Crystal structures of the two major aggrecan degrading enzymes, ADAMTS4 and ADAMTS5

LIDIA MOSYAK,¹ KATY GEORGIADIS,² TANIA SHANE,¹ KRISTINE SVENSON,¹ TRACY HEBERT,¹ THOMAS MCDONAGH,¹ STEWART MACKIE,¹ STEPHANE OLLAND,¹ LAURA LIN,¹ XIAOTIAN ZHONG,¹ RONALD KRIZ,¹ ERICA L. REIFENBERG,² LISA A. COLLINS-RACIE,³ CHRISTOPHER CORCORAN,³ BETHANY FREEMAN,³ RICHARD ZOLLNER,³ TOD MARVELL,³ MATTHEW VERA,¹ PHAIK-ENG SUM,¹ EDWARD R. LAVALLIE,³ MARK STAHL,¹ AND WILLIAM SOMERS¹

¹Department of Chemical and Screening Sciences, Wyeth Research, Cambridge, Massachusetts 02140, USA

²Women's Health and Musculoskeletal Biology, Wyeth Research, Cambridge, Massachusetts 02140, USA

³Department of Biological Technologies, Wyeth Research, Cambridge, Massachusetts 02140, USA

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Abstract

Aggrecanases are now believed to be the principal proteinases responsible for aggrecan degradation in osteoarthritis. Given their potential as a drug target, we solved crystal structures of the two most active human aggrecanase isoforms, ADAMTS4 and ADAMTS5, each in complex with bound inhibitor and one wherein the enzyme is in apo form. These structures show that the unliganded and inhibitor-bound enzymes exhibit two essentially different catalytic-site configurations: an autoinhibited, nonbinding, closed form and an open, binding form. On this basis, we propose that mature aggrecanases exist as an ensemble of at least two isomers, only one of which is proteolytically active.

Keywords: protein structure; enzymes; metalloproteins; aggrecanases

Supplemental material: see www.proteinscience.org

Osteoarthritis (OA) is a progressive disease that results in degradation of articular cartilage and chronic pain. The extracellular matrix is composed of two major components, aggrecan and collagen. Aggrecan is a large multidomain proteoglycan that provides cartilage with compressibility and elasticity by swelling and hydrating the collagen network (Vertel and Ratcliffe 2000). Loss of aggrecan is considered a critical early event in OA, occurring initially at the joint surface and progressing to the deeper zones. This is followed by degradation of collagen fibrils and mechanical failure of the tissue (Nagase and Kashiwagi 2003). Aggrecanase-1 (ADAMTS4) and

aggrecanase-2 (ADAMTS5), members of the ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) gene family, cleave aggrecan at a unique site termed the "aggrecanase site" (Abbaszade et al. 1999; Tortorella et al. 1999). ADAMTS4 and ADAMTS5 are expressed in human normal and OA cartilage (Yamanishi et al. 2002) and in OA synovium, and contribute to the structural damage that characterizes human OA (Powell et al. 2007; Song et al. 2007). However, there is no consensus in the literature as to which aggrecanase is the most important in human OA. In mice, ADAMTS5 (but not ADAMTS4) is responsible for disease progression in a surgically induced model of OA (Glasson et al. 2004, 2005). ADAMTS4/ADAMTS5 double knockout mice are physiologically normal (Majumdar et al. 2007) and also protected from developing OA. Given the normal phenotype of the double knockout mice, dual inhibition

Reprint requests to: Lidia Mosyak, Department of Chemical and Screening Sciences, Wyeth Research, 200 Cambridge Park Drive, Cambridge, MA 02140, USA; e-mail: Imosyak@wyeth.com; fax: (617) 665-8993.

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of ADAMTS4 and TS5 is a reasonable strategy to inhibit “aggrecanase” activity in human OA.

