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Structure of a low-melting-temperature anti-cholera toxin: llama V_HH domain

Variable heavy domains derived from the heavy-chain-only antibodies found in camelids (V_HH domains) are known for their thermal stability. Here, the structure of A9, an anti-cholera toxin V_HH domain ($K_d = 77 \pm 5$ nM) that has an unusually low melting temperature of 319.9 ± 1.6 K, is reported. The CDR3 residues of A9 form a β -hairpin that is directed away from the former V_H-V_L interfacial surface, exposing hydrophobic residues to the solvent. A *DALI* structural similarity search showed that this CDR3 conformation is uncommon.

1. Introduction

Single-domain antibodies (sdAbs) can be generated by recombinant expression of variable heavy domains of heavy-chain-only antibodies found in camelids (V_HH domains) or of conventional V_H domains (Holliger & Hudson, 2005). SdAbs utilize three hypervariable regions known as complementarity-determining regions (CDRs) in antigen recognition, and at ~ 15 kDa are about one tenth of the size of conventional antibodies. Many sdAbs attain high affinities for their targets, leading to a diverse array of applications (Eyer & Hruska, 2012).

Often, V_HH domains prove to be more thermally stable than antibody fragments from conventional antibodies (Goldman *et al.*, 2006; Harmsen & De Haard, 2007; Pérez *et al.*, 2001). V_HH domains have adapted to the absence of the light chain by modifying residues on the former interfacial V_H-V_L surface (Riechmann & Muyldermans, 1999). In addition, the long CDR3 loop in V_HH domains can pack against the former V_H/V_L interfacial surface to prevent aggregation (Ewert *et al.*, 2002; Perchiacca *et al.*, 2011, 2012; Perchiacca & Tessier, 2012), to enhance solubility (Barthelemy *et al.*, 2008) and to stabilize the V_HH-domain fold (Bond *et al.*, 2003; Ewert *et al.*, 2002).

A large number of structures of variable domains (871 structures contain v-set domains) have been deposited in the PDB, but very few of these structures are accompanied by their melting temperatures, making correlations between structure and stability difficult to define (Supplementary Fig. S1[†]). V_HH A9 was selected from a non-immune semisynthetic llama library and shown to bind the B subunit of cholera toxin (Goldman *et al.*, 2006). Here, we examine the structural features of this low-melting-temperature V_HH domain and show that the conformation of its CDR3 loop is uncommon.

2. Materials and methods

2.1. Protein production

The protein sequence of the previously described V_HH A9 was inserted into pET-22 expression vector (Novagen). Protein production was essentially as described previously (Goldman *et al.*, 2006); a detailed description is included in the Supplementary Material.

2.2. Crystallization, data collection, structure determination and refinement

The A9 construct was crystallized in sitting drops using condition No. 8 of Index (0.1 M sodium acetate trihydrate pH 4.5, 3.0 M NaCl; Hampton Research). Several crystals grew within a week and were cryoprotected in mother liquor supplemented with 1–5% PEG 400.

Diffraction data were collected using a MicroMax-007 HF rotating-anode generator (Rigaku) equipped with a VariMax HF (Osmic) monochromator and a Saturn 994 (Rigaku) CCD detector. Diffraction images were indexed, integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997). The structure was solved by molecular replacement using *AMoRe* (Navaza, 2001) with PDB entry 3cfi (Lam *et al.*, 2009) as the starting model. After rigid-body refinement and simulated annealing in *CNS* v.1.1 (Brünger *et al.*, 1998), the model was refined using *REFMAC5* (Murshudov *et al.*, 2011). Model building and solvent addition were performed with *Coot* (Emsley *et al.*, 2010; Emsley & Cowtan, 2004).

2.3. Circular dichroism

Thermal denaturation was monitored by circular dichroism (CD) as described previously (Goldman *et al.*, 2011). The protein was diluted to 20 µg ml⁻¹ in deionized water; the average melting temperature (T_m) and standard deviation were calculated from three measurements using protein from two different preparations. Data were fitted to a four-parameter logistic function using *GraFit 5* (Erithacus Software) to determine the T_m .

