Journal of Structural Biology 169 (2010) 349–359

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Solution structure of the complex of VEK-30 and plasminogen kringle 2

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ARTICLE INFO

Article history:
Received 20 August 2009
Accepted 16 September 2009
Available online 30 September 2009

Keywords:
NMR structure
Protein domains
Kringle modules
M-protein function
Bacterial virulence factors

ABSTRACT

The solution structure of the complex containing the isolated kringle 2 domain of human plasminogen ($K2_{Pg}$) and VEK-30, a 30-amino acid residue internal peptide from a streptococcal M-like plasminogen (Pg) binding protein (PAM), has been determined by multinuclear high-resolution NMR. Complete backbone and side-chain assignments were obtained from triple-resonance experiments, after which structure calculations were performed and ultimately refined by restrained molecular simulation in water. We find that, in contrast with the dimer of complexes observed in the asymmetric unit of the crystal, global correlation times and buoyant molecular weight determinations of the complex and its individual components showed the monomeric nature of all species in solution. The NMR-derived structure of $K2_{Pg}$ in complex with VEK-30 presents a folding pattern typical of other kringle domains, while bound VEK-30 forms an end-to-end α-helix (residues 6–27) in the complex. Most of the VEK-30/$K2_{Pg}$ interactions in solution occur between a single face of the α-helix of VEK-30 and the lysine binding site (LBS) of $K2_{Pg}$. The canonical LBS of $K2_{Pg}$, consisting of Asp54, Asp56, Trp60, Arg69, and Trp70 (kringle numbering), interacts with an internal pseudo-lysine of VEK-30, comprising side-chains of Arg17, His18, and Glu20. Site-specific mutagenesis analysis confirmed that the electrostatic field formed by the N-terminal anionic residues of the VEK-30 α-helix, viz., Asp7, and the non-conserved cationic residues of $K2_{Pg}$, viz., Lys43 and Arg55, play additional important roles in the docking of VEK-30 to $K2_{Pg}$. Structural analysis and kringle sequence alignments revealed several important features related to exosite binding that provide a structural rationale for the high specificity and affinity of VEK-30 for $K2_{Pg}$.

1. Introduction

Over the past two decades, much evidence supporting a structure of plasminogen (Pg) that comprises a number of independent domains has emerged. Among the modules that constitute this protein are a series of highly homologous kringle domains. Five of these ~80 residue triple-disulfide linked polypeptides, and the intervening linker regions, form the noncatalytic chain of Pg (Sottrup-Jensen et al., 1978). This polypeptide chain is sequentially followed by an activation peptide region and a latent serine protease domain. Cleavage of one peptide bond in the activation peptide module results in conversion of the zymogen, Pg, to plasmin (Pm), a serine protease that represents the major extracellular protease found in blood.

Kringle domains are responsible for the binding of Pg and Pm to effector molecules, among which include lysine and its analogs (Menhart et al., 1991; Hoover et al., 1993; McCance et al., 1994; Nilsen et al., 1999). While binding of Pg to free lysine is likely of limited significance in vivo, these kringle lysine binding sites (LBS) in Pg, and its activated product, plasmin (Pm), interact with proteins containing COOH-terminal lysine residues, leading to important functional consequences. As one example, binding of kringle domains of Pg/Pm to fibrin clots that develop COOH-terminal Lys/Arg during lysis further enhances the degradation of fibrin by Pm catalysis, and also stimulates activation of Pg by its activators (Fleury et al., 1993a,b). Of additional significance, similar kringle-mediated binding of Pg/Pm to cell surface receptors results in the assembly of Pg/Pm on cells (Miles et al., 1988). Such cell-bound proteolytic capabilities are of importance to extracellular-related processes, e.g., extracellular matrix degradation, that can facilitate tumor cell invasion and metastasis (DeVries et al., 1996).

It is now clear that assembly of the Pg/Pm system components on some microorganisms, is vital to their pathogenesis. One notable example includes the receptors for Pg/Pm on some strains of Gram-positive Group *A* streptococci (GAS) (Lottenberg et al., 1987), a major cause of skin and mucosal infections in humans (Carapetis et al., 2005). One such Pg/Pm receptor has been identified on subclasses of bacterial M-proteins (*emm* genes) that contain the *Pg*-binding group *A* streptococcal *M*- or M-like protein (PAM).