To elucidate the structural and mechanistic features of these enzymes and provide a starting point for the structure-based design of aggrecanase-specific inhibitors, we have determined the X-ray structures of truncated mature human ADAMTS4 at a resolution of 2.8 Å, both in apo form and in complex with the potent biphenyl inhibitor compound 1 (Fig. 1A), and of truncated mature human ADAMTS5 bound to the broad-spectrum metalloprotease inhibitor batimastat (Fig. 1B), at a resolution of 2.6 Å. The crystallized proteins are each composed of an N-terminal metalloprotease catalytic domain and a C-terminal disintegrin-like domain. The present structures provide the first atomic insight into inhibitor aggrecanase-active site interactions and have played an important role in the design of the next generation of dual ADAMTS4/-TS5 inhibitors.

Results and Discussion

Structures of ADAMTS4 and ADAMTS5 are highly homologous

The structures of the two closely related proteins ADAMTS4 and ADAMTS5 (with 48% sequence identity outside the pre- and prodomains) present a common fold comprising an N-terminal metalloprotease domain and a C-terminal disintegrin-like domain joined through an extended linker and stabilized by four disulfide bonds

each (Fig. 1C). Overall, the structures are highly homologous, with a root mean square (rms) deviation of 1.5 Å for 275 equivalent C- α pairs, allowing a common description for each. The metalloprotease catalytic domain (residues 214–428 in ADAMTS4 and residues 264–476 in ADAMTS5) contains the active site, the catalytic zinc, and two calcium-binding sites and has an α/β structure characteristic of the metzincin family (reprolysin type). The main feature of this domain, which immediately distinguishes it from other metalloproteases, is in the dynamic nature of its active site, which exhibits two essentially different conformations, as described in detail below, depending on the presence of ligand. After completing the N-terminal metalloprotease domain, the chain continues with a 9-residue extended linker at the back and then returns to the front side to form the smaller C-terminal disintegrin-like domain (residues 438–508 in ADAMTS4 and residues 486–555 in ADAMTS5). This arrangement places the latter on the prime side of the active site, where it shields the deep S1' pocket and, to a lesser extent, the S3' pocket from solvent (we use the common protease nomenclature in Fig. 2).

The disintegrin-like domain of ADAMTS4 and ADAMTS5 shares 25%–45% sequence similarity to snake venom disintegrins that bind to platelet integrins. It does not contain, however, the characteristic, atrolysin-like cysteine arrangement and the putative integrin-binding motif ECD seen in the disintegrin-like domain of some ADAMs, such as fertillins. In the structures reported here, it forms a unique fold of two α -helices, two β -sheets, and several loops throughout. A search through the PDB for the canonical “true” disintegrins revealed that, of the two, the C-terminal β -sheet shows structural similarity to the small C-terminal portions of ADAM10 (Janes et al. 2005) and snake venom trimestatin (Fuji et al. 2003). Superposition of the latter brings the long protruding RGD-loop of trimestatin on top of the very short loop of aggrecanase (⁴⁹⁷PAQ⁴⁹⁹ in ADAMTS4 and ⁵⁴⁴KGR⁵⁴⁶ in ADAMTS5). This loop is conserved in length but, unlike the variant of either the RGD or ECD integrin-binding motif, is divergent in sequence (see Supplemental Fig. 1). Despite this similarity, there is no published evidence to support association with integrins, and the function of the disintegrin-like domain remains unclear. In combination with the thrombospondin type I domain, the disintegrin-like domain was proposed to play an important role in recognition of substrate folding topology (Lauer-Fields et al. 2007). Given its close proximity to the active site in the three-dimensional structure, it is reasonable to suggest that the disintegrin-like domain could represent an auxiliary substrate-binding surface and thus be directly involved in substrate recognition. It may function, for example, as an exosite that guides substrate specificity for aggrecanases.

