ADAMTS-4. Overall triple-helical topology is generally recognized by diverse proteases and in some cases is a crucial feature of enzyme specificity. Further supporting this hypothesis, the type V collagen α1(V) 436–450 sequence is readily cleaved by MMP-2 and MMP-9 when in triple-helical conformation but only slowly hydrolyzed by MMP-9 when presented in a single-stranded form (9).

The recognition of triple-helical structure by a diverse group of proteases is consistent with the presence of collagen-like proteins in a great variety of organisms. Collagens have been described for vertebrates and numerous invertebrates, including mussels, worms, sponges, annelids, hydra, and corals (60). In addition, the Streptococcus Scl1 and Scl2 proteins have been shown to form collagen-like triple helices (61). However, efficient triple-helical peptidase activity does not always equate to collagenolytic activity. Of the proteases studied here, only MMP-1 and MMP-13 exhibited efficient activity toward type I collagen. Interruption of the triple-helical motif, as seen in type IV collagen, allows for efficient thermolysin activity and a small amount of ADAMTS-4 activity. Denaturation of collagen to individual poly(Pro) II helical strands (gelatin) allows for more general proteolysis. Therefore, some general proteases can cleave through a three-stranded protein that possesses poly(Pro) II structures but not through a continuous collagen triple helix. Even a subtle relaxing of the triple helix, as is seen in type III collagen compared with type I, allows for more general proteolysis (42–44). Thus, a critical question that remains is why do some triple-helical peptidases, such as ADAMTS-4, trypsin, and thermolysin, have little or no collagenolytic activity especially compared with MMP-1 (40, 44)? Two scenarios can be considered: (a) enzymes require regions beyond the active site (exosites) to bind, orient, and distort/unwind collagen for hydrolysis to occur, and (b) subtle differences in substrate dynamics and hydration influence triple helix hydrolysis. It is possible that many proteases of distinct mechanisms possess triple-helical peptidase activity and that convergent evolution of exosites led to a few proteases possessing collagenolytic activity. Even within the MMP family, the evolutionary relationship of collagenolytic MMPs indicates a convergent process (62). The roles of enzyme exosites and substrate dynamics in triple helix hydrolysis need to be further explored.

The examples presented here indicate that topological specificity may well be an overall guiding principle for proteolytic behavior. For some proteases, topological specificity is complimentary to sequence specificity (MMP-1); for others, topological specificity dictates sequence specificity (ADAMTS-4). There are numerous cases, beyond triple-helical structure, where substrate topology may serve as a determinant for protease specificity. Serum protease inhibitors, such as the human α₂-macroglobulin and its homologs, entrap a variety of proteases with a similar “bait” region via unique three-dimensional structures (63–65). Linear peptide models of α₂-macroglobulin cleavage site sequences are hydrolyzed much less efficiently by MMP-1 than linear models of collagen cleavage sites, whereas the opposite is true for the native proteins (64, 66). In
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some cases, αr-macroglobulin cleavage sites are not even predicted based on phage display peptide library experiments (67), suggesting protease recognition of this serum protease inhibitor is directed by substrate topology. In a similar fashion, the α-secretase activity against amyloid precursor protein has little sequence specificity (68, 69), and thus specificity has been attributed to the distance between the membrane-bound protease and substrate and the α-helical conformation of the substrate (68–70). The enzyme that cleaves thyrotropin hormone receptors also lacks sequence specificity, and its action has been attributed to a “molecular ruler” mechanism based on the distance between where the enzyme binds and cleaves (71). In the case of thyrotropin hormone receptors, the initial binding event could well be dictated by the three-dimensional structure of the substrate (68–70). The enzyme that cleaves thyrotropin hormone converting enzyme shedding of a great variety of cell surface exosites to allow for catabolism of macromolecular substrates.

Protease topological preferences could work in concert with exosites to allow for catabolism of macromolecular substrates. Such a precedent has been established previously for γ-secretase where hydrolysis of transmembrane domains is regulated by exosite binding to an α-helical structure (74–76). It should be possible to identify proteases that recognize specific topologies and design substrates based on these topologies.

Topological specificity ultimately represents a unique opportunity for protease inhibitor design via exploitation of exosites (74, 77, 78). This could be critical in cases where selectivity is difficult to achieve within a protease family, such as MMPs (79–82). It is possible that the topological substrates developed here may be applied for high throughput screening of aggrecanase exosite inhibitors. Such an assay would be the first for high throughput screening of aggrecanases that is non-aggre-can-based (24, 83–85).

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