Disulfide linkage engineering for improving biophysical properties of human V_H domains

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To enhance their therapeutic potential, human antibody heavy chain variable domains (V_Hs) would benefit from increased thermostability. The highly conserved disulfide linkage that connects Cys23 and Cys104 residues in the core of V_H domains is crucial to their stability and function. It has previously been shown that the introduction of a second disulfide linkage can increase the thermostability of camelid heavy-chain antibody variable domains (V_HHs). Using four model domains we demonstrate that this strategy is also applicable to human V_H domains. The introduced disulfide linkage, formed between Cys54 and Cys78 residues, increased the thermostability of V_H by 14–18°C. In addition, using a novel hexa-histidine capture technology, circular dichroism, turbidity, size exclusion chromatography and multilangle light scattering measurements, we demonstrate reduced V_H aggregation in domains with the Cys54–Cys78 disulfide linkage. However, we also found that the engineered disulfide linkage caused conformational changes, as indicated by reduced binding of the V_Hs to protein A. This indicates that it may be prudent to use the synthetic V_H libraries harboring the engineered disulfide linkage before screening for affinity reagents. Such strategies may increase the number of thermostable binders.

Keywords: aggregation/antibody variable domains/disulfide linkage engineering/synthetic V_H libraries/thermostability

Introduction

Protein stability is a key to the performance of protein therapeutics. Unstable proteins often aggregate or partially unfold. This leads to undesirable consequences in vivo such as increased susceptibility to degradation, decreased serum half-life, immunogenic reactions or non-specific antigenic recognition (Mitraki and King, 1992; Hurle et al., 1994; Wetzel, 1994; Wörn and Plückthun, 2001; Horwich, 2002; Frokjaer and Otzen, 2005). The end result is reduced therapeutic efficacy.

Human V_H domains form an important class of biologics. Aggregation was recognized as a major drawback of V_Hs, as early as 1989 (Ward et al., 1989); however, significant advances since then have made the generation of non-aggregating V_Hs a routine exercise. For example, selection approaches have been developed that allow for efficient screening of synthetic human V_H libraries for V_H binders that are non-aggregating and have high thermodynamic stability (Jespers et al., 2004; To et al., 2005; Famm et al., 2008; Arbabai-Ghahroudi et al., 2009a,b, 2010). Site-specific mutagenesis approaches for improving the non-aggregation character of V_H domains have also been developed (Arbabai-Ghahroudi et al., 2009a, 2010). Novel complementary and/or generic mutagenesis approaches that render V_Hs non-aggregating are desirable; more desirable are the approaches that also stabilize V_H domains in a multifaceted manner.

The sole disulfide bond connecting β-strands B and F of V_H domains is highly conserved (Amzel and Poljak, 1979; Williams and Barclay, 1988). Linking Cys23 and Cys104 residues in the core of V_Hs, its loss leads to a dramatic decrease in the thermodynamic stability, misfolding and non-functionality (Goto and Hamaguchi, 1979; Proba et al., 1998; Ciaccio and Laurencıe, 2009). It follows that adding extra disulfide linkage(s) at optimal positions should increase V_H stability as it has been shown in several instances, including Ig domains (Wetzel et al., 1988; Matsumura et al., 1989; Betz, 1993; Young et al., 1995; Davies and Riechmann, 1996; Mansfeld et al., 1997; Hashihara et al., 2007; Chan et al., 2008; Saerens et al., 2008; Gong et al., 2009; Hussack et al., 2011; Govaert et al., 2012; Wozniak-Knopp et al., 2012). Disulfide linkages stabilize proteins by reducing their conformational entropy through limiting the number of possible conformers that lead to unfolded states (Fersht, 1997; Mason et al., 2002).

Recently, it was shown that the engineering of a second Cys54–Cys78 disulfide linkage, which bridges β-strands C’ and D, into camelid heavy-chain antibody variable domains, V_HHs, increased their thermal and chemical stabilities (Hashihara et al., 2007; Chan et al., 2008; Saerens et al., 2008; Hussack et al., 2011). Here, we show that the application of the same disulfide linkage engineering approach to V_Hs leads to increases in the thermostability of V_Hs as well, with additional improvements in V_H non-aggregation.

Materials and methods

Cloning, expression and mass spectrometry analysis

Mutant V_Hs, huVHAm302S, huVHAm427S, huVHAm431S and huVHPC235S with Cys substitutions at positions 54 and
78 were created from their corresponding wild-type VHs, huVHAm302, huVHAm427, huVHAm431 and huVHPC235, respectively, by the splice overlap extension-polymerase chain reaction approach (Ho et al., 1989; Arbabi-Ghahroudi et al., 2010). VHs were cloned, expressed and purified by immobilized metal ion affinity chromatography (IMAC) as described (Arbabi-Ghahroudi et al., 2009a). The purity of VHs was subsequently assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

To verify the presence/absence of the Cys54–Cys78 disulfide linkage, VHs were digested with trypsin and chymotrypsin and the generated peptides were subjected to mass spectrometry (MS) analysis as described in the legend of Supplementary Fig. S2.