Figure 1. (A) Chemical structure of compound 1. (B) Chemical structure of batimastat. (C) Stereo cartoon diagram of ADAMTS4. The metalloprotease and disintegrin-like domains are colored according to secondary structure: cyan for α -helices and magenta for β -strands. Compound 1 is shown with yellow carbons, in stick mode. Zinc and calcium ions are labeled and are shown as spheres. Two α -helices that are part of the active sites are labeled.

Figure 2. Molecular surfaces of the active sites with inhibitor bound and detailed view of the interactions involved. (A) Stereo surface representation of the ADAMTS4 active site colored in cyan with the bound inhibitor denoted in stick mode, with yellow carbons. (B) Stereo surface representation of the ADAMTS5 active site colored in blue with the bound batimastat shown in stick mode, with white carbons. The disintegrin-like domain is colored yellow for each molecule. Zinc and calcium ions are labeled and are shown as spheres. The active site pockets are labeled using a common protease nomenclature, and the S1' and S2' loops are shown as ribbons.

Active sites with inhibitor bound

The active sites of ADAMTS4 and ADAMTS5 share many conserved residues and assume virtually identical configurations. In the inhibitor bound state, the catalytic site is a deep, solvent-exposed cleft running parallel to the helix α D and between the walls of two pockets, S1' and S2' (Fig. 2A,B). At the center of this cleft is the catalytic zinc, key to protease activity, coordinated by three conserved histidines. The main differences compared with the MMPs (matrix metalloproteases) that affect the overall shape and chemical environment of the active site involve the outward facing S2' and the inward facing S1' pocket. The S2' pocket is defined by a short disulfide-containing loop with the unifying motif CGxxxCDTL—something not observed in any of the MMPs; we call it the S2'-loop (residues 322–330 in ADAMTS4 and 371–379 in ADAMTS5, see Supplemental Fig. 1). Unlike the large loop region present in all the MMPs, the S2'-loop adopts a compact β -turn structure some distance away from the catalytic zinc. This arrange-

ment is stabilized by the disulfide bond and by interaction with calcium ion. Another difference is in the shape of the deep end of the S1' pocket, which is afforded by a compact S1'-loop (residues 389–403 in ADAMTS4 and residues 437–451 in ADAMTS5). In the present structures, this loop defines about half of the inner face of the S1' pocket, with residues from the α B and α D helices forming the rest. In the MMPs, on the other hand, the corresponding loop region encircles almost the entirety of the S1' pocket and thus essentially defines the shape of this cavity. These are the principal structure differences that can be explored to meet selectivity requirements for aggrecanases versus MMPs.

Although the active sites of ADAMTS4 and ADAMTS5 have the conserved structural features just described, differences in four amino acids (Ala²⁵², Val³⁹⁰, Met³⁹⁵, and Val³⁹⁸ are replaced by Leu³⁰¹, Leu⁴³⁸, Leu⁴⁴³, and Ile⁴⁴⁶ in ADAMTS5) result in a roomier S1' pocket in ADAMTS4. The latter is in fact a channel that spans ~ 17 Å and is completely filled by the inhibitor (see below and Fig. 2A). Not surprisingly then, differences in the relative size of

these pockets seem to be a major determinant of ADAMTS inhibitor selectivity. We found that compounds with bulky P1' moieties display a significant decrease in inhibition of the ADAMTS5 protease activity compared with ADAMTS4. For example, and as observed from the FRET (fluorescence resonance energy transfer) measurements (see Materials and Methods), compound 1 has an IC_{50} of 0.1 μ M for ADAMTS4 and only 27 μ M for ADAMTS5, accounting for the 270-fold difference in inhibition.

