High Resolution Crystal Structure of the Catalytic Domain of ADAMTS-5 (Aggrecanase-2)*

Received for publication, July 18, 2007, and in revised form, November 5, 2007. Published, JBC Papers in Press, November 8, 2007, DOI 10.1074/jbc.M705879200

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Aggrecanase-2 (a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5)), a member of the ADAMTS protein family, is critically involved in arthritic diseases because of its direct role in cleaving the cartilage component aggrecan. The catalytic domain of aggrecanase-2 has been refolded, purified, and crystallized, and its three-dimensional structure determined to 1.4 Å resolution in the presence of an inhibitor. A high resolution structure of an ADAMTS/aggrecanase protein provides an opportunity for the development of therapeutics to treat osteoarthritis.

ADAMTS-4* (1) and ADAMTS-5 (2), also referred to as aggrecanase-1 and -2, respectively, play a key role in the pathological breakdown of the cartilage extracellular matrix observed in osteoarthritis. These proteinases belong to the recently discovered ADAMTS family, which includes 19 members and belongs to the metzincin clan of MEPS (mainly clan MA(M) according to MEROPS), which encompasses protease families containing an extended zinc binding consensus sequence, HEXXHXXXGXX (3). Both ADAMTS-4 and -5 are glutamyl endopeptidases and cleave aggrecan, the major proteoglycan in articular cartilage responsible for resisting compressive forces in the joint, at five specific sites: Glu373→Ala374, Glu1545→Gly1546, Glu1714→Gly1715, Glu1819→Ala1820, and Glu1919→Leu1920 bonds (human sequence) (4, 5), resulting in cartilage destruction. Protection from cartilage destruction has been observed in short term inflammatory arthritis (6) and in surgically induced osteoarthritis models in ADAMTS-5 knock-out, but not ADAMTS-4 knock-out, mice (7, 8), suggesting a critical role for ADAMTS-5 in murine arthritis. Designing potent and selective inhibitors of ADAMTS-5 as a potential osteoarthritis therapeutic is an ongoing effort but has been difficult because ADAMTS members share little sequence homology with the well studied and related matrix metalloproteinase (MMP) (9) and ADAM (10) metzincin family members. The limited expression levels and low solubility of catalytic domain constructs of ADAMTS proteins has thus far precluded the determination of a crystal structure of any member of this family. To overcome these obstacles, we used site-directed mutagenesis, high pressure refolding, and thermal unfolding studies to prepare pure and active catalytic domain of ADAMTS-5 (cataADAMTS-5) from Escherichia coli and determined its crystal structure at 1.4 Å resolution in the presence of an inhibitor. Although the fold of the protein resembles other metalloproteases, the shape of its substrate binding site is unique, and a novel structural calcium binding site was discovered for this family. In our structure, a pan-protease inhibitor binds at the catalytic site and protrudes into the unique S1′-subsite of the enzyme. Elucidation of a high resolution three-dimensional structure of the catalytic domain of an ADAMTS metalloproteinase enables detailed study of substrate specificity and the potential for rational design of selective inhibitors of this important class of enzymes.

EXPERIMENTAL PROCEDURES

Cloning and Expression of the Catalytic Domain of ADAMTS-5—A full-length cDNA clone identical to the published sequence of human ADAMTS-5 (SwissProt/TrEMBL accession number NM 007038) was isolated from a human placenta cDNA library. Using the ADAMTS-5 cDNA clone as a template, the DNA sequence for the catalytic domain of ADAMTS-5 (amino acids Se(i)362 to Ile(489)) was amplified by PCR, causing the addition of Met-Ala residues at the amino terminus of the protein. Mutagenesis was performed using the QuickChange kit (Stratagene) to incorporate the L282K mutation into the L282K mutation. The resulting coding region was inserted into an expression vector (Preclac, specR) downstream of a modified recA promoter/lac operator. A 10-liter fermentation was performed using the resulting plasmid and E. coli host strain MON208 (W3310, rpoH358, lacIQ, ompT::kan). Bacterial cells were harvested by centrifugation 4 h after induction. The cell paste was resuspended in 5.0 ml of 10 mM Tris/15 mM EDTA per gram of cell paste. The cell suspension was passed through a chilled microfluidizer twice at 10,000–15,000 psi. Inclusion bodies were isolated from the cell lysate by centrifugation.

