The von Willebrand Factor A3 Domain Does Not Contain a Metal Ion-dependent Adhesion Site Motif*

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von Willebrand factor (vWF) is a multimeric plasma protein that mediates platelet adhesion to exposed subendothelium at sites of vascular injury. The A3 domain of vWF (vWF-A3) forms the principal binding site for collagens type I and III. We report here the crystal structure of the vWF-A3 domain at 2.2-Å resolution. As expected, the structure is similar to the integrin I domain but with several novel features. Sequence alignments had suggested that the domain contained an integrin metal-ion-dependent adhesion site (MIDAS) motif, but the crystal structure shows that the motif is modified and that no metal ion is bound. We have introduced mutations into the vestigial MIDAS motif and report that, unlike the I domain of integrin αβ1, vWF-A3 continues to bind collagen after disruption of the motif. We conclude that collagen recognition by vWF-A3 occurs by a mechanism different from that of the integrin αβ1.

von Willebrand factor (vWF) is a multimeric plasma protein that mediates platelet adhesion to exposed subendothelium at sites of vascular injury and also transports the coagulant factor VIII (1). Abnormalities in vWF lead to von Willebrand disease, the most common congenital bleeding disorder. Clinical bleeding occurs when the plasma vWF level falls below the normal level of 5–10 μg/ml (type I disease) or when dysfunctional molecules are produced (type II disease) (2).

Mature vWF contains 2050 residues (~240 kDa) and contains four types of repeated domains (A–D) present in 2–4 copies each, in the order D3-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2. vWF forms dimers and multimers that enhance its biological activity (4). Dimer formation involves interchain disulfide bonding between C-terminal domains, whereas higher order multimers are formed by disulfide bonding between D3 domains (5). Electron microscopy reveals a tadpole-shaped vWF protomer with a large bilobal body (~250 × 50 Å), thought to include the N terminus, and a filamentous tail (~350 long × 20–30 Å thick), presumed to end at the C terminus. The protomer makes tail-to-tail (“dimer” contact) and head-to-head (“multimer” contact) with other protomers (6, 7).

Initial platelet adhesion is mediated by sequences within the A1 and A3 domains of multimeric vWF. The A1 domain (residues 479–717 of mature vWF) binds to platelet glycoprotein Ib/IX, heparin, and cell surface sulfatides. Although both the A1 and A3 domains can bind to collagen, the A3 domain contains the principal collagen-binding site (8). The adhesive properties of vWF are tightly regulated so that plasma vWF does not normally interact with circulating platelets. vWF, however, binds to subendothelial collagen that has been exposed following vascular injury, leading to a conformational change in vWF that allows the A1 domain to bind to platelet glycoprotein Ib/IX.

A large number of proteins contain vWF A-type domains, including the α-subunits of leukocyte integrins (9), where the domains are generally called I domains. Crystal structures have previously been reported for the I domains of integrins αMβ2 and αLβ2 (10, 11). The I domain from the integrin αβ1 is its principal collagen recognition domain (12), and its crystal structure has also been solved recently. In all cases, the integrin I-domain adopts the classic “dinucleotide binding” fold with a central hydrophobic parallel β-sheet, flanked on two sides by amphipathic helices (10). The domain also contains a metal-binding site at the top of the β-sheet which is critical for its adhesive function (14). It has been called the MIDAS (Metal Ion Dependent Adhesion Site) motif (10), and recent mutagenesis studies support the proposal that the upper surface of the I-domain forms the integrin-ligand interface (15, 16).

We report here the crystal structure of the vWF-A3 domain, which we determined as a first step in understanding the molecular basis of vWF-ligand interactions. Since the integrin α2-I domain also binds collagen, it had been suggested that the two domains would have similar binding motifs. However, our results show that the vWF-A3 domain, unlike the α2-I domain, does not bind metal and that the collagen binding properties of vWF-A3 persist even after the disruption of its MIDAS-like motif by site-specific mutagenesis.