Inhibitor binding

Compound 1 and batimastat both possess zinc-chelating groups, and both insert between the walls of S1' and S2' but bind in a rather different way (Fig. 2A,B). This is due to the fact that batimastat is essentially a peptide-based inhibitor and, as such, interacts with the protein in an extended, β -sheet-like conformation. With the exception of its complex with atrolysin C, batimastat binds in a similar way to the various metalloproteases. This common binding mode is also observed in the batimastat-ADAMTS5 complex: It chelates the Zn ion through the hydroxamic group with its three side chains packing against S1, S1', and S2' (Fig. 2B). In contrast, compound 1, which chelates the Zn ion through the carboxylic group, binds mainly to the S1' site by protruding its long P1' group deeply into the S1' pocket and making extensive hydrophobic and some polar contacts with ADAMTS4 residues (Fig. 2A).

Active site of unliganded aggrecanase

The major difference between the apo and inhibitor-bound ADAMTS4 structures is found in the position and conformation of the S2'-loop. In the unliganded state, this loop moves from its "open" position toward the catalytic zinc by ~ 8 Å (Fig. 3A). It does not form a β -turn structure but instead folds into the active site in a "closed," Ω -shaped-like conformation, resulting in the removal of bound calcium and a markedly different positioning of the disulfide bridge. The conserved residues in the $^{322}\text{CGxxxxCDTL}^{330}$ motif appear to be key to the observed changes (the configurations start to diverge at Cys³²² and do not realign until Leu³³⁰). Perhaps the most unexpected feature of this arrangement is that residues Asp³²⁸ and Thr³²⁹ place their side chains around the catalytic zinc so that Thr³²⁹ fills the space at the mouth of S1' while the side-chain carboxylate of Asp³²⁸ chelates the zinc, contributing the strong chelating bond interaction. Unlike the open conformation seen in the inhibitor-bound enzyme (Fig. 3B), access to the catalytic site in the free enzyme closed conformation is blocked. Hence, the S2'-loop appears to be an "autoinhibitory" element in two aspects. First, it can impair enzyme sensitivity to substrates by sterically impeding their

Figure 3. Molecular surfaces of the two different active-site configurations. (A) Molecular shape of the active site as seen in the apo ADAMTS4 structure. The side chains of Thr³²⁸ and Asp³²⁹ are visible through the transparent surface. (B) Molecular shape of the active site as seen in the liganded ADAMTS4 structure with the inhibitor omitted from the view. The surfaces of the active site and the disintegrin-like domain are colored cyan and yellow, respectively. The S2' region $^{322}\text{CGxxxxCDTL}^{330}$ is depicted in red ribbons with the differences in the conformation (discussed in the text) highlighted in red. The insert shows the ribbon superposition of the two loop configurations: The closed conformation is depicted in red, the open conformation is shown in blue; the disulfide bond Cys³²²-Cys³²⁷ and the side chain of Asp³²⁹ are depicted in stick mode, highlighting differences in positioning.

access into the S1' and S2' pockets. Second, Asp³²⁸ prevents the zinc from becoming enzymatically active until Asp³²⁸ is removed. We surmise that the energetic cost of loosening this chelating bond in the complex structures is in part compensated for by a new interaction with calcium. It seems, therefore, that the aggrecanase-active site adapts itself to the ligand by forming new intramolecular contacts to balance the energetic cost of those broken during the conformation transition. This observation is in stark contrast to a rather preformed and exposed active site derived from crystal structures of majority of MMPs. Importantly, there are no apparent crystal contacts that might account for the "insertion" of the S2'-loop into the active site, and the eight independent copies of the ADAMTS4 molecules in the crystallographic asymmetric unit have virtually identical conformations even in different packing environments.

Hypothetical substrate binding model

Conformational changes associated with ligand binding can be described in general by two models—the induced-fit and

the conformer selection model. The latter model postulates that proteins likely present to the incoming ligands an ensemble of binding site conformations rather than a unique fold (Kumar et al. 2000; Ma et al. 2002). Because apo ADAMTS4 is observed in an autoinhibited closed form and because no specific activator or inducer molecule has been reported for mature aggrecanases, we suggest that, in the absence of ligand, the closed state of this protease may not be the only conformation that exists in solution. Instead, and in accord with the conformer selection theory, the open form may coexist in equilibrium with the nonbinding closed form, albeit as the minor population. In the process of binding, a given ligand will bind selectively to an active, most complementary form, shifting the equilibrium in favor of the binding conformation and finally changing the average structure of the enzyme. The reported static crystal structures each captures a weighted average of the ensemble but illustrates that the enzyme can exist as a dynamic equilibrium between at least two observed states.

