Improving solubility and refolding efficiency of human V_Hs by a novel mutational approach

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The antibody V_H domains of camelids tend to be soluble and to resist aggregation, in contrast to human VH domains. For immunotherapy, attempts have therefore been made to improve the properties of human V_Hs by camelization of a small set of framework residues. Here, we have identified through sequence comparison of well-folded llama V_H domains an alternative set of residues (not typically camelid) for mutation. Thus, the solubility and thermal refolding efficiency of a typical human V_H, derived from the human antibody BT32/A6, were improved by introduction of two mutations in framework region (FR) 1 and 4 to generate BT32/A6.L1. Three more mutations in FR3 of BT32/A6.L1 further improved the thermal refolding efficiency while retaining solubility and cooperative melting profiles. To demonstrate practical utility, BT32/A6.L1 was used to construct a phage display library from which were isolated human V_Hs with good antigen binding activity and solubility. The engineered human V_H domains described here may be useful for immunotherapy, due to their expected low immunogenicity, and in applications involving transient high temperatures, due to their efficient refolding after thermal denaturation.

Keywords: human V_H/immunogenicity/mutation/phage display library/solubility and refolding

Introduction

Although the utility of V_Hs (i.e. IgG heavy chain variable domains) as recombinant antibodies was demonstrated in 1989 (Ward et al., 1989), it was not until the discovery of camelid heavy chain antibodies (HCAs) (Hamers-Casterman et al., 1993) that V_Hs, in particular human V_Hs, began to receive serious attention (Holliger and Hudson, 2005). This is mostly because HCAs provided a solution for poor V_H solubility (Ward et al., 1989), which had triggered a setback in pursuing applications of V_H proteins. For therapeutic applications, human V_Hs are preferred over the V_Hs derived from HCAs (i.e. V_Hs) because they are potentially less immunogenic (Holt et al., 2003). However, in contrast to V_Hs, which are highly soluble and exist essentially as monomeric proteins, human V_Hs typically form high molecular weight aggregates in solution (Ward et al., 1989; Davies and Riechmann, 1995; Jespers et al., 2004a). Moreover, human V_Hs demonstrate far less efficient refolding following thermal denaturation than V_Hs (Perez et al., 2001; Ewert et al., 2002). Amino acid substitutions V37F/Y, G44E/Q, L45R/C and W47G/S/L/F play a prominent role in V_H solubility (Harmsen et al., 2000; Muyldermans et al., 2001). It was suggested that the efficient thermal refolding efficiency of V_Hs is mediated by the same substitutions that are responsible for V_H solubility (Ewert et al., 2002). In terms of stability, as measured by melting temperatures (T_m) and denaturant-induced unfolding profiles, V_Hs are comparable to V_Hs and, in fact, those based on human consensus V_H3 domain show significantly higher stability than V_Hs (Davies and Riechmann, 1996a; Perez et al., 2001; Dumoulin et al., 2002; Ewert et al., 2002).

Camelization involving mutation of the residues at positions 44, 45 and 47 in human V_Hs to those commonly found in V_Hs has generated soluble human V_H proteins (Davies and Riechmann, 1994; Tanha et al., 2001). This strategy, however, does not always result in soluble V_Hs (Martin et al., 1997; Voordijk et al., 2000) and it is becoming clear that residues at other positions and in the CDR3 sequence also play a role in V_H/V_H solubility (Spinelli et al., 1996; Reiter et al., 1999; Tanha et al., 2002; Vranken et al., 2002; Dottorini et al., 2004). In addition, it is not known whether camelization would also generate V_Hs with good thermal refolding efficiencies. Such refoldable V_Hs would have high efficacy in settings where they experience transient denaturing temperatures (Holt et al., 2003).