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doi:10.1016/j.jsb.2009.09.011

This protein binds Pg with high affinity, after which Pg is activated to Pm by GAS-secreted streptokinase (SK), thus providing the bacteria with surface protease activity that allows it to invade surrounding tissue (Sodeinde et al., 1992; Wistedt et al., 1995). A 30-residue internal polypeptide of PAM, VEK-30, represents the major binding determinant of the PAM/Pg interaction. Interestingly, while VEK-30 binding to Pg requires an intact LBS for maximal binding efficiency, VEK-30 does not contain a C-terminal lysine residue, in contrast to many other Pg/Pm receptors. In addition, the binding of VEK-30 to Pg is highly specific for the kringle 2 domain of Pg ($K2_{Pg}$), despite the fact that $K2_{Pg}$ displays the weakest affinity and specificity for lysine analogs in comparison to the other lysine-binding kringles of Pg.

We previously solved the X-ray crystal structures of the complexes, VEK-30/$K2_{Pg}$ and VEK-30/angiostatin($K1_{Pg}$–$K2_{Pg}$–$K3_{Pg}$), and both revealed that an internal "pseudo-lysine", formed from positive (Arg17 and His18) and negative (Glu20) side-chains, inserts into the LBS of $K2_{Pg}$ (Rios-Steiner et al., 2001; Cnudde et al., 2006). However, this unusual binding modality alone cannot explain the high affinity of the binding, nor the specificity of VEK-30 for $K2_{Pg}$. Indeed, while the structures illuminated numerous exosite interactions which would appear to contribute to the specificity of VEK-30 for $K2_{Pg}$, their importance to the binding energy is unknown. Furthermore, examination of the crystal packing in both VEK-30/$K2_{Pg}$ and VEK-30/angiostatin revealed that the asymmetric units of both complexes exist in dimeric form. To further identify the potential roles of the exosite interactions in the docking of VEK-30 to $K2_{Pg}$, and to obtain information on the dynamic aspects of this interaction, we have employed NMR-based methodologies. This communication presents a report of our findings.

2. Experimental

2.1. Protein preparation

Human $K2_{Pg}$(C4G/E56D/L72Y), a triple variant of $K2_{Pg}$ that displays enhanced affinity for lysine analogs and VEK-30 compared to wild-type-$K2_{Pg}$, was expressed in *Pichia pastoris* GS115 cells as described in detail previously (Nilsen et al., 1999). For ^{15}N labeling of the peptide, the recombinant yeast cultures were grown in medium containing $(^{15}NH_4)_2SO_4$ (99%, Cambridge Isotope Laboratories, Andover, MA). For ^{15}N and ^{13}C labeling, the medium for ^{15}N feeding was used, and glucose and methanol were replaced by [^{13}C]-glucose (99%; Isotec, Champaign, IL) and [^{13}C]-methanol (99%, Isotec). Overall recoveries for the unlabeled and labeled products were 100 mg/l and 80 mg/l, respectively.

To prepare the functional internal peptide, VEK-30, a $(His)_6$-B1 immunoglobulin-binding domain of streptococcal protein G (GB1)-tagged fusion expression system was employed. Briefly, the DNA fragment containing the VEK-30 cDNA was inserted into the bacterial expression vector pET-15b (Novagen, Gibbstown, NJ)/GB1 (Cheng and Patel, 2004), and expressed in *Escherichia coli* BL21 (DE3). The final construct contained, sequentially, an ATG initiation codon, a purification $(His)_6$ tag, the GB1 fusion partner for increased solubility, a 9-residue linker, and a thrombin cleavage site, LVPR'GS (' representing the site of thrombin-catalyzed cleavage), all sequentially inserted into the parent plasmid (Cheng and Patel, 2004). A synthetic gene encoding VEK-30 and a stop codon were inserted immediately downstream of the thrombin cleavage site.

After induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37 °C, the fusion protein was purified using a Ni^{2+}-Sepharose affinity chromatography column (HisTrap HP; GE Healthcare, Piscataway, NJ). The target fusion protein fractions were pooled and dialyzed against 100 mM NH_4HCO_3 at 4 °C, and lyophilized. The purified fusion protein (~20 mg), viz., GB1-VEK-30, was then cleaved with 100 U thrombin (Enzyme Research Laboratories, South Bend, IN). The cleaved fragments were further separated using a 1 ml HiTrap HP affinity column (GE Healthcare). The flow-through fractions contained VEK-30 (with a GS sequence at the amino-terminus that remained after thrombin cleavage), and were pooled and dialyzed (molecular weight <2,000 cut-off dialysis membrane), against 50 mM Tris–HCl, pH 7.4/500 mM NaCl overnight at 4 °C. After dialysis, the mixture was applied to a HiTrap-benzamidine FF column (GE Healthcare) to remove the thrombin. The flow-through fraction containing purified VEK-30 was collected and pooled. Finally, the pooled fractions were dialyzed against 100 mM NH_4HCO_3, 4–6× at 4 °C, and lyophilized. Isotopic labeling was achieved in M9 mineral medium containing $^{15}NH_4Cl$ (99%, Cambridge Isotope Laboratories, Andover, MA) as the sole nitrogen source and/or ^{13}C glucose (99%, Isotec, Champaign, IL) as the sole carbon source. The VEK-30 variants were obtained by site-directed mutagenesis based on pET-15b/GB1/VEK-30, and were over-expressed and purified using the same method as above.

The integrity of all protein and peptides were determined by MALDI-TOF mass spectrometry on an Autoflex III (Bruker Daltonics, Bremen, Germany). For all uniformly labeled ^{15}N and $^{13}C/^{15}N$ species, single mass peaks were obtained of the correct molecular weights, indicating nearly complete incorporation of the heavy isotopes.

2.2. Surface plasmon resonance (SPR)

Binding of recombinant VEK-30 variants to $K2_{Pg}$ was performed using a BIAcore-X SPR system (BIAcore, Uppsala, Sweden) at 25 °C. A 1:1 mixture of 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M *N*-ethyl-*N*-dimethylaminopropylcarbodimide (EDC) (provided in the amine coupling kit; BIAcore AB), were added to the sensor chip (CM-5). Subsequently, $K2_{Pg}$, diluted to a final concentration of 100 μg/ml and redissolved in 0.1 M NaOAc buffer, pH 4.0, was injected to the flow cell 2 (FC2) for immobilization on the sensor surface. After coupling, 1 M ethanolamine was injected to deactivate the sensor chip surface. All binding experiments were conducted by injecting various concentrations (0.2–5 μM) of VEK-30 and its variants in HBS-EP buffer (10 mM Hepes/150 mM NaCl/3 mM EDTA/0.005% polysorbate 20, pH 7.4) over the $K2_{Pg}$-coupled chip surface. At the end of each cycle, regeneration of the surfaces was achieved by injecting 20 μl of washing buffer (0.1 M glycine, pH 2.5), which, as shown by control experiments, did not change the binding properties of $K2_{Pg}$ bound to the CM-5 chip.

Sensorgrams were analyzed using BIAevaluation software, version 3.0. The binding data were subtracted from those obtained using a reference flow cell prepared by the same method, with the exception that $K2_{Pg}$ was not immobilized on the chip. The apparent equilibrium dissociation constants (K_D) were calculated from the ratio of the dissociation (k_d) and association rates (k_a).

2.3. Circular dichroism (CD) measurements

CD spectra of the VEK-30 peptides were measured (200–250 nm) with a Jasco J-815 spectrometer using a quartz cell of 1 mm light path at room temperature. The concentrations of VEK-30 were adjusted to 30 μM in 100 mM sodium phosphate, pH 7.4. Protein and peptide concentrations were determined by UV absorption at 280 nm. The α-helical content was determined from mean residue ellipticities at 222 nm using the empirical relationship, $f_\alpha = (-[\theta]_{222} - 2340)/30{,}300$) (Chen et al., 1972).

2.4. Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed as described previously (Cnudde et al., 2006) in a Beckman XL-I analyt-