Owing to the strong sequence similarity between ADAMTS4 and ADAMTS5 in the active site and in the S2' region CGxxxCDTL, in particular, it seems likely that these findings may be of general significance for aggrecanases and be a unifying aspect for this family. It is unclear why this concept of conformational diversity does not seem to apply to other metalloproteases for which crystal structures are known. We suggest the possibility that conformational mobility of the active site is an additional feature of mature aggrecanases, which may be required for high affinity association with the substrate and may be coupled to the dynamic behavior of proteoglycan. We also cannot rule out the possibility that this state of dynamic equilibrium may be subject to regulation either by substrate or by another protein or by another domain of the multi-domain aggrecanase.

Materials and Methods

Protein expression and purification

Both ADAMTS4 and ADAMTS5 proteins were expressed in Chinese hamster ovary (CHO) cells: Constructs encoding residues 213–520 of the active site mutant human ADAMTS4 (Glu369Gln) were produced as a C-terminal Flag-tagged (sequence DYKDDDDK) protein, and constructs encoding residues 262–628 of human ADAMTS5 were expressed as a C-terminal Strep-tagged (sequence GSAWSHPQFEK) protein. To aid in structure determination, the same ADAMTS4 protein but labeled with seleno-methionine was produced in CHO cells as described by the authors (U.S. patent 20070105207). Conditioned CHO media expressing ADAMTS4 were diluted into 25 mM HEPES (pH 6.8), 5 mM CaCl₂, and 10 μ M ZnCl₂; bound to a Poros HQ column; and eluted with linear gradient 50 mM–1 M NaCl. A polypropyl aspartamide hydrophobic interaction column, followed by gel filtration, anti-Flag M2 affinity, and ion-exchange (Mono Q) chromatography yielded protein sam-

ples suitable for crystallography studies. For crystallization, the protein sample was concentrated to 8 mg/mL in 25 mM HEPES (pH 6.8), 300mM NaCl, 10 μ M ZnCl₂, and 5 mM CaCl₂. The seleno-methionine-labeled material was prepared using the same protocol. Conditioned media expressing ADAMTS5 were diluted into 20 mM Tris-Cl (pH 8.0), 5 mM CaCl₂, 10 μ M ZnCl₂, 50 and mM NaCl; bound to Poros HS anion exchange column; and eluted with NaCl gradient up to 1 M. The protein was purified by a combination of Strep-Tactin (IBA GmbH) affinity chromatography and gel filtration (Superdex-200). For crystallization, the resulting ADAMTS5 containing fractions were pooled and concentrated to 5 mg/mL in 20 mM Tris (pH 8.5), 5 mM CaCl₂, 10 μ M ZnCl₂, and 50 mM NaCl.

Crystallization

Crystals of both forms of ADAMTS4, unliganded and bound to compound 1, were obtained by vapor diffusion at 18°C using 10% PEG 4K, 0.1 M MES (pH 6.0) as a precipitating solution. Optimized crystals were grown by seeding with addition of 8–15 mM L-cysteine, and crystals of the seleno-methionine-substituted protein were grown using a similar technique. Crystals of liganded ADAMTS4 belong to the monoclinic space group *P*2₁, with four molecules per crystallographic asymmetric unit. Crystals of apo ADAMTS4 have the same *P*2₁ symmetry but different unit cell parameters (see Supplemental Table 1) and eight molecules in the asymmetric unit. Crystals of the ADAMTS5–batimastat complex were obtained by vapor diffusion at 18°C using 10% PEG 8K, 0.2 M ammonium sulfate, and 0.1M MES (pH 6.5) as a precipitate. They belong to the space group *P*3₁2₁, with one complex per asymmetric unit. For data collection, all crystals were transferred to the solution containing crystallization reagent plus 25% glycerol, and then flash-frozen in the liquid nitrogen.