We recently showed that several V_Hs, derived from llama IgGs, had characteristics associated with camelid V_Hs in that they were highly soluble and demonstrated reversible thermal denaturation, despite the absence of ‘camelid’ residues at positions 37, 44, 45 and 47 (Tanha et al., 2002; Vranken et al., 2002) or a W103R mutation (Conrath et al., 2001). Based on framework region (FR) sequence comparison of these V_Hs and the human V_H3 family consensus sequence (Riechmann and Muyldermans, 1999), to which the llama V_H3 showed the highest sequence homology, five amino acid substitutions, E6A (primer-forced mutation), S74A, R83K, A84P and L108Q, were proposed to be responsible for the high solubility and refolding efficiency of these llama V_Hs. Here we show that V_H mutations, involving the above five substitutions, can be used to create human V_H proteins with improved solubility and thermal refolding efficiency and good antigen-binding activity.

Materials and methods


Standard PCR, which utilized mutagenic primers, was employed to construct BT32/A6.L1 and BT32/A6.L2 from the human antibody BT32/A6 (Tanha et al., 2001). BT32/A6.L1 has five mutations with respect to BT32/A6 (S23A, S82AN, V93A, E6A, T108Q) and BT32/A6.L2 has three additional mutations with respect to BT32/A6.L1 (S74A, R83K, A84P) (Figure 1). The mutants were cloned in pSJF2 expression plasmid (Tanha et al., 2003), followed by transformation of Escherichia coli strain TG1 using standard cloning techniques.
The numbering system is used (Kabat et al., 1989). Clones harboring the BT32/A6.L1 and BT32/A6.L2 genes were identified by PCR and DNA sequencing.

**Library construction and panning**

To construct the BT32/A6 library, the BT32/A6 gene was used as the template in PCR to amplify a shorter fragment using FR1- and FR3-specific primers. The product was gel purified using the QIAquick Gel Extraction™ kit (QIAGEN Inc., Mississauga, ON, Canada) and extended by PCR using the FR1-specific and 5'-GCCCCAGATATCAA[A/(A/C)NN]25TTTCCACACGATAA-3' primers. The resultant PCR fragments with randomized CDR3s were purified using the QIAquick PCR Purification™ kit (QIAGEN Inc.) and the full-length V_H genes were constructed by PCR using FR1-specific primer and a primer that added the FR4 codons. In the case of the BT32/A6.L1 library, the BT32/A6.L1 gene was used as the initial template, and CDR3 was randomized using the primer 5'-CCCTTGCCCAGATATCAA-A/(A/C)NN]5GTAATAACCACTACTATC-3'. Cloning of the library was confirmed by batch sequencing of VH genes. For the BT32/A6.L1 library V_H genes in a phage vector, transformation, library size determination, and phage propagation and purification were performed as described (Tanha et al., 2002). The integrity of the library was confirmed by batch sequencing of V_H genes. Panning and subsequent screening of the phage clones by enzyme-linked immunosorbent assays (ELISA) and DNA sequencing were performed as described (Tanha et al., 2002). For panning with human alpha-thrombin, the thrombin active site was blocked with an excess amount of D-Phe-Pro-Arg-CH2Cl (PPACK) (Calbiochem, Mississauga, ON, Canada). Specific elution of phages that bound to the fibrinogen-recognition site of thrombin was carried out by competitive binding with the hirudin-derived peptide hirudin54-65 at 1 mM concentration (Bachem, King of Prussia, PA). Subcloning of the V_H fragments from the phage vector into pSJF2 expression plasmid for expression purposes was performed as described (Tanha et al., 2003).

**Protein analyses**

V_Hs were expressed and purified (Tanha et al., 2001) and their concentrations were determined by OD280 measurements using molar absorption coefficients calculated for each protein (Pace et al., 1995). Gel filtration chromatography using a Superdex 75 column (GE Healthcare, Baie d’Urfe, QC, Canada) was performed as described (Deng et al., 1995). ts/50 were determined at a protein concentration of 2 µg/ml as described for PTH50 (Zhang et al., 2004). Refolding experiments were performed in 10 mM sodium phosphate buffer pH 7.0 using circular cuvettes with 1 cm pathlengths. Spectra were recorded and processed as described for ts/50 measurements (Zhang et al., 2004).