EXPERIMENTAL PROCEDURES

Crystal Structure Determination—Recombinant vWF-A3 containing residues 908–1111 of mature vWF was expressed in Escherichia coli using the pQE9 expression vector and purified as described previously (8). Crystals were grown by hanging drop vapor diffusion from 24 to 32% PEG 8000, 100 mM Tris, pH 8.5, and 200 mM MgCl2 at 22 °C. Crystals belong to space group P21 with cell dimensions a = 42.5 Å, b = 66.8 Å, c = 57.8 Å, and β = 101.9°. The asymmetric unit contains two

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1 The abbreviations used are: vWF, von Willebrand factor; MIDAS, metal ion-dependent adhesion site; r.m.s., root mean square.
vWF-A3 Structure

| Table I | Summary of crystallographic analysis |
|---------|-------------------------------------|
|         | The space group and cell dimensions are: P2₁, a = 42.5 Å; b = 66.8 Å, c = 57.8 Å; β = 101.9°. Crystals contain two molecules/asymmetric unit and 34% solvent. The refinement statistics were as follows: resolution 8.0–2.3 Å; Riso = 21.5%; Rwork = 15.8%; number of waters, 169; r.m.s. bond length, 0.010 Å; r.m.s. bond angle, 1.58°; Rmin = $\sum F_{obs}/\sum F_{calc}$, where $I = \langle F_{obs} \rangle / \langle F_{calc} \rangle$, where $F_{obs} = \pm |F_{iso}|$; $F_{calc}$ = protein structure factor amplitude, $F_{iso}$ = heavy atom derivative structure factor amplitude. Phasing power = root mean square ($|F_\ell|/\langle E \rangle$), where $|F_\ell|$ = heavy atom structure factor amplitude, and $E$ = residual lack of closure. FOM is the mean figure of merit. The r.m.s. bond lengths and angles are the deviations from ideal values. |

|        | dmin | Unique % | Redundancy | Riso | Rwork | Sites | Phasing Power | FOM |
|--------|------|----------|------------|------|-------|------|---------------|-----|
| Native | 2.2  | 15,112 (96) | 2.5        | 5.6  |       |      |               | 0.55|
| Hg     | 2.7  | 10,182 (96) | 2.1        | 12.8 | 24.3  | 2    | 1.5           |     |
| Gd     | 2.5  | 9,419 (96)  | 2.2        | 9.0  | 23.4  | 6    | 0.9           |     |

a

**vWF-A3 Structure and has a low solvent content (34%).** Magnesium is not required for crystal growth but affects the growth rate. Crystallization from 0, 10, 100, and 200 mM MgCl₂ took 2 months, 1 month, 7 days, and 2 days, respectively, to produce crystals suitable for diffraction. A second crystal form was also observed under the same conditions in space group P2₁ with unit cell parameters $a = 58.0$ Å, $b = 65.1$ Å, $c = 84.5$ Å, and $β = 101.8°$ but has not been further studied. A mercury derivative was obtained by soaking a crystal in 10 mM Gd(NO₃)₃ for 3 days. Data from native and derivative crystals were collected on a MarResearch Imaging Plate and a Rigaku rotating anode generator with focusing mirrors at room temperature. Data were reduced with DENZO and scaled with SCALEPACK (17). The positions of six gadolinium atoms and two mercury atoms were readily determined by inspection of difference Pattersons and Fouriers using phases calculated by HEAVY (18). A heavy atom refinement and phasing was performed using the CCP4 suite (19). Phases were refined using MLPHARE with the anomalous signal included. An electron density map calculated at 3-Å resolution could be partly interpreted by manually steering a model of an integrin A domain into the electron density. The first round of refinement used a polyalanine model of the central $β$-sheet and helices 1–5. This partial model was refined using XPLOR (20), and the model phases were recombined with MIR phases using SIGMAA to produce a new map from which the loops, helix 6 and side chains were constructed. The $R$ factor at this stage was 31%. After additional rounds of refinement, model building, and extension of the data to 2.2-Å resolution, weak density emerged for the C-terminal helix, a8 (residues 178–197), and the disulfide bridge. A round of non-crystallographic symmetry averaging was performed to resolve the ambiguities in this region. The final $R$ factor was 16% for all data between 8 and 2.2 Å ($R_{free} = 21.5%$) with restrained individual atomic B factors. The final model contains two molecules of vWF-A3 and 169 water molecules. The geometry is excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.010 Å and 1.58° for bond angles. All main chain geometries are excellent with r.m.s. difference from ideal bond lengths of 0.01...
BamHI and HindIII and used to transform the expression vector, pQE9, as described previously (8). The following primers were constructed (desired nucleotide substitution underlined). Reverse primers: 5'-GG-
GAAACGGGAGGAGCC-3' (Ser938Arg); 5'-GGTGGTGATGTCTCC-
ATACTG-3' (Ser974Asp); 5'-CCCCGATTTGGACGGGGCCTCCC-3'
(Ser1005Val). Forward primers: 5'-TCCTGGTCACGGCCGTCTCTG-
TGG-3' (Asp1039Ala); 5'-TCCTGGTCACGCAGGTCTCTGTG-3'
(Asp1039Gln). Mutant proteins were expressed and purified as de-
scribed previously for the wild-type domain (8).