Melting temperature and fraction refolded determinations

Circular dichroism (CD) spectra and melting temperature (Tm) measurements were obtained as described by Kim et al. (2012) using a Jasco J-815 spectropolarimeter equipped with a Peltier thermoelectric-type temperature control system (Jasco, Easton, MD, USA). The determination of the reversibility of the temperature-induced denaturation and the estimation of the fraction of refolded protein recovered following thermal denaturation were carried out according to previously described protocols (Barthelmy et al., 2008). Purified monomeric VHs (50 μg/ml in 0.1 M sodium phosphate buffer, pH 7.4) obtained by size exclusion chromatography (see below and Supplementary Fig. S3) were used for CD, Tm and fraction refolded measurements, with CD parameters set as follows: bandwidth = 1 nm; temperature range = 30–96°C; temperature ramp = 1°C/min; data collection = every 1°C; DIT = 4 s; and accumulations = 1. Ellipticity changes at 210 nm (huVHAm431 and huVHAm431S), 205 nm (huVHAm427, huVHAm427S, huVHAm302 and huVHAm302S), 220 nm (huVHPC235) and 200 nm (huVHPC235S) were used for the construction of thermal unfolding curves and subsequent calculation of Tm, m, and fraction refolded values. With the huVHAm427S and huVHAm431S unfolding transition curves, which had no clear lower plateau, ellipticity values at 96°C were taken as the lower plateaus for constructing melting curves and subsequent determination of Tms. Therefore, the Tm,s for both huVHAm427S and huVHAm431S are estimated minimum Tm,s.

Size exclusion chromatography and multiangle light scattering analyses

Two hundred microlitres of VHs (0.25 mg/ml) dialyzed in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4) plus 0.5 mM ethylenediaminetetraacetic acid (PBS/EDTA)—and free of any insoluble aggregates as determined by turbidity measurements at 360 nm—were applied at a flow rate of 0.5 ml/min to a Superdex™ 75 10/300 GL column (GE Healthcare, Baie d’Urfé, QC, Canada) equilibrated with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline containing EDTA and polysorbate 20 buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.005% P20 surfactant, pH 7.4). Size exclusion experiments were carried out at room temperature and chromatograms were obtained. For each chromatogram, background absorbance was subtracted and all peaks were normalized with respect to the monomeric peak, which was arbitrarily set at 100%. Peaks to the left of monomeric peaks were considered to be aggregate peaks. Peak areas, A, were determined by integration using AKTA FPLC (GE Healthcare) operating software UNICORN (version 5.1 for Windows). %Aggregate was determined by the formula: %Aggregate = ((AAggregate)/(AAggregate + Amonomer)), where AAggregate is the area of aggregate peaks and Amonomer is the area of the monomer peak. %Aggregate was not determined for huVHAm427 as its monomeric peak was not considered authentic due to peak tailing. %Aggregate values are recorded in Table II. %Recovery was determined as described in the legend of Supplementary Fig. S4 and recorded in Table II.

Clear VH samples (0.55–0.8 mg/ml) were subjected to size exclusion chromatography as described above with PBS as the equilibration buffer, and the molecular weights of the monomeric species were confirmed by multangle light scattering using a tri-angle light scattering detector (miniDawn Treos; Wyatt Technology, Santa Barbara, CA, USA). Experiments were performed in duplicates.

Turbidity analysis

In brief, to measure turbidity, VHs were dialyzed in PBS/EDTA, centrifuged in a microfuge at 13,000 rpm for 1 min, and their concentrations were adjusted to 100 μg/ml. Sample absorbance (A360nm) was taken at room temperature in a spectrophotometer using a 100-QS, 1 mm path length Absorbance Cell (Hellma Analytics, Müllheim, Germany). The samples were then heated to 80°C for 20 min, cooled at room temperature for 20 min, centrifuged for a few seconds to collect condensation, pipetted up and down a few times and their A360nm was measured once again. All treated and untreated samples, including a non-aggregating llama VH H control (A4.2; Hussack et al., 2011) had the same A360nm as the background sample (PBS/EDTA) indicating the absence of turbidity. Turbidity measurements were performed in duplicates.