To determine the refolding efficiency, RE, protein samples were equilibrated at 30°C (native) then at 85°C (fully unfolded) for 20 min and the spectra were obtained in each instance. The samples were cooled to room temperature for 70 min, re-equilibrated to 30°C and the spectrum was obtained. The RE was calculated from

\[ RE = \frac{R - U}{U - N} \times 100 \]

where, N and U represent the CD signals of the native and fully unfolded proteins at 30°C and 85°C, respectively, and R is the CD signal of the protein obtained at 30°C following its unfolding at 85°C. Refolding efficiencies were calculated as the average of two measurements at 235 nm.

The binding kinetics for the interaction of the purified FlagL1 V_H to immobilized anti-FLAG M2 monoclonal antibody (Sigma, Oakville, ON, Canada) were determined by surface plasmon resonance using a BIACORE 3000 biosensor system (Biacore, Inc., Piscataway, NJ). A total of 1900 resonance units of M2 and 598 resonance units of ovalbumin as a reference surface were immobilized on a research grade column chromatography to remove any trace of aggregates prior to the binding analysis. All measurements were carried out at 25°C in 10 mM HEPES buffer pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% P20 at a flow rate of 40 µl/min. Surfaces were regenerated by washing with running buffer. Data were evaluated using the BIAsevaluation 4.1 software (Biacore, Inc.).

**Fibrinogen clotting assays**

Fibrinogen clotting assays were performed with 0.1% bovine plasma fibrinogen (Sigma, Oakville, ON, Canada) in 50 mM Tris–HCl, pH 7.6, 100 mM NaCl, 0.1% poly(ethylene glycol) 8000, at 37°C. Assay mixtures contained different concentrations of V_H and were incubated at 37°C for 2 min. The reaction was initiated by the addition of human α-thrombin

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Fig. 1. Amino acid sequences of BT32/A6 V_H and its derivatives. The dots in the sequence entries indicate amino acid identity with BT32/A6. The Kabat numbering system is used (Kabat et al., 1991).
15N-labeled VHs were produced in bacterial cultures grown in NMR spectroscopy medium containing 1.0 g/l of [15N]ammonium sulfate and 2.0 g/l of glucose, supplemented with 1 mM MgSO₄, 0.15 mM CaCl₂, 0.00005% vitamin B1 and 100 µg/ml ampicillin. Purified proteins were concentrated and exchanged into PBS buffer, pH 6.5 by ultrafiltration. Protein samples contained ~0.08 mM of uniformly 15N-labeled protein and 10% D₂O. NMR experiments were performed at 298 K on a Bruker Avance-500 NMR spectrometer equipped with a 5 mm triple-resonance CryoProbe. Two-dimensional 1H–15N HSQC spectra were acquired using solvent suppression via the WATERGATE method (Sklenar et al., 1993). NMR data were processed and analyzed using the Bruker XWINNMR software package. For HSQC titration experiments, aliquots of PPACK-thrombin at a concentration of 3.5 mg/ml were added to the VH protein samples.