Collagen Binding Assay—A final concentration of 2 mg/ml homoge-
nized insoluble bovine type I collagen (Sigma) or 1% bovine serum albumin was added to microtiter wells in 65 mM sodium phosphate buffer, pH 7.2, for 90 min at 37 °C. After washing three times with Tris-buffered saline, pH 7.4, to remove unadsorbed collagen, residual binding sites were blocked by the addition of 3% bovine serum albumin, 0.1% Tween 20 in Tris-buffered saline for 60 min. Concentrations varying from 1 to 4 μM of either wild type or each of the vWF-A3 mutant proteins was added to the wells and incubated for 60 min at room temperature. Wells were then washed with Tris-buffered saline and the remaining bound vWF-A3 detected with an enzyme-linked immunosorbent assay. Wells were incubated with a 1:2000 dilution of monoclonal anti-His antibody (Qiagen, La Jolla, CA) for 1 h at 37 °C. After washing three times, a secondary anti-mouse antibody that was conjugated with horseradish peroxidase was added for 1 h and bound antibody visual-
ized by the addition of o-phenylenediamine. Nonspecific binding was
determined by subtracting peroxidase-conjugated antibody bound to
wells coated only with bovine serum albumin and subtracted from total binding.

**Fig. 2.** a, main chain schematic of the vWF-A3 domain, with β-strands as arrows and helices as coils (drawn with MOLSCRIPT (25)). Residues comprising the vestigial MIDAS motif are shown as ball-and-stick. b, stereo Ca plot comparing vWF-A3 (solid lines) with the integrin α2β1 I domain (dashed lines). The two molecules have been superimposed on the main chain atoms of the central β-sheet (r.m.s. difference = 0.61 Å). Labeling as in Fig. 1a.

**Fig. 3.** Stereo diagram of the vWF-A3 vestigial MIDAS motif, with schematic main chain and ball-and-stick side chains. The water molecule is labeled W, with hydrogen bonds as dashed red lines. Drawn with MOLSCRIPT (25), RASTER3D (26), and RENDER (27).
RESULTS

Structure of the vWF-A3 Domain—We crystallized a recombinant vWF-A3 domain and solved its structure at 2.2-Å resolution using two heavy atom derivatives (Table I). The final structure contains two molecules of vWF-A3 and 169 water molecules. All of the main chain is well defined, with the exception of residues at the N and C termini (923–925 and 1098–1109 (α8)) in both molecules, for which the electron density is weak. The disulfide bridge linking the N and C termini (Cys\textsuperscript{923}–Cys\textsuperscript{1109}) is visible and delimits the ordered residues. The overall fold is, as expected, very similar to the integrin I domain, with a central hydrophobic parallel β-sheet flanked on two sides by amphipathic helices (Figs. 1 and 2). The domain is globular with a height of 40 Å and a width (across the β-sheet) of 30 Å. The main chain atoms of the central β-sheet overlap with the integrin αM I domain with a r.m.s. deviation of 0.5 Å.