2.4. Surface plasmon resonance

Antigen binding affinity was measured by surface plasmon resonance (SPR) using a ProteON XPR36 (Bio-Rad). Cholera toxin was purchased from Calbiochem (San Diego, California, USA). One lane of a GLC chip was coated with cholera toxin (20 µg ml⁻¹ in 10 mM sodium acetate pH 5.0) using the standard EDC coupling protocol provided by the manufacturer; the other lanes served as negative controls. All experiments were performed at 298 K. The binding affinity of V_HH A9 was measured by flowing dilutions ranging from 1000 to 0 nM over the chip surface at 50 µl min⁻¹ for 60 s. The data were analyzed with the *ProteOn Manager* 3.1.0 software and were

Figure 1
(a) Structure of the anti-cholera toxin V_HH A9. The CDR1 loop is colored red, the CDR2 is colored green and the CDR3 is colored blue. (b) Overlay of A9 and NbGspD-7, the most structurally similar V_HH (PDB entry 3ezj) identified by DALI.

Table 1

X-ray crystallographic data-collection and refinement statistics for anti-cholera toxin V_HH A9 (PDB entry 4idl).

Values in parentheses are for the outermost resolution shell.

Space group	C2
Unit-cell parameters (Å)	$a = 56.44$, $b = 51.86$, $c = 46.48$
Wavelength (Å)	1.54
Resolution range (Å)	44.253–2.09 (2.16–2.09)
Unique reflections	7931
$R_{\text{merge}}^{\dagger}$	0.065 (0.185)
$\langle I/\sigma(I) \rangle$	20.8 (5.9)
Completeness (%)	98.92 (86.27)
Multiplicity	3.6 (3.2)
Refinement statistics	
Resolution (Å)	26.87–2.09 (2.14–2.09)
No. of reflections	7235 (465)
R factor ‡	0.206
$R_{\text{free}}^{\S}$	0.222
No. of atoms	
Protein	941
Solvent	61
Other	—
Average B factors (Å ²)	
Protein	18.8
Solvent	20.9
R.m.s.d. from ideal geometry	
Bond lengths (Å)	0.010
Bond angles (°)	1.092
Ramachandran plot, residues in	
Most favored regions (%)	93.3
Additional allowed regions (%)	6.7
Generously allowed regions (%)	0.0
Disallowed regions (%)	0.0

† R_{merge} was calculated as $\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. ‡ R for the working set of reflections was calculated as $\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$. § R_{free} was calculated using a test set consisting of 5% of the total reflections.

corrected by subtraction of the zero antibody concentration column. The binding constants were determined using the equilibrium binding model of the software. The average K_d and standard deviation were calculated from seven complete sets of binding curves.

3. Results and discussion

3.1. Structure

The structure of the anti-cholera toxin V_HH A9 is shown in Fig. 1. Table 1 contains the data-collection and refinement statistics. Density was observed for residues Lys1–Ser123. V_HH A9 has the general v-type immunoglobulin fold (Bork *et al.*, 1994) comprised of a two-layer β -sandwich. While most v-set domains contain nine β -strands (four over five), V_HH A9 contains ten β -strands. Immunoglobulin variable domains contain a conserved disulfide between Cys22 and Cys95. In the V_HH A9 structure, density for the disulfide bond indicated that the bond was fully formed in the periplasmically expressed protein.

CDR3 of llama V_HH is typically around 15 residues (Conrath *et al.*, 2003), which is slightly longer than the average CDR3 of human V_H (11.6 residues; Davies & Riechmann, 1994); the V_HH A9 described here has a 15-residue CDR3 that expands the β -sheet by forming a β -hairpin. CDR2 also forms a β -hairpin. CDR1 contains a rare 3_{10} -helical segment formed by Thr28–Ala29–Ser30. Examination of the A9 structure shows that Ser30 (of CDR1) and Tyr110 (of CDR3) are within hydrogen-bonding distance (2.76 Å); this interaction may potentially stabilize the CDR3 conformation.