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) analysis of VH binding to a Ni2+-NTA sensorchip was carried out. A control llama VH monomer (A4.2) and a control VH homodimer (Baral et al., 2012) were also included in the analysis. All VH/VH domains had His6 tags at their C-termini. The VH homodimer is formed by the non-covalent association of two VH monomer units and as a result has two C-terminal His6 tags. VH domains huVHAm302S, huVHAm427S and huVHAm431S were not included in the analysis as, unlike the control domains, they had His6 tags. SPR experiments were carried out with protein fractions corresponding to the dimeric peak in the case of the VH dimer control and monomeric peaks for the remaining VH/VH domains. Resonance units (RU) from duplicate data sets were averaged and then normalized to obtain %RU. The binding of VHs/VH to an activated NTA sensorchip was determined by SPR using a BIACORE 3000 (GE Healthcare). In each cycle, 0.5 mM NiCl2 was injected at 5 μl/min for 2 min, followed by an injection of 50 nM VH/VH in running buffer (10 mM HEPES, 150 mM NaCl, 0.005% P20 surfactant, 50 μM EDTA, pH 7.4) at a flow rate of 5 μl/min with an injection time of 5 min and a dissociation time of 10 min. Sensorgrams were run in duplicates. The NTA chip was regenerated with 350 mM EDTA in 10 mM HEPES, 150 mM NaCl at pH 8.4 for 3 min before the next cycle. Analyses were carried out at 25°C in running buffer. Dissociation rate
constants \((k_{offs})\) were obtained over 60 s time periods between 315–375 s and 780–840 s. Data were analyzed with BIAevaluation 4.1 software (GE Healthcare). SPR analyses of \(V_H\) binding to protein A were carried out as described in the legend of Supplementary Fig. S6.

**Results**

**Mutant \(V_H\)s with Cys54 and Cys78 substitutions are readily expressed in soluble forms**

We chose four human \(V_H\)s for this study: huVHAm302, huVHAM427, huVHAM431 and huVHPC235, all with protein A binding activity (Fig. 1a). huVHAm302, huVHAM427 and huVHAM431 additionally bind to \(\alpha\)-amylase (Arbabi-Ghahroudi et al., 2009b), whereas the antigen specificity of huVHPC235 is not known. By size exclusion chromatography, the first three \(V_H\)s were aggregation prone, whereas huVHPC235 appeared to be non-aggregating. Employing the splice overlap extension-polymerase chain reaction approach, we created the mutant versions by introducing Cys pairs at positions 54 and 78 (Fig. 1). The mutants, designated as huVHAm302S, huVHAM427S, huVHAM431S and huVHPC235S, were cloned, expressed and purified by IMAC. Results showed that the mutant \(V_H\)s had expression yields comparable with their wild-type counterparts (Supplementary Fig. S1a), indicating that the engineered disulfide linkage had no adverse effect on the expression of \(V_H\). We observed that the three aggregating wild-type \(V_H\)s eluted significantly later than their corresponding mutant versions, indicating tighter binding of the wild-type \(V_H\)s to the purification column. This could be due to the fact that the aggregating wild-type \(V_H\) may additionally interact with the column materials through non-specific interactions and/or coordinate tighter to the column nickel due to the presence of multiple His-tags on aggregates. We also noticed that the mutant \(V_H\)s migrated slower than their wild-type counterparts on non-reducing SDS-PAGE gels, with migration differences disappearing under reducing conditions (Supplementary Fig. S1b). Such SDS-PAGE mobility patterns have been seen in the case of \(V_H\)s as well, and it was suggested that this indicates Cys54–Cys78 disulfide linkage formation in extra Cys mutants (Hussack et al., 2011).

**Cys54 and Cys78 residues form a disulfide linkage in mutant \(V_H\)s**

Next, we aimed to verify by MS if the engineered Cys54 and Cys78 in mutant \(V_H\)s formed a disulfide linkage. Wild type and mutant \(V_H\)s were digested with trypsin or a trypsin–chymotrypsin combination and the generated proteolytic peptides were subjected to MS analysis (Supplementary Fig. S2) (Wu et al., 2009; Hussack et al., 2011; Kim et al., 2012), huVHAm302, huVHAM302S and huVHPC235S were readily digested with trypsin (Supplementary Fig. S2a). From our MS results with trypsinized huVHAm302S and huVHPC235S, we successfully identified disulfide linked peptides (Supplementary Fig. S2; Table I). The identification coverage of each protein from the analysis of their tryptic digests using nano-RPLC-MS with data-dependent analysis (DDA) was more than 30%, where the disulfide-linked peptide ions appeared prominent in the survey scan of the
were analyzed for matching ions to guide subsequent manual de novo sequencing. In the case of huVHAm427 and huVHAm427S, a prominent ion at m/z 964.04 (3+) was sequenced as GLEWVCAISSSGGSTYYADSVK (P1) disulfide-linked to GLEWVCAISSSGGSTYYADSVK (P2) as shown (Supplementary Fig. S2b), indicating the existence of a Cys54–Cys78 disulfide linkage. A complete disulfide-linked fragment ion series was obtained from P1 with P2 linked via a disulfide linkage, which remained intact under collision-induced dissociation (Supplementary Fig. S2; Table I). The formation of the Cys54–Cys78 disulfide linkage was also verified for huVHPC235S (Table I; MS² spectrum not shown). Disulfide linkages between Cys23 and Cys104, and Cys111 and Cys112.1 were also identified by MS for both huVHAm302 and huVHAm302S (Table I), huVHAm427, huVHAm427S, huVHAm431 and huVHAm431S were highly protease resistant, and therefore, had to be treated with a higher amount of trypsin (huVHAm427 and huVHAm427S) or a higher amount of trypsin plus chymotrypsin (huVHAm431 and huVHAm431S) for the MS analyses. Following protease digestions, the existence of disulfide linkages was verified by de novo sequencing with the assistance of the DBond program. DBond v2.07 was used to generate the potential disulfide-linked peptide ions corresponding to non-specifically cleaved or miscleaved peptides (Choi et al., 2010). The resulting output was parsed for disulfide-linked peptides with match-quality scores >15 and the corresponding raw data were analyzed for matching ions to guide subsequent manual validation and targeted MS² experiments to obtain MS² spectra for de novo sequencing. The results showed the existence of the Cys54–Cys78 disulfide linkage in huVHAm427S and huVHAm431S (Table I; MS² spectra not shown). In addition, the conserved Cys23–Cys104 disulfide linkage was also verified by MS in all four wild type and mutant V_Hs. Both huVHAm427/huVHAm427S and huVHAm431/huVHAm431S pairs also have a pair of Cys at complementarity-determining region 1 (CDR1) position 38 and CDR3 positions 107 or 116, respectively (Fig. 1a). However, only in the case of the huVHAm431/huVHAm431S pair, were we able to show the existence of an inter-CDR1–CDR3 disulfide linkage (Cys38–Cys116). In addition, we could not determine whether Cys111 and Cys112.1 in huVHAm431 and huVHAm431S V_Hs (Fig. 1a) formed disulfide linkages. The slower non-reducing SDS-PAGE mobilities compared with corresponding wild-type V_Hs is consistent with the presence of the extra Cys54–Cys78 disulfide linkage in mutant V_Hs (see above).

