Supplementary Information

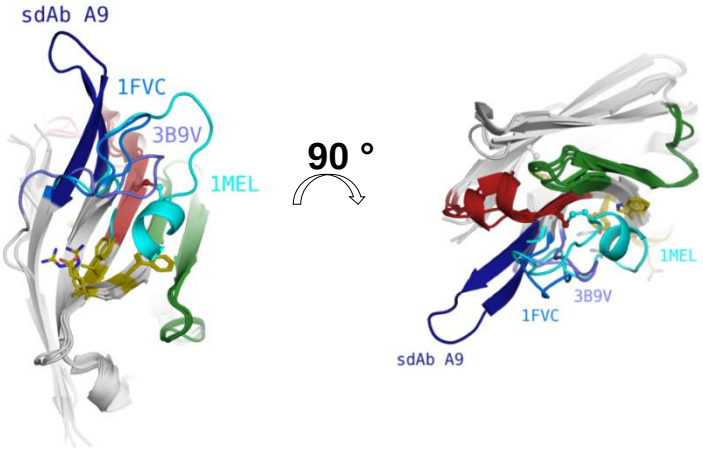
Protein preparation

The protein sequence of the previously described A9 V_HH (Goldman, Anderson, Liu, Delehanty, Sherwood, Osborn, Cummins, & Hayhurst, 2006) was inserted into a pet22 expression vector (Novagen) using the following primers: 5'-GAGATATACATAT GAAATACCTATTGCCTACG-3' and 5'GCAAGCTTAGTGATGGTGATGGTGATGTGA GGAGACGGTGACCTGGG-3'. The resulting plasmid was verified by restriction analysis and sequencing.

The pET22 plasmid was cotransformed with a pRARE vector into BL21(DE3) *E. coli* cells. A single colony was used to inoculate 5 ml of Terrific Broth (TB) supplemented with 50 mg/ml ampicillin and 34 mg/ml chloramphenicol in the morning and was left to grow at 30°C during the day. In the evening, the 5 ml culture was added to a 50 ml TB overnight culture with the same antibiotics and grown at 30 °C. The next morning, four 2 L flasks filled with 500 ml TB were inoculated with the 50 ml overnight culture and shaken at 30 °C until the optical density (O.D.) 600 reached ~1.5. At this point, the expression of the sdAb was induced by adding 1 mM IPTG to each culture and allowed to continue to shake at 30 °C. When the O.D. 600 began to decrease, the cells were harvested by centrifugation at 5,000 rpm for 15 minutes. The cell pellets were then either frozen at -80 °C for storage or used for immediate purification. The cell pellets were resuspended in 20 mM Tris-HCl (pH 7.5), 300 mM NaCl, and the Complete EDTA-free protease inhibitor cocktail (Roche). French press lysis of the resuspended cells was followed by centrifugation at 20,000 rpm for 1 hour for lysate clarification. In parallel, 3 ml Ni-NTA resin (Qiagen) was equilibrated with the suspension buffer. The filtered supernatant was applied to the pre-equilibrated Ni-NTA resin and allowed to bind in batch at 4 °C for at least 2 hours. The protein-bound resin was emptied into a gravity column and washed with at least 100 ml of 20 mM Tris-HCl (pH 7.5) and 300 mM NaCl followed by another 50 ml of the same buffer supplemented with 20 mM imidazole. The sdAb protein was eluted with the buffer and 250 mM imidazole. The Ni-NTA resin purification was followed by size-exclusion chromatography using the Superdex S75 16/60 (GE Healthcare), eluting the purified protein with 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl.

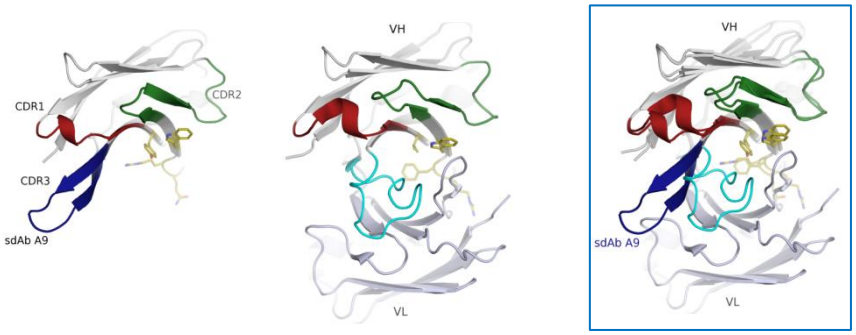
Supplementary Figure S1. **(A)** Overlay of V_HH A9 with three other structures with reported melting temperatures. **(B)** Location of the CDR3 loop of A9 relative to the former V_H/V_L interfacial surface. Structure of a scFv heterodimer (V_H and V_L domain) (PDB 3JUY) (Clark, et al, *Protein Sci.* (2009) 18(12):2429) superposed with A9. In V_HH A9 the former V_H/V_L interfacial surface is not covered by its CDR3 and the surface of the beta sheet is exposed. **(C)** One mutation in 3B9V, H35G, enables the CDR3 loop to fold over the β sheet to a greater degree altering the packing and conformation of the Trp-95 side chain of the CDR3 with Gly-35 of the beta sheet (Barthelemy, et al, *J. Biol. Chem*. 283(6):3639). A similar Gly/Trp hydrophobic packing interaction was implicated in the stabilization of human V_H HEL4 (Jespers, et al, *J. Mol. Biol*. 337(4):893). Gly-35 is also present in sdAb A9, but in V_HH A9 the Gly-35 is distant (~22 Å) from any Trp residue of the A9 CDR3 loop residue underscoring the importance of examining the tertiary structure and secondary structural elements of CDRs while attempting to identify or incorporate potentially stabilizing residues by site-directed mutagenesis in other V_HH.