The conformation of the V_HH A9 CDR3 loop is uncommon. A DALI structural similarity search (Holm & Rosenström, 2010) showed that other close structural homologues all had varying degrees of coverage of the V_H–V_L interfacial surface by the CDR3

loop (Supplementary Fig. S2), including the most similar V_HH, NbGspD-7 (PDB entry 3ezj; Korotkov *et al.*, 2009). These two V_HH domains could be aligned with an r.m.s.d. of 0.61 over 107 C α atoms (of 123). V_HH domains predominantly differ only in their CDR sequences; thus, these V_HH domains are 62% identical in sequence but have low sequence identity within the CDRs (Supplementary Fig. S3). Comparison of the two V_HH domains, which bind structurally unrelated antigens, shows that although varied in sequence, their CDR1 and CDR2 regions superpose and are structurally similar. The residues within the CDR3 of the two V_HH domains differ significantly in sequence and length. In the antigen–V_HH complex, NbGspD-7 predominantly contacts its antigen through CDR3. Three other cholera-binding V_HH clones identified during our library screening possessed identical CDR3 sequences to A9 (Goldman *et al.*, 2006), suggesting that V_HH A9 utilizes residues within CDR3 for antigen recognition.

The 'V_HH tetrad' refers to four residues located at the former V_H–V_L interface which differ between conventional V_H and camelid V_HH domains (Barthelemy *et al.*, 2008). V_HH A9 contains one atypical residue in its tetrad: Trp47. Residue 47 is typically a Trp in human V_H but is substituted by Gly, Ser, Leu or Phe in V_HH; this exposed hydrophobic residue may contribute to the low *T_m* of A9 and its propensity to aggregate.

3.2. Melting temperature

Typically, the *T_m* values of sdAbs range from 324 to 351 K (Barthelemy *et al.*, 2008; Bond *et al.*, 2003; Dumoulin *et al.*, 2002; Goldman *et al.*, 2011). However, a V_HH with a *T_m* of 358 K has been identified by our group (Graef *et al.*, 2011), and Barthelemy and coworkers reported a human V_H with a *T_m* of 356 K (Barthelemy *et al.*, 2008). Highly stable sdAbs are of value to field applications as

they may not require refrigeration or cold chain transport. Regardless of *T_m*, most sdAbs reversibly refold after thermal denaturation (Bond *et al.*, 2003; Pérez *et al.*, 2001) with minimal irreversible aggregation (Perchiacca *et al.*, 2012; Perchiacca & Tessier, 2012). The V_HH A9 was found to have an unusually low *T_m* of 319.9 ± 1.6 K and did not reversibly refold after thermal denaturation, indicating that irreversible processes (*e.g.* aggregation, oxidation, deamination *etc.*) were likely to be linked to unfolding (Patro *et al.*, 2002; Wang, 2005; Fig. 2).

3.3. Binding affinity

Although many V_HH domains show high affinity for their target antigens, those derived from non-immune libraries often do not bind their targets as strongly as V_HH domains derived from immunized animals. SPR was used to determine the dissociation constant (*K_d*) of V_HH A9; the *K_d* was determined to be 77 ± 5 nM by SPR (Fig. 2).

4. Conclusions

There is currently a very weak basis of experimental data correlating *T_m* and structural motifs in sdAbs. We observed that the conformation of CDR3 in the low-melting-temperature V_HH A9 differs from most variable domains in that it extends a β -strand and expands the sheet but does not cover the former V_H–V_L interfacial surface. Additional comparative analysis is needed in order to determine to

Figure 2 (a) Thermal denaturation and renaturation curves monitored by CD. The melting temperature is taken to be the inflection point in the slope (*T_m* = 319.9 ± 1.6 K). Changes in the high-tension voltage (HTV) versus temperature are shown in the subplot below. (b) SPR curves generated when the indicated concentration of V_HH A9 was flowed over a cholera toxin-functionalized surface; the results were interspot and buffer corrected. (c) Plot of the SPR signal (expressed in response units; RU) versus concentration from which the *K_d* was calculated.

what extent the low melting temperature of V_HH A9 is a consequence of its CDR3 conformation. By identifying structural features which differ between high- and low-*T_m* sdAbs, it may be possible to engineer and/or predict structural features which impart high thermal stability.

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