### Mutant V_Hs are far more thermostable than the wild-type counterparts

To assess the effect of the engineered disulfide linkage on the thermostability of V_Hs, we determined the thermal unfolding midpoint temperatures (T_m) of the V_Hs by CD spectrometry. All V_Hs exhibited the two-state sigmoidal melting curves typical of single-domain antibodies. We found that mutant V_Hs had significantly higher T_m increases compared with their corresponding wild-type counterparts (Fig. 2a and b; Supplementary Fig. S3; Table II) (paired t-test, two-tailed, P = 0.0002). The wild-type V_Hs had T_m increases of 53.8–73.0°C which increased to 71.4–89.2°C for mutant V_Hs. This corresponds to T_m increases (ΔT_m) of 13.9–17.6°C. By comparing disulfide linkage patterns of mutant vs. wild-type V_Hs (Table I), it is clear that the T_m increases are due to the presence of the extra Cys54–Cys78 disulfide linkage. Unlike huVHAm302S and huVHPC235S, huVHAm427S and huVHAm431S had unusually high T_m increases of 89.2 and 87.5°C, respectively, most likely due to the presence of a second non-canonical, inter-CDR1–CDR3 disulfide linkage (Fig. 1a; Tables I and II), in addition to the conserved Cys23–Cys104 disulfide linkage common to all V_Hs. Previous studies showed that inter-CDR1–CDR3 disulfide linkages stabilized (e.g. thermostabilized) human V_Hs and cameldid V_Hs (Davies and Riechmann, 1996; Arbabi-Ghahroudi et al., 2009b; Govaert et al., 2012). The high T_m increases of huVHAm427S and huVHAm431S explain why these two

### Table I. Disulfide linkage determination of V_Hs by MS

<table>
<thead>
<tr>
<th>V_H</th>
<th>Tryptic peptides*</th>
<th>MW_{for} (Da)</th>
<th>MW_{exp} (Da)</th>
<th>ΔMW{sup} (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>huVHAm302</td>
<td>S{sup}C{sup}OTSLC{sup}TSTTR</td>
<td>1284.54</td>
<td>1284.55</td>
<td>−0.01</td>
</tr>
<tr>
<td></td>
<td>L{sup}S{sup}C{sup}A{sup}S{sup}G{sup}D{sup}T{sup}V{sup}S{sup}D{sup}E{sup}S{sup}M{sup}T{sup}W{sup}V{sup}R</td>
<td>3630.55</td>
<td>3630.52</td>
<td>0.03</td>
</tr>
<tr>
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<td>1284.54</td>
<td>1284.56</td>
<td>−0.02</td>
</tr>
<tr>
<td></td>
<td>GLEW{sup}V{sup}C{sup}A{sup}I{sup}S{sup}S{sup}G{sup}S{sup}G{sup}S{sup}T{sup}Y{sup}Y{sup}A{sup}D{sup}S{sup}V{sup}K</td>
<td>2889.28{sup}b</td>
<td>2889.30{sup}b</td>
<td>−0.02{sup}b</td>
</tr>
<tr>
<td>huVHAm427</td>
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<td>1225.57</td>
<td>1225.64</td>
<td>−0.07</td>
</tr>
<tr>
<td></td>
<td>V{sup}C{sup}A{sup}I{sup}S{sup}S{sup}G{sup}S{sup}T{sup}Y</td>
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<td>1740.81{sup}b</td>
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<td></td>
<td>V{sup}C{sup}V</td>
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<td>901.44{sup}b</td>
<td>−0.06{sup}b</td>
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<tr>
<td></td>
<td>TV{sup}S{sup}E{sup}C{sup}M</td>
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<td>1147.47</td>
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<td>1144.50</td>
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<td></td>
<td>V{sup}C{sup}A{sup}I{sup}S{sup}S{sup}G{sup}S{sup}T{sup}Y</td>
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<td>1623.62{sup}b</td>
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<tr>
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<td>huVHPC235S</td>
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<td>2889.28{sup}b</td>
<td>2889.30{sup}b</td>
<td>−0.02{sup}b</td>
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<td></td>
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<td>3331.51</td>
<td>3331.47</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Major tryptic or tryptic/chymotryptic peptides containing disulfide linkages are shown, with connecting cysteine residues underlined and boldfaced (see Supplementary Fig. S2 for experimental details). Non-specific or miscleavage occurred during the trypsin/chymotrypsin digestion of huVHAm427 and huVHAm431 and their mutant versions. Spaces within peptide doublets denote sequence discontinuity.