There are several surprising features of the structure that could not have been readily predicted from homology modeling using the integrin αM and αL I domains. First, the α2-helix is replaced by a loop and helix α3 is extended by 5 residues. Second, at the top of the domain, the βE-α6 loop adopts a different conformation. Third, the α7-helix is only two turns long and is followed by an abrupt 90° turn before forming a new 8th helix, ending with the C-terminal cysteine Cys\textsuperscript{1109}.

The Vestigial MIDAS Motif—The MIDAS motif in integrin I domains consists of three closely apposed loops which together form the metal-binding site. In vWF-A3, these three loops have a very similar conformation, and two of the loops have identical or analogous residues as follows: the βA-α1 loop contains the DXSXS consensus sequence (residues Asp\textsuperscript{934}–Ser\textsuperscript{938}) and the α3-α4 loop contains a serine (Ser\textsuperscript{1005}) in place of the threonine in the integrin I domains. However, in the third loop, βD-α5, the position of the MIDAS aspartic acid (Asp\textsuperscript{1039} in αM(2)) is taken by Thr\textsuperscript{1038}. Sequence comparisons had suggested that the next residue, Asp\textsuperscript{1039}, might be analogous to the MIDAS aspartic acid, but the crystal structure shows that the sequence alignment was incorrect.

Although crystallization occurred in the presence of 200 mM Mg\textsuperscript{2+}, no metal is bound to the vestigial MIDAS motif (Fig. 3). There is a buried water molecule that hydrogen-bonds to the side chains of Asp\textsuperscript{934}, Ser\textsuperscript{1005}, and Tyr\textsuperscript{572}, it also hydrogen-bonds to the backbone nitrogens of Asp\textsuperscript{1039} and Thr\textsuperscript{1038}, which would not be possible for a metal ion. The water molecule corresponds most closely in position to one of the metal-coordinating water molecules in the integrin I domain, not to the metal ion itself.

At the top of the βA-α1 loop, a phenylalanine side chain (Phe\textsuperscript{928}) points out into solution, creating a pocket occupied by three water molecules (in the integrin I domains, the analogous residue is buried in the hydrophobic core). The buried MIDAS aspartate (Asp\textsuperscript{934}) lies at the base of this pocket. This water-filled cavity is sealed at the other end by the side chain of Arg\textsuperscript{1000}, although 8 Å distant, the positive charge, together with the solvent channel, may help to stabilize the buried aspartate.

Dimer Contact—The two molecules in the asymmetric unit of the crystal form a dimer related by a 2-fold axis of symmetry. The dimer interface comprises parts of the βC-α3 turn, and the α4-βD turn is chiefly hydrophobic and buries 1220 Å\textsuperscript{2} of surface area, more than twice the area of the next largest contact. The two molecules can be superimposed very closely; the overall r.m.s. difference for backbone atoms is 0.65 Å, 0.17 Å for the central β-sheet (Fig. 1b). Curiously, there is a breakdown of symmetry at the dimer interface where the βC-α3 turn adopts a quite different conformation in the two molecules. In one conformation, Asn\textsuperscript{963} and Val\textsuperscript{985} are involved in dimer contact, whereas Val\textsuperscript{984} packs against the backbone of the loop. In the second conformation, Asn\textsuperscript{963} substitutes for Val\textsuperscript{984}, and Val\textsuperscript{984} and Val\textsuperscript{985} form the dimer contact. At opposite ends of the interface there are two pairs of closely apposed histidine side chains. In one case, a pair of aspartates can form salt bridges with the histidines. Despite the intriguing crystal structure, we do not yet have independent evidence that the dimer has physiological relevance. The interaction is not strong, since recombinant A3 runs as a monomer on a gel filtration column at concentrations up to 50 μM.