Structure determination

The structure of liganded ADAMTS4 was determined with phases obtained by multiwavelength anomalous diffraction (MAD) from crystals of seleno-methionine-substituted protein. MAD data and native data were collected on beamline 5.0.2 at the Advanced Light Source (ALS; Berkeley, CA). All data sets were integrated and scaled with MOSFLM and SCALA (Collaborative Computational Project, Number 4 1994). Selenium sites were located with SHELXD (2001 Bruker-AXS, XM, version 6.12). Refinement of anomalous scatterer parameters, phase calculation, and density modification were performed with SHARP (De La Fortelle and Brigogne 1997). Experimental maps were used to build an initial model in QUANTA, with subsequent rounds of rebuilding and refinement against native data using REFMAC (Winn et al. 2001). The crystal structure of unliganded form was solved by molecular replacement method with AMORE (Collaborative Computational Project, Number 4 1994), using the refined structure of the inhibitor-bound form. Data from crystals of unliganded enzyme were collected at ALS, beamline 5.0.2. Rebuilding and refinement of unliganded ADAMTS4 model was performed in REFMAC (Winn et al. 2001). Diffraction data from a crystal containing the ADAMTS5–batimastat complex were collected at Advanced Photon Source, Argonne National Laboratory, beamline 22-ID of SER-CAT, and processed and reduced with HKL2000 (Otwinowski and Minor 1997). The structure was determined by molecular replacement method with PHASER (McCoy et al. 2005), using the ADAMTS4

structure as a search model. Model rebuilding and refinement were performed in COOT (Emsley and Cowtan 2004) and REFMAC (Winn et al. 2001). Although the amino acid sequence of the crystallized protein encompasses three domains—catalytic, disintegrin-like domain, and thrombospondin-like domain—the latter domain, residues 557–628, has no supporting electron density, presumably because of disorder. Experimental details of data collection and model refinement for all three structures are given in Supplemental Tables 1 and 2.

ADAMTS4 and ADAMTS5 FRET assay

A continuous assay was used in which the substrate was a synthetic peptide containing the fluorescent group o-amino-benzyl (Abz) that was quenched by energy transfer to a 2,4-dinitrophenyl group (Dnp). Human recombinant ADAMTS4 (Flannery et al. 2002) or ADAMTS5, prepared as described above, were used at final concentrations of 5 µg/mL. The peptide sequence was Abz-TEGEARGSVI-Dap(Dnp)-KK (Anaspec) and derived from the aggrecanase cleavage site of aggrecan. The final concentration of substrate in the assay was 25 µM. The buffer used in this assay was 50 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM CaCl₂, 0.1% CHAPS, and 5% glycerol. Compounds (in duplicate) were serially diluted from 2 mM to 0.01 µM in 100% DMSO. The total reaction volume was 100 µL. The buffer and enzyme were added first followed by addition of 10× inhibitor. Enzyme and buffer alone samples were included in order to obtain the maximal rate of substrate cleavage. The reaction was allowed to stand for 15 min at 30°C. The substrate was added and mixed and the reaction monitored for 40 min at 30°C in kinetic mode using the following wavelengths: λ_{ex}, 340 nm; λ_{em}, 420 (GeminiXS Molecular Devices).

Data deposition

The atomic coordinates have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, New Jersey (2RJF, 2RJQ, and 3B27).

Electronic supplementary material

Supplementary material contains the alignment of metalloprotease and disintegrin-like domain sequences of human ADAMTS4 and ADAMTS5 and details of the crystallographic data collection, phasing, and refinement for the three reported structures.

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