Refolding and Purification of the Catalytic Domain of ADAMTS-5—Isolated inclusion bodies were resuspended to a final concentration of ~5 mg/ml in buffer A containing 50 mM glycine, pH 10.0, 10 mM CaCl2, 500 μM ZnCl2, 2% protease...
inhibitor mixture (P-8849; Sigma), 50 mM arginine, 100 mM reduced glutathione. The suspension was delivered to the cavity of a High Hydrostatic Pressure Apparatus (11, 12) (Barofold Inc., Boulder, CO), pressurized at 32,630 psi for 14–16 h. Following depressurization, the inclusion body extract was directly exchanged into a buffer containing 50 mM HEPEs, pH 8.0, 2 mM oxidized glutathione, 50 mM arginine, 10 mM CaCl₂, 200 μM ZnCl₂, and 1% protease inhibitors. The protein was allowed to refold at room temperature until catalytic activity peaked. The refolded material was then clarified by filtration, concentrated, and passed over a 26/60 Sephacryl S-200 HR size exclusion chromatography column (GE Healthcare). CatalysisADAMTS-5 fractions were pooled based on SDS-PAGE purity greater than 95% and size exclusion chromatography migration as a monomeric protein.

**ADAMTS-5 Crystal Structure**

**ADAMTS-5 Activity Assay**—Direct kinetic analysis of ADAMTS-5 catalytic domain activity was determined using the 6-Fam/QSY-9 (6-carboxyfluorescein, QSY-9-maleimide; Molecular Probes, Eugene, OR) fluorescently quenched peptide K(6-Fam)-DVQEFRGVTTAVIRC(QSY-9)-KGK (13). Briefly, a 1 μM solution of peptide in buffer (100 mM Tris-Cl, 100 mM NaCl, 10 μM CaCl₂, 0.05% Brij, pH 7.5) was incubated with ~450 nM of cатаADAMTS-5 at 37°C in a total volume of 100 μL. Fluorescence at 519 nm was monitored over a 60-min period with an excitation of 495 nm. Fitting of dose-response data for titration and inhibitor potency determinations was done using commercial non-linear curve-fitting software (GraFit version 5; Erithacus Software, Ltd.). The supplied 4-parameter equation for determining IC₅₀ was modified to allow the inclusion of no enzyme controls, and the Morrison equation for tight binding inhibitors (14) was added as a user-defined equation.

**Thermal Stability Assay**—The Tₘ of cатаADAMTS-5 in complex with various inhibitors and substrate peptides was assessed using an iCycler (Bio-Rad) and monitoring Sypro Orange fluorescence at 540 nm (15). For these studies, 105 μM concentration of known ligands was added to the protein samples, and the Tₘ values and curve shapes were compared across samples.

**Crystallization and Structure Determination of the Catalytic Domain of ADAMTS-5**—Crystals of the complex were grown by vapor diffusion using 10 mg/ml protein and a well solution of 25% (v/v) polyethylene glycol 3350, 200 mM ammonium acetate, 100 mM Tris, pH 8.5. Needle crystals appeared in 1 day and matured to a size of 0.3 × 0.03 × 0.03 mm by 7 days. Diffraction data were collected on an ADSC Quantum-210 detector at APS (Argonne, IL) beamline 17-1D. Data were integrated and scaled using the HKL2000 suite (16). The crystal belonged to space group P2₁, with unit cell dimensions a = 53.06, b = 44.49, c = 76.38 Å, and β = 90.07° and two protein molecules per asymmetric unit. Although the cell dimensions resemble an orthorhombic lattice, this and all other crystals tested are in fact monoclinic. The structure of the complex was determined by molecular replacement methods using AMORE (17) and a combination of five superimposed structures, ADAM-17 (1bkc) (18), ADAM-33 (1r55) (10), atrolysin-C (1atl) (19), astatatin-A (1bsw) (20), and adamalysin II (4aig) (21). The ADAM-33 protein was deconvoluted from the solution and used as the starting point for model building in O (22) and subsequent refinement using X-plo (23) Refmac (17). The final structure has been refined at 1.40 Å resolution to an R_free = 21.4% with excellent stereochemistry (root mean square deviation of bond lengths from ideality of 0.008 Å (Table 1). Non-crystallographic symmetry restraints were not utilized. Superimposing the two chains yields a significant root mean square deviation of 0.55 Å on 212 backbone Cα atoms. The final structure of ADAMTS-5 is comprised of a signal sequence (SS), pro-domain (Pro), catalytic domain (Cata), disintegrin-like domain (Dis), spacer region, and a thrombospondin motif (TS) and submotif (TS'). The filled circles show the relative positions of glycosylation sites. The mutation site, L282K, is also indicated.