{sup}bThe very close match between MW_{for} (formula molecular weight) and MW_{exp} (experimental molecular weight) indicates the presence of the Cys54–Cys78 disulfide linkage.

{sup}ΔMW = MW_{for} − MW_{exp}.

{sup}The wild-type huVHPC235S contains only one pair of Cys, the conserved Cys23 and Cys104 residues which always form a disulfide linkage.
Thermostability and aggregation state of VHs. (a) Representative example showing the thermal unfolding curves of huVHAm431 and huVHAm431S (see Supplementary Fig. S3 for unfolding curves for all VHs and experimental details). The upper and lower thermal unfolding curves correspond to ellipticity measurements obtained upon first and second heating, respectively. $T_{m,s}$ (the midpoint temperatures of unfolding curves) and fraction refolded values are recorded in Table II. (b) Graph comparing the $T_{m,s}$ of wild type and corresponding mutant VHs. (c) Aggregation state of VHs determined by analytical Superdex™ 75 size exclusion chromatography. The elution volumes for monomeric peaks (marked by arrowheads) were 12.7 ml (huVHAm302), 12.4 ml (huVHAm302S), 15.3 ml (huVHAm427), 14.6 ml (huVHAm427S), 12.3 ml (huVHAm431), 12.2 ml (huVHAm431S), 13.3 ml (huVHPC235) and 13.6 ml (huVHPC235S). Peaks to the left of monomeric peaks were considered to be aggregate peaks. huVHAm427 monomeric peak shows tailing (marked by an arrow). (d) SPR analysis of VH binding to a Ni²⁺-NTA sensorchip. A control llama VHH monomer (A4.2; Hussack et al., 2011) and a control VH dimer (Baral et al., 2012) were also included in the analysis.

Table II. Biophysical properties of VHs

<table>
<thead>
<tr>
<th>VH</th>
<th>$T_m$ (°C)</th>
<th>$\Delta T_m$ (°C)</th>
<th>Fraction refolded</th>
<th>$K_D$ (μM)</th>
<th>%Aggregate</th>
<th>%Recovery</th>
<th>MW_fwd (kDa)</th>
<th>MW_exp (kDa)</th>
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</thead>
<tbody>
<tr>
<td>huVHAm302</td>
<td>53.8 ± 0.1</td>
<td>17.6 ± 0.22</td>
<td>0.60 ± 0.12</td>
<td>3</td>
<td>13.9 ± 0.02</td>
<td>69 ± 0.6</td>
<td>15.56 ± 0.04</td>
<td>18.33 ± 0.04</td>
</tr>
<tr>
<td>huVHAm302S</td>
<td>71.4 ± 0.2</td>
<td>0.46 ± 0.07</td>
<td>10</td>
<td>4</td>
<td>4.1 ± 0.15</td>
<td>76 ± 0.1</td>
<td>15.43 ± 0.03</td>
<td>16.73 ± 0.75</td>
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<td>huVHAm427</td>
<td>73.0 ± 0.3</td>
<td>16.2 ± 2.6</td>
<td>0.84 ± 0.04</td>
<td>1.6</td>
<td>3.8 ± 2.9</td>
<td>NA</td>
<td>14.52 ± 0.03</td>
<td>16.35 ± 0.03</td>
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<tr>
<td>huVHAm427S</td>
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<td>0.75 ± 0.11</td>
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<td>5</td>
<td>6.6 ± 0.76</td>
<td>78 ± 0.3</td>
<td>14.39 ± 0.03</td>
<td>15.27 ± 0.53</td>
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<td>huVHAm431</td>
<td>71.4 ± 0.4</td>
<td>16.1 ± 0.45</td>
<td>0.45 ± 0.02</td>
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<td>5.5 ± 0.08</td>
<td>82 ± 0.5</td>
<td>15.43 ± 0.03</td>
<td>17.95 ± 0.62</td>
</tr>
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<td>87.5 ± 0.2</td>
<td>0.89 ± 0.07</td>
<td>8</td>
<td>4</td>
<td>4.3 ± 0.14</td>
<td>100 ± 1.9</td>
<td>15.62 ± 0.03</td>
<td>17.05 ± 0.30</td>
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<td>huVHPC235</td>
<td>59.1 ± 0.2</td>
<td>13.9 ± 0.20</td>
<td>0.58 ± 0.05</td>
<td>0.3</td>
<td>0 ± 0.02</td>
<td>70 ± 3.7</td>
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<td>15.03 ± 0.09</td>
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<tr>
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<td>3</td>
<td>0</td>
<td>0.6 ± 0.09</td>
<td>105 ± 3.6</td>
<td>14.97 ± 0.03</td>
<td>16.11 ± 0.3</td>
</tr>
</tbody>
</table>