Results

It was recently shown that a set of llama VHs demonstrated high solubility and reversible thermal denaturation (Tanha et al., 2002; Vranken et al., 2002). These characteristics were attributed to five amino acid substitutions, E6A, S74A, R83K, A84P and L108Q, based on FR sequence comparison of these VHs and the human VH3 family consensus sequence (Riechmann and Muyldermans, 1999) to which the llama VHs showed the highest sequence homology. We decided to investigate whether the aforementioned mutations would create human VH proteins with improved biophysical properties. For our studies we chose the VH from the human immunoglobulin M monoclonal antibody BT32/A6 (Dan et al., 1995) since it belongs to the human VH3 family (Figure 1). In addition, the solution properties of BT32/A6 VH and a number of its camelized derivatives have been studied extensively (Tanha et al., 2001). However, an FR-based sequence comparison of BT32/A6 VH and the human VH3 consensus sequence revealed that BT32/A6 VH deviated from the consensus sequence at positions 23 in FR1 and 82a and 93 in FR3. Consequently, the human VH3 consensus sequence was restored in BT32/A6 through S23A, S82aN and V93A mutations. Two more mutations, E6A and T108Q, were introduced to construct the first mutated version, BT32/A6.L1 (Figure 1). Previously, a VH mutation from Gln at position 108 in a murine scFv resulted in a 30-fold increase in the soluble product and significantly higher stability during storage (Kipriyanov et al., 1994). As controls, BT32/A6.ERI, a camelized derivative of BT32/A6 with G44E/L45R/Y47I mutations (Figure 1), and PTH50, a llama VH (Zhang et al., 2004) were included. The solution properties of BT32/A6.ERI had been shown to be excellent as it remained soluble at high concentration and exhibited sharp NMR spectra (Tanha et al., 2001). However, its refolding efficiency, although higher than the wild type, is significantly lower than the mutated versions BT32/A6.L1 and BT32/A6.L2, i.e. 27%. PTH50, as expected, shows the highest refolding efficiency, 95%. The modest increase in refolding efficiency of BT32/A6.ERI relative to the wild type is inconsistent with the hypothesis that attributes the high thermal refolding efficiencies of VHs to the presence of ‘camelid’ residues at positions 37, 44, 45 and 47 (Ewert et al., 2002). At a lower VH concentration (2 µg/ml), the refolding efficiencies of the VHs significantly increased and there was no longer a significant difference between BT32/A6.L1 and BT32/A6 refolding efficiencies: BT32/A6, 58%; BT32/A6.L1, 94%; BT32/A6.L2, 90%. This increase in refolding efficiencies (increase in production of the native fold) at the lower concentration is presumably due to a concomitant decrease in the amount of multimer and aggregate VHs whose formation follows second- and higher-order reaction kinetics and thus is very dependent on the substrate (unfolded VH) concentrations. The Ts values of VHs increased, although marginally, with the same substitutions that also increased solubility and refolding efficiency. In all instances, a single denaturation transition indicated a two-state behavior. The Ts values increased from 62.4°C for the wild type to 63.4°C for BT32/A6.L1.
and to 64.4°C for BT32/A6.L2 (Figure 2c). The $T_m$ for BT32/A6.ERI was intermediate between BT32/A6.L1 and BT32/A6.L2 (63.7°C). PTH50, which had the highest refolding efficiency, had the lowest $T_m$ (59.7°C), confirming the previous finding that VHHs are characterized by high thermal refolding efficiencies but not necessarily high $T_m$s (Ewert et al., 2002).

To assess its suitability as library scaffold, BT32/A6.L1 was used to construct a synthetic phage display library in which CDR3 residues were randomized. The choice of BT32/A6.L1 as opposed to BT32/A6.L2 as the library scaffold was based on the fact that while BT32/A6.L1 was not quite as soluble as BT32/A6.L2, it had fewer of its original residues replaced with ‘non-human’ residues. Consequently, being ‘more human’ and thus potentially less immunogenic, BT32/A6.L1 should be more favorable for immunotherapy. A reference library based on the BT32/A6 scaffold was also constructed. To assess the integrity of the libraries, colony PCR and DNA sequencing of the library clones were performed. In the instance of the BT32/A6 library, out of 36 colonies analyzed by PCR and agarose gel electrophoresis, 16 did not have insert, 5 had truncated inserts and 15 had full-length inserts with authentic open reading frames as determined by DNA sequencing. For the BT32/A6.L1 library, 38 out of the 40 colonies analyzed had complete VH sequences with 2 having no VH. After normalization for full-length VHs, the sizes of both libraries were determined to be $2.5 \times 10^8$.

In the case of the BT32/A6 library, up to four rounds of panning under different conditions were performed against two protein targets including anti-FLAG M2 a monoclonal antibody which recognizes the FLAG motif XYKXXD (Tanha et al., 2001). For each target, PCRs on 150 colonies from various rounds showed enrichment for truncated VHs. The predominance of the truncated VHs over full-length VHs is thought to be due to their growth advantage. This predominance is further accentuated by the instability of the full-length VHs which compromises their functionality and hence selection during the binding steps of panning.