**RESULTS AND DISCUSSION**

**Production of Properly Folded, Active cатаADAMTS-5**—Attempts to express soluble ADAMTS-5 protein in multiple bacterial and eukaryotic expression systems have resulted in low expression levels and the inability to purify the enzyme to homogeneity. In addition, traditional methods for refolding proteins from inclusion bodies that succeeded for metalloproteases, including members of the MMP and ADAM families, have not succeeded to date for aggrecanases. We designed several point mutants and variations of the length of the catalytic domain constructs to improve both expression yield and solubility. These studies led us to focus on optimizing the expression of cатаADAMTS-5 in E. coli inclusion bodies using a construct containing residues 262–480 with a single point mutation (L282K), converting a surface hydrophobic residue into a charged amino acid (Fig. 1). This protein accumulated in inclusion bodies and was successfully made soluble using the new technology of high hydrostatic pressure refolding (11, 12); it was subsequently purified to homogeneity by size exclusion chromatography.
chromatography (Fig. 2a). The pure catalaADAMTS-5 protein efficiently cleaved the peptide substrate K-(6FAM)-DVQE ↓ FRGVTAVIRC(Qsy9)-KGK; (↓ indicates the cleavage site, Fig. 2b) (13). The addition of tissue inhibitor of matrix metalloproteinase 3 (Fig. 3a, TIMP-3), the natural inhibitor of ADAMTS-5 activity (24), blocked peptide cleavage in a concentration-dependent manner. Tight binding inhibitor analysis of these data yielded a 

$K_i$ of 38 nM and an active enzyme concentration of 332 nM. This active concentration was 73% of the protein concentration established by absorbance at 280 nm suggesting that most of the catalaADAMTS-5 protein is catalytically competent. A hydroxamic acid-based small molecule inhibitor of ADAMTS-5, Compound 1 (Fig. 3b), had an IC$_{50}$ of 0.29 $\mu$M.

Crystallization of catalaADAMTS-5—Apo-catalaADAMTS-5 could not be crystallized by surveying an extensive matrix of conditions. We and others have observed that a number of proteins are more prone to crystallize with inhibitors that produce a significant increase in protein unfolding temperature ($T_m$). Analysis of the shift in protein unfolding temperature ($T_m$) as a function of different buffer components and known ligands identified a combination of conditions that increased the thermal stability of the protein. The most significant change in its $T_m$, an increase of 27°C ($T_m = 43^\circ C$ → $T_m = 70^\circ C$), was observed when catalaADAMTS-5 was in complex with Compound 1 (Fig. 4). Addition of this inhibitor enabled crystals of the complex to be grown. The crystal belongs to the space group P2$_1$ with unit cell dimensions a = 53.06, b = 44.49, c = 76.38 Å, and $\beta = 90.07^\circ$ and two protein molecules in each asymmetric unit. Molecular replacement methods, using a searching model of a combination of five metalloproteinase structures including ADAM-17, ADAM-33, atrolysin-C, acetolysin-A, and adalamysin II, led to an unambiguous solution. The three-dimensional structure was refined at 1.4 Å resolution with an $R_{free}$ value of 21.4% (Table 1).

**Structural Analysis**—Amino acid sequences and three-dimensional structures of the catalytic domains of various members of three metalloproteinase families, including MMP, venom matrix metalloproteinase, and ADAM, are known (10, 18–20, 25). An amino acid sequence alignment of members of these families reveals that outside its own family, ADAMTS-5 is most homologous to ADAM-33 with a sequence identity of $\sim$30%, followed by several venom matrix metalloproteinases, including acetolysin-A 25%, atrolysin-D 23%, adalamysin-II 22%, and atrolysin-C 19%, and MMPs <10% (MMP-3 8%, MMP-13 6%, and MMP-8 5%). Although TACE (tumor necrosis factor-α-converting enzyme, ADAM-17) only has 17% sequence identity with catalaADAMTS-5, like ADAM-33 it shows notable similarities in the defined secondary structural regions within catalaADAMTS-5.