*Mean ± SEM.
*bEstimated minimum $T_m$.
*cEstimated minimum $\Delta T_m$.
*dFraction refolded is the fraction folded value for the VH at 30°C following thermal denaturation at 96°C.
*eRecovery was determined as described in Supplementary Fig. S4 legend.
*fMW measurement was performed once.

NA, not applicable.
V1Hs were more protease resistant than huVHAm302S and huVHPC235S. Previous studies showed that V1Hs became protease resistant with Cys54–Cys78 disulfide linkage mutations, and that there was a positive correlation between protease resistance of V1Hs and their Tms (Hussack et al., 2011).

**Mutant V1Hs are less aggregation prone than the wild-type counterparts**

Next, we investigated if the mutant V1Hs with improved thermostability were also less aggregation prone. The effect of Cys54–Cys78 disulfide linkage mutation on protein aggregation has not been explored in the case of camelid V1Hs, presumably because aggregation is not an issue with V1Hs. We assessed the aggregation behavior of V1Hs by Superdex™ 75 size exclusion chromatography. Typically, aggregating V1Hs form significant amounts of multimeric/aggregating species which are distinguishable from monomeric species by their lower elution volumes on Superdex™ 75 size exclusion chromatograms and/or have a tendency to ‘tail’ to the chromatography system surfaces, e.g. column materials, resulting in lower elution recoveries and/or monomeric profiles characterized by ‘tailing’. Non-aggregating V1Hs, on the other hand, display single, symmetrical monomeric peak profiles. We found that the engineered disulfide linkage reduced V1H aggregation (Fig. 2c; Table II). The wild-type V1Hs and huVHAm302 and huVHAm431 aggregated at 13.9% ± 0.02% and 5.5% ± 0.08%, respectively, which was reduced to 4.1% ± 0.15% and 4.3% ± 0.14% for the mutants (unpaired t-test, two-tailed, P = 0.0002 [huVHAm302/huVHAm302S pair]; P = 0.0181 [huVHAm431/huVHAm431S pair]). In the instance of huVHAm427/huVHAm427S pair, although the mutant did not show significant improvement in non-aggregation over the corresponding wild type (6.6% ± 0.76% vs. 3.8% ± 2.9%; unpaired t-test, two-tailed, P = 0.4470), the severe tailing seen in the case of the wild-type V1H was rectified by the introduction of the disulfide linkage in the mutant which displayed a symmetrical monomeric peak (Fig. 2c). Clearly, this tailing cannot be due to the presence of other proteins in the V1H preparation as it can be inferred from the SDS-PAGE profile of huVHAm427 (Supplementary Fig. S1). Moreover, %recovery (of monomeric species) significantly increased from 69% ± 0.6% (huVHAm302) and 82% ± 0.5% (huVHAm431) to 76% ± 0.1% and 100% ± 1.9% for mutant versions, respectively, indicating the mutants had significantly reduced stickiness (unpaired t-test, two-tailed, P = 0.0070 [huVHAm302/huVHAm302S pair] and P = 0.0107 [huVHAm431/huVHAm431S pair]) (Supplementary Fig. S4; Table II). Even in the case of huVHPC235, which displayed a purely monomeric profile (Fig. 2c), the introduction of the non-canonical disulfide linkage increased %recovery from 70% ± 3.7% (wild type) to 105% ± 3.6% (mutant) (unpaired t-test, two-tailed, P = 0.0212).