A

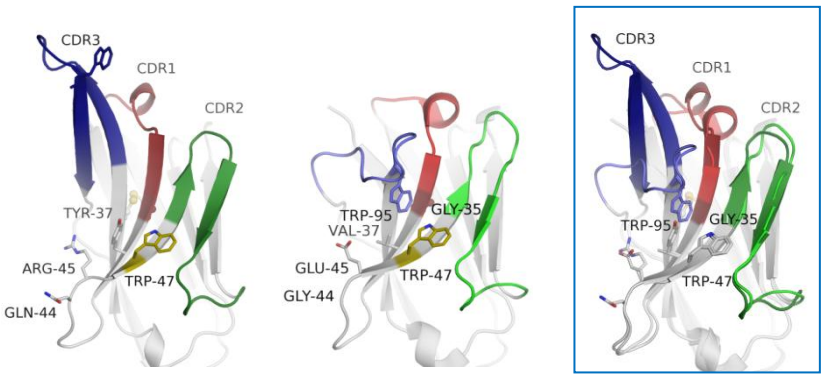


PDB	T_m (ºC)
V_HH A9	47
V_HH 1MEL	69
V_H 1FVC	58
V_H 3B9V	79

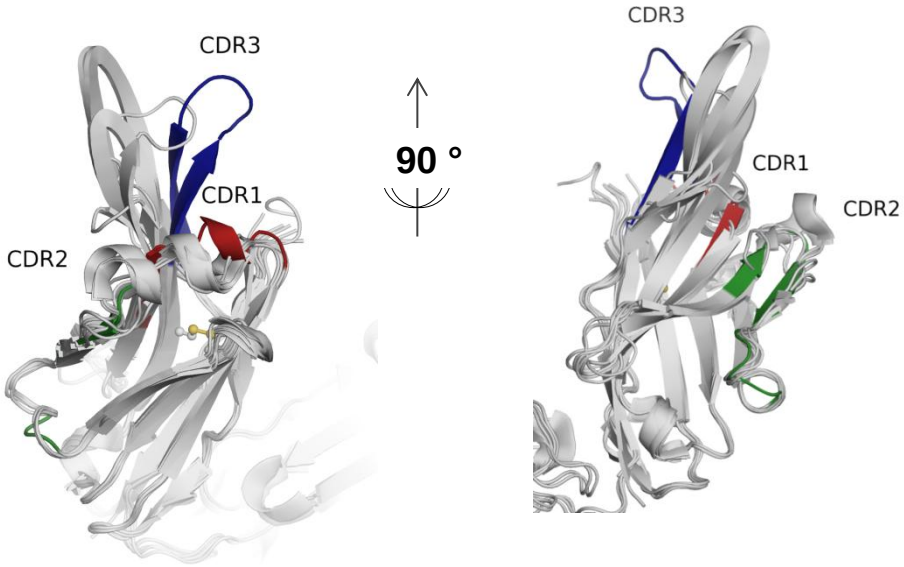
B



C



Supplementary Figure S2. Results of a Dali structural similarity search. The CDR3 conformation of A9 is unusual. Alignment of the V_H and V_HH which contained beta stranded regions in their CDR3 sequences. The CDR3 conformation of A9 is the most dissimilar and has a convex curvature and did not superpose with any of the structurally similar hits identified by Dali.



Supplementary Figure S3. Sequence alignment of A9 with its closest structural homologue, NbGspD-7 (PDB 3EZJ), abbreviated as Nb7. Framework regions are colored black, CDR1 is in red, CDR2 in green, and CDR3 in blue. The conserved cystines are highlighted in yellow; the tetrad residues are highlighted in cyan. The 'V_HH tetrad' refers to four residues present at the former V_H/V_L interface that typically differ between conventional V_H and camelid V_HH. Changes between V_H and V_HH are typically V37 to F or Y; G44 to E or Q; L45 to R or C and W47 to G, S, L, or F.