The overall fold of the catalaADAMTS-5 protein resembles the α/β structure of other metalloproteinases with their characteristic central five-stranded β-sheet (Fig. 5) formed by four strands (II, I, III, V) in a parallel configuration and a short fifth one (IV) in an antiparallel configuration. The central β-sheet is flanked with two helices, A and C, on its concave side and one long helix, B, on its convex side. Although helices A and C are shared with other metalloproteinases, such as venom matrix metalloproteinase, ADAM,
ADAMTS-5 Crystal Structure

FIGURE 4. Thermal unfolding assay identifies stabilization for cataADAMTS-5 in the presence of an inhibitor. Addition of Compound 1 to the protein raises the $T_m$ from 43 °C in its absence (○) to 70 °C in its presence (●). At elevated temperatures the dye bound to the protein is diminished as a result of protein aggregation/precipitation occurring as the temperature is increased, and therefore the fluorescence is decreased.

and MMP structures, known MMP structures totally lack the long helix B. In addition, cataADAMTS-5 distinguishes itself from all other metalloprotease structures reported to date in having the helix B tether itself to the strand V by a novel disulfide bond between residues 342 and 394 in the sequence (Fig. 5). In total, the catalytic domain of ADAMTS-5 contains four disulfide linkages, compared with three in ADAM33 as well as ADAM17 and zero in all known MMP structures.

The central β-sheet and its flanked α-helices constitute the N-terminal part of the protein chain. The remaining 65 C-terminal residues harbor the last Zn$^{2+}$-chelating His$^{320}$ residue, enclose the S1'-pocket, and allow residue Asp$^{474}$ to complete a unique calcium cluster site. The surface flexible loop comprising residues Gly$^{322}$-Asp$^{323}$-Lys$^{324}$-Asp$^{325}$-Lys$^{326}$ (highlighted in red and shown in Fig. 5) is the only flexible area in this structure as well as other cataADAMTS-5 structures that we determined and shows little or no electron density in the Fourier maps. It may represent an antibody recognition site.

Recently, the structure of the catalytic domain of ADAMTS-1 was reported at the 53rd Annual Meeting of the Orthopaedic Research Society (26). The coordinates of the ADAMTS-1 structure have not been made available to the public; thus, an exact comparison with cataADAMTS-5 is not possible. However, judging from the abstract by Gerhardt et al. (26) and comparing the printed structure of ADAMTS-1 with our structure of ADAMTS-5, it can be concluded that the secondary structures of both enzymes are similar. This is expected, because both the catalytic domain and the residues around the active site of these two enzymes share 60% sequence identity.

The mutation site of the present cataADAMTS-5 structure, L282K, highlighted in red in Fig. 5, is located near the end of the first short helix A1, which is at the surface and sits in a water channel. Its polar side chain of the first molecule, molecule-A, of the asymmetric unit forms a charged interaction directly with Asp$^{469}$ of a neighboring molecule, whereas the polar side chain of the molecule-B of the asymmetric unit forms a hydrogen bond with a bridged water, which in turn interacts with Asp$^{469}$ of a neighboring molecule. This site is quite far from the active site and likely has minimal impact on catalytic activity but may contribute to the packing of the lattice in this crystal form. In addition, mutation of this site is crucial for crystallization of the wild-type cataADAMTS-5, refolded and purified under the same conditions used for the mutated one, has failed to yield any crystals under a wide range of crystallization conditions, including the one producing crystals for the L282K mutant.

A superposition of representative structures from three different families of metalloproteases, i.e. cataADAMTS-5 for ADAMTS, ADAM-33 (protein code 1r55) for ADAM, and MMP-3 (1d7x) for MMP, is shown in Fig. 6. ADAMTS-5 and ADAM-33 show significant overlap in the secondary structure (sheets and helices) but to a much lesser extent in the loop regions. In contrast, the MMP-3 structure loosely overlays in the 5-strand sheet and central helices A and C, whereas the rest...
of the structure is quite deviated when compared with ADAMTS-5 or ADAM-33. The flexible S1’-loops in all three structures are quite different, and this family-dependent specificity is further illustrated below.