We confirmed by SPR and multiangle light scattering analyses that the monomeric peaks were indeed so despite their wide elution volume (Ve) variations (Fig. 2c: Ve = 12.2–15.3 ml). The SPR analysis was based on the affinity of the His tag for Ni²⁺ and the observation that, because of avidity effects, proteins with more than one His tag have much slower koff than those with one His tag (Nieba et al., 1997; Khan et al., 2006). Thus, a monomeric His₆-tagged V1H (with one His₆ tag) is expected to have a faster koff from a Ni²⁺ surface than a multimeric His₆-tagged V1H (with multiple His₆ tags). In SPR control experiments, a purely monomeric llama V1H with one C-terminal His₆ tag gave koff of 2.71 ± 0.01 × 10⁻³ and 2.18 ± 0.01 × 10⁻³ at early and later windows of the dissociation phase when passed over a Ni²⁺-immobilized sensorchip, while a purely dimeric V1H with two C-terminal His₆ tags (Baral et al., 2012) gave koff of 6.83 ± 1.04 × 10⁻⁵ and 4.21 ± 3.92 × 10⁻⁶ for the same dissociation phase windows, reflecting a 250- to 2000-fold slower koff (Fig. 2d). All the C-terminally His₆-tagged V1Hs corresponding to monomeric peaks on size exclusion chromatograms gave koff very similar to that of the monomeric V1H control, confirming their monomeric state (Fig. 2d). The SPR results were further confirmed by multiangle light scattering experiments, where it was shown that the calculated molecular weights associated with monomeric peaks (MWexp) were very close to their corresponding expected, formula molecular weights (MWfo) (Table II). The molecular weights of the monomer and dimer controls were also confirmed by multiangle light scattering experiments (16.50 ± 0.35 kDa vs. 15.73 kDa [MWfo] for the monomer; 35.83 ± 1.12 kDa vs. 31.05 kDa [MWfo] for the dimer).

Reversibility of thermal unfolding, measured in terms of fraction refolded values (Fig. 2a; Supplementary Fig. S3; Table II), was not compromised by the introduction of the engineered disulfide linkage for three of four V1Hs (unpaired t-test, two-tailed; P = 0.4333 for huVHAm302 vs. huVHA m302S; P = 0.5412 for huVHAm427 vs. huVHAm427S; P = 0.2169 for huVHPC235 vs. huVHPC235S), and in the case of huVHAm431S, was significantly improved from 0.45 (huVHAm431) to 0.89 (huVHAm431S) (P = 0.0268), indicating huVHAm431S acquired reduced tendency to aggregate (Barthelemy et al., 2008). Thermal unfolding of mutant V1Hs though not completely but to a large extent was reversible (fraction refolded = 0.46–0.89). However, it should be noted that fraction refolded values may be an overestimation as they may have contributions from non-active misfolded V1Hs. Voltage values obtained on V1H samples during CD measurements and visual inspection of the samples following CD measurements indicated the absence of any insoluble aggregates (Supplementary Fig. S5) (Benjwall et al., 2006). The incomplete reversibility of thermal unfolding may therefore have to do, at least in part, with the formation of soluble aggregates. We also investigated the tendency of V1Hs to form insoluble aggregates by turbidity analysis (Dudgeon et al., 2012). Turbidity measurements showed that similar to a non-aggregating V1H control (A4.2), all heat-treated (80°C, 20 min) wild type and mutants V1Hs completely resisted (insoluble) aggregation. However, the formation of soluble aggregates and/or misfolded species cannot be excluded as suggested by the size exclusion chromatography and thermal unfolding data.

**Mutant V1Hs have altered conformations compared with the wild-type counterparts**

Previously, it was shown that the binding affinity and specificity of V1Hs were altered with Cys54–Cys78 disulfide linkage mutations, suggesting that the engineered disulfide linkage altered the V1H conformational structures (Chan et al., 2008; Saerens et al., 2008; Hussack et al., 2011). To verify if the same is true for V1Hs, we used protein A to probe V1H conformation (Starovasnik et al., 1999; Graille et al., 2000) by determining the protein A equilibrium dissociation constants (Kₐₐₕ) for wild type and mutant V1Hs. We
found by SPR experiments that, compared with wild-type V18S, mutant V18S bound to protein A with lower affinity, indicating that the engineered disulfide linkage altered VH conformation (Fig. 3; Supplementary Fig. S6; Table II). The affinity reductions ranged from 2-fold for the huVHAm431/huVHAm431S pair to 10-fold for the huVHPC235/huVHPC235S pair. As reported previously for VHHs, the huVHAm431 pair to 10-fold for the huVHPC235


di- 

meric capacity, indicating that similar to the conserved Cys23–Cys104 disulfide linkage, the formation of the Cys54–Cys78 disulfide linkage is not interfered with by the presence of CDR1 and CDR3 cysteines with disulfide linkage forming capacity, indicating that similar to the conserved Cys23–Cys104 disulfide linkage, the formation of the disulfide linkage between Cys54 and Cys78 is highly favorable (Table I). We have also shown that the introduction of the Cys54–Cys78 disulfide linkage significantly increases the thermostability of V18S, as shown by T_m increases of at least 14°C, without adversely affecting expression yields. Similar thermostability gains were also seen in the case of several mutant V18HS with the same non-canonical disulfide linkage (Hagihara et al., 2007; Chan et al., 2008; Saerens et al., 2008; Hussack et al., 2011). In a more recent publication, it was shown that the presence of Cys54–Cys78 disulfide linkage additionally improved the protease resistance of V18Hs (Hussack et al., 2011). Thus, it is very likely that the same beneficial effect exists in the case of VH domains. From the point of view of engineering for stability, the present approach appears to be generally applicable not only to V18S and V18HS, but also to V18S, as the introduction of Cys pairs at equivalent positions in V18S also led to the formation of the intended disulfide linkage and significant increases in thermostability and protease resistance (manuscript in preparation).