Collagen Binding—We have previously shown that the principal recognition site for types I and III collagen resides in the A3 domain of vWF (8). To explore the role of the MIDAS motif in vWF-A3 binding to collagen, we introduced a series of mutations into the MIDAS-like sequence, expressed the recombinant mutant proteins, and examined their ability to bind to collagen by two independent methods. The mutations were either to homologous residues in vWF-A1 (Ser\textsuperscript{938} → Arg and Ser\textsuperscript{1005} → Thr) or to other residues (Asp\textsuperscript{934} → Ala and Asp\textsuperscript{1039} → Glu). All five of the mutant proteins were readily expressed in E. coli and purified to homogeneity. As shown in Fig. 4, none of the five substitutions perturbed collagen binding.

Comparison with the αβ1 I Domain—The integrin αβ1 binds collagen via the homologous I domain. This led to the suggestion that vWF and αβ1 might bind collagen via similar motifs. The crystal structure of αβ1 I domain has recently been determined\textsuperscript{2}; it contains, as expected, an authentic MIDAS motif with bound Mg\textsuperscript{2+}. Two groups have shown that collagen binding to αβ1 is metal ion-dependent, being supported by Mg\textsuperscript{2+} and Mn\textsuperscript{2+}, but not by Ca\textsuperscript{2+} (21, 22). In addition, point mutations in residues that comprise the MIDAS motif abolish collagen binding, implicating the upper surface of the domain (23). The two domains are different in other respects also: the αβ1 I domain contains an insertion at the top of strand βE which forms a prominent α-helix that protrudes from the MIDAS face of the domain. vWF-A3 contains no such insertion (Fig. 2b). The surface charge distribution on the MIDAS face is also quite different; in vWF-A3 the upper surface is largely acidic, and in α2-I, the metal ion creates a region of positive charge (Fig. 5). One similar feature is the lack of helix α2 and the extension of helix α3 compared with the leukocyte (αM and
\( \alpha_L \) I domains, which creates a flat hydrophobic surface at one edge of the \( \beta \)-sheet (Fig. 2b).

**DISCUSSION**

The crystal structure of the vWF-A3 domain shows that the domain does not bind metal even though the vestigial MIDAS motif contains only one non-conservative change (Asp to Thr). The lack of metal binding to the isolated vWF-A3 domain did not preclude the possibility that metal bound only to the A domain-collagen complex, providing a bridge between them (as suggested for integrin I-domain-ligand interactions [10]). However, our mutagenesis studies demonstrate that residues comprising the vestigial MIDAS motif are not critical for collagen binding, effectively ruling out a role for metal in vWF-A3 collagen interactions.

The lack of metal binding may be related to the unusual conformation of the phenylalanine residue that follows the DXSXS motif. Its side chain sticks out into solution, rather than packing into the hydrophobic core as the analogous residues do in the integrin I domains. This opens a water channel to the buried aspartic acid, Asp\(^{934} \). In the integrin I domains, the negative charge is neutralized by the metal ion. The water channel may thus be important for the stability of the folded conformation of vWF-A3, obviating the need for metal. In vWF-A1, one of the MIDAS serines is replaced by an arginine (Arg\(^{524} \)), which may play a role analogous to the metal ion in stabilizing the buried aspartic acid (Asp\(^{520} \)).

Comparison with the structure of the \( \alpha_2\beta_1 \) I domain shows that although they have similar three-dimensional folds, there are no obvious shared structural features that could define a common collagen binding motif. The metal ion dependence of \( \alpha_2\beta_1 \)-collagen binding and the existence of a conventional MIDAS motif make it seem very likely that the mode of collagen binding is different and therefore that the requirement for specific collagen sequences will be different.

The A/I domain fold is a member of a larger family called the dinucleotide binding fold, which is present in many intracellular phosphoryl transfer enzymes [24]. In enzymes with this class of fold, the top of the central \( \beta \)-sheet (which contains the MIDAS motif in integrin I domains) always plays a functional role. Our results do not rule out a role for the upper surface of the vWF-A3 domain in collagen binding. Alternatively, vWF-A3 may employ this surface for another purpose, such as binding to the A1 or A2 domains, with collagen binding to a different surface. Our crystal structure will allow the design of experiments to test these hypotheses.

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