The catalytic site in this cataADAMTS-5 structure has the shape of a funnel, as shown in Fig. 7, that opens up at the zinc site at its widest point and then forms an L-shaped, hydrophobic channel through the S1’-site and out to solvent at its distal end. The side chain of Leu$^{433}$ forms the roof of the channel, but this residue is solvent-exposed and may be flexible. Three key interactions between the inhibitor and the protein define their binding mode. The hydroxamate head group chelates to the Zn$^{2+}$ ion and hydrogen bonds to Glu$^{411}$, the sulfone group hydrogen bonds to the backbone of Leu$^{379}$, and the phenyl-O-phenyl group has a strong hydrophobic interaction with the highly lipophilic and specific S1’-pocket. The pocket can be defined by three elements: the base of the strand IV defined by amino acids 379–382 with Leu$^{379}$ as a marker, a portion of the helix C including amino acids Leu$^{402}$ to Glu$^{411}$, and the loop consisting of amino acids Arg$^{437}$ to Ile$^{446}$ (Fig. 8, cyan). This loop in the pocket will undergo a certain degree of conformational change upon binding to inhibitors of different sizes and shapes. A structural alignment of this pocket is seen in Fig. 8 with four structures, cataADAMTS5 (this study), ADAM-33, TACE (ADAM-17, 2ddf), and MMP-3. The MMP-3 structure was chosen here for the comparison because MMP-3 has a rather big and deep S1’-pocket like cataADAMTS-5. The strand IV, marked by Leu$^{379}$, is totally conserved in all four structures. The beginning of helix C, marked by Leu$^{402}$ of these structures, is very different, except between ADAM-33 and TACE, but they start to converge after two residues and extend the convergence toward the Zn$^{2+}$ binding site. The first half of the loop, containing residues 437–446, is conserved among the various structures, described in Fig. 8, but diverges at the middle point of the loop and then goes off in different directions. For clarity, some variations in the loop conformation for MMP-3 and TACE were not included in the figure; however, the above assessments hold for those variations. This description relates only to the protein backbone, and if the side chains as well as residue types are also considered in the S1’-pocket, even more variations are observed.

Besides the catalytic zinc binding site, there are two calcium binding sites critical for maintaining the overall structural integrity of the protein. One site is occupied by a single calcium, and the other contains two calcium ions (Fig. 5, Ca-cluster site). Both sites are adjacent to disulfide linkages, the former by Cys$^{371}$-Cys$^{376}$ and the latter by Cys$^{388}$-Cys$^{471}$. The Ca-cluster conjoins the N-terminal, C-terminal ends of the protein and the loop connecting the helix B and the strand III, which is on the opposite end of the zinc binding site. One calcium ion at this site has seven oxygen ligands, whereas the other has eight oxygen ligands, provided either by protein atoms or solvent molecules. The protein ligands involve residues Glu$^{270}$, Asp$^{353}$, Asp$^{360}$, and Asp$^{474}$. Because the corresponding residues in ADAM-33, TACE, and several venom matrix metalloproteinases are Glu, Leu, Asp, and Asn, they can only accommodate one calcium ion. This Ca-cluster site does not exist in any
known metalloprotease structure and appears to be unique to the ADAMTS.

In addition, the present crystal has a Matthews coefficient $V_m$ (27) of 1.84, which corresponds to a 37% solvent content in the crystal. For such low solvent content, a number of intermolecular contacts and surface overlapping are expected and consequently the cataADAMTS-5 molecules in the crystal are tightly packed (Fig. 9). Roughly on the same plane, the first molecule in the asymmetric unit (molecule-A) (overall surface 8780 Å$^2$) is surrounded by six of its symmetric mates with buried surface areas ranging from 550 to 1140 Å$^2$. On the top of this plane, molecule-A is surrounded by the second molecule in the asymmetric unit (molecule-B) and two of its symmetric mates cover the molecule-A from the back. These eight molecules make contacts with molecule-A, and 80% of the surface of molecule-A is buried. Such high molecular contacts are probably due to the unique molecular shape and surface polarity (or nonpolarity) of cataADAMTS-5. This versatility for molecular interactions may increase the protein aggregation; in this case, it would contribute to the difficulties encountered in early attempts to refold and purify this protein.

Evidence that ADAMTS-5 knock-out mice are protected from cartilage destruction (6, 7) points to a critical role for ADAMTS-5 in murine arthritis and may suggest a similar role in human osteoarthritis. The 1.4 Å resolution structure of ADAMTS-5 complexed to Compound 1 reported here provides insights into the pattern of interactions between the protein and its inhibitor at a very good level of detail and confidence. It offers the potential for rational design of potent and selective inhibitors of ADAMTS-5 as potential osteoarthritis therapeutics that could offer an important advancement in treating this disease.

Acknowledgments—Use of the IMCA-CAT beamline 17-ID (or 17-BM) at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with the Center for Advanced Radiation Sources at the University of Chicago. Use of the Advanced Photon Source was supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract W-31-109-Eng-38.

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ADAMTS-5 Crystal Structure

JANUARY 18, 2008 • VOLUME 283 • NUMBER 3

JOURNAL OF BIOLOGICAL CHEMISTRY

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