We found that the engineered disulfide linkage reduced aggregation of VH domains, a significant finding considering that VH domains have the general drawback of being aggregation prone. It is likely that the improved non-aggregation of VH mutants compared with wild-type V18S is due to their increased thermodynamic stability and/or having aggregation resistant unfolded states (Hussack et al., 2011). However, the VH domains in this study were based on the same VH framework regions and shared a very high percentage of sequence identity. Thus, it remains to be seen if the beneficial effect of the engineered disulfide linkage in terms of improving non-aggregation is general across VH domains with differing framework regions. Barthelemy et al. (2008) identified non-aggregating VH domains whose non-aggregation was independent of CDR3 sequence and derived from mutations in the V_i interface. However, it is very unlikely that solubilizing framework region mutations alone, including those presented in this study, can accommodate the diversity of CDR sequences (or even just CDR3 sequences) encountered in VH libraries. In other words, non-aggregation (of VHs) is a function of both framework region sequence and CDR sequence (Martin et al., 1997; Ewert et al., 2003; Christ et al., 2007), and it is very likely that synthetic VH libraries would always be populated to various degrees with aggregating V18S. Thus, coupling selection for affinity to selection for non-aggregation during the panning stage of library selections (Jespers et al., 2004; Famm et al., 2008) is advisable even when dealing with VH libraries enriched for non-aggregating domains (Christ et al., 2007), as the approach increases the likelihood of obtaining non-aggregating VH binders.

Using protein A as a structural sensor, we found that the introduction of the Cys54–Cys78 disulfide linkage led to conformational changes in the backbone of mutant V18S. This is conceivable as the engineered disulfide linkage connects, and possibly alters, the β-strands (C’ and D) which are presumably involved in protein A binding (Riechmann and Davies, 1995; Starovasnik et al., 1999). The differential effect of the engineered disulfide linkage on VH affinity, as also seen previously with V18HS (Hussack et al., 2011), suggests that the Cys54–Cys78 disulfide linkage alters the structure of V18S to a different extent. We cannot comment with certainty on the effect of the engineered disulfide linkage on antigen affinity of V18S since we were not able to obtain affinity values for wild-type V18S: in the case of huVHPC235 the antigen was not known, and in the case of amylase
binders huVHAm302, huVHAm427 and huVHAm431 (Arbabi-Ghahroudi et al., 2009b), the tendency of V$_{H}$S to aggregate precluded reliable affinity measurements by SPR. However, it is likely that the conformational changes observed through protein A measurements would transmit through paratopes leading to affinity and specificity compromises in V$_{H}$ binders as has been demonstrated with VHHs (Chan et al., 2008; Saens et al., 2008; Hussack et al., 2011). Thus, to avoid this drawback, one may start the selection for binders from synthetic V$_{H}$ libraries—which are, after all, the source of V$_{H}$ binders—that already have the Cys54–Cys78 disulfide linkage feature. Such V$_{H}$ libraries which would be generated by CDR randomization on stable scaffolds with the Cys54–Cys78 disulfide linkage mutation should also be a richer source of binders with characteristics such as high expression, thermostability, protease resistance and non-aggregation compared with the same libraries built on the wild-type scaffolds. Furthermore, the efficiency of isolating binders with such desirable characteristics would increase, should the selection be performed under stability pressure as has been successfully demonstrated (Jespers et al., 2004; Famm et al., 2008; Arbabi-Ghahroudi et al., 2009b).

We demonstrated that an SPR-based assay based on the Ni$^{2+}$–His$_6$ tag interaction, and involving flowing His-tagged V$_{H}$S over Ni$^{2+}$-immobilized sensorchip surfaces, can be employed to distinguish between monomeric and multimeric V$_{H}$S. The approach can be used as a complement or alternative to size exclusion chromatography for assessing the aggregation status of V$_{H}$S or other antibody fragments. As the screening may be applied directly to un-purified VH samples in cell extracts since the His-tagged protein at a concentration as low as 50 nM, it should be applicable to high-throughput screening of non-aggregating VHs (or other His-tagged proteins) expressed on a small scale. Moreover, the screening may be applied directly to un-purified V$_{H}$ samples in cell extracts since the His-tagged protein purification should occur as the cell extracts flow through the Ni$^{2+}$ sensorchip surfaces.

Taken together, our study presents a novel approach for eficacy engineering of V$_{H}$-based biologics. Given the structural similarities between V$_{H}$S and V$_{L}$S, the approach should be applicable to V$_{L}$-based biologics as well. Furthermore, libraries based on the V$_{H}$ scaffold with the Cys54–Cys78 disulfide linkage feature—in particular those based on the highly thermodynamically stable human V$_{H}$3 family sequences (Ewert et al., 2003)—should provide a wider depth and breadth in terms of affinity and specificity range compared with the same libraries based on the wild-type V$_{H}$ Scaffold due to the higher stability and the reduced loss of library members to aggregation.

**Supplementary data**

Supplementary data are available at PEDS online.

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**References**


Stable V_{H} domains with extra disulfide linkage