By contrast, the library based on BT32/A6.L1 was very stable as there was enrichment for full-length VH binders during panning against two single-chain antibodies H11 and Yst9.1 (Reilly et al., 2001; Tanha et al., 2002), M2 monoclonal antibody and human thrombin with a blocked active site. Following three rounds of panning against the two single-chain antibodies, more than 90% of the phage clones tested positive in phage ELISA. The binding, measured in terms of OD$_{450}$, was 0.4–1.7 for the positive clones compared to
Table 1. CDR3 sequences of anti-Yst9.1 and anti-H11 V_H binders identified by panning the BT32/A6.L1 library

<table>
<thead>
<tr>
<th>Yst9.1 binders</th>
<th>H11 binders</th>
</tr>
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<tbody>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>SRWSEFGDI</td>
<td>YSFSSPFDI</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>SNWSSFGDI</td>
<td>NLFSFPFDI</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>SRWSSFGDI</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>RRRWMEGFDI</td>
<td>SEFSSPFDI</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>REWMSGFDI</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>KFAKSSFDI</td>
<td>DWGSWFDI</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>FNKRLFDFI</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>SDNIGFDI</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>AVMGNFDI</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>GMLSTTFDI</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>TERSNTFDI</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>SEKLSGFDI</td>
<td>DRLKVEYYDSSGYY</td>
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<tr>
<td>DRLKVEYYDSSGYY</td>
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</tr>
<tr>
<td>TLPGDFDI</td>
<td>DRLKVEYYDSSGYY</td>
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<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
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<tr>
<td>GLGGTFFDI</td>
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</tr>
<tr>
<td>NGLNSTFDI</td>
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</tr>
<tr>
<td>DRLKVEYYDSSGYY</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
<tr>
<td>?QPSPEFDI</td>
<td>DRLKVEYYDSSGYY</td>
</tr>
</tbody>
</table>

The randomized region is shown in bold. Dashes denote deletion and ‘?’ sequence ambiguity.

0.030–0.040 for the phage clones displaying no V_H. Batch sequencing revealed more than a dozen different V_H binders in each instance with several V_H sets having the same motif (Table I). For example, the sequence motif SRWSS/EG exists in the first four Yst9.1 binders and the sequence FSSP is part of the paratope of the first five H11 binders in Table I.

In the case of M2, 12 different V_H binders with the expected XYKXXD recognition motif were identified (Tanha et al., 2001). One of the binders, FlagL1 V_H with the recognition sequence DYKRFD, was subcloned into an expression vector and purified in milligram quantities (Figure 3a). Analysis by size exclusion chromatography showed that the protein was completely monomeric (Figure 3b inset). The V_H was characterized by surface plasmon resonance and the data for the binding of FlagL1 to immobilized M2 were fitted to a simple 1:1 interaction model. Global analysis of the data gave an association rate constant of $4 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ and a dissociation rate constant of $2 \times 10^{-1} \text{s}^{-1}$. From these rate constants the dissociation constant of the interaction was determined to be 5 $\mu$M. This compares with an anti-M2 V_H previously isolated from a cameldized human V_H library with a $K_D$ of 1 $\mu$M (Tanha et al., 2001), and where the tighter binding is likely due to a better binding sequence (EYKDFD).

In the case of thrombin, 64% (general elution) and 36% (specific elution) of the third round clones tested were positive in ELISA. Of the five different V_H binders that were identified and subsequently expressed as soluble V_Hs, four were obtained by specific elution (Figure 4a, PEPC1-4). These had at least two acidic residues separated by bulky hydrophobic residues in their CDR3s, a sequence feature characteristic of protein cofactors or inhibitors targeting the fibrinogen-recognition exosite of thrombin (Rose and Di Cera, 2002; Pechik et al., 2004). When tested for anti-thrombin activity in a thrombin-induced fibrinogen clotting assay, two of the four V_Hs, i.e. PEPC2 and PEPC3, caused significant delays of the fibrinogen clotting time at micromolar concentrations (Figure 4a and b). For example, at a concentration of 50 $\mu$M, PEPC2 and PEPC3 prolonged the clotting time from 25 to 45 s, indicating $IC_{50}$ values of significantly less than 100 $\mu$M. These relatively weak interactions were further characterized by NMR titration using HSQC to ascertain that the V_Hs had specific contacts with thrombin. The $^{1}H-$HN HSQC spectrum of PEPC2 responded to the titration of thrombin, wherein a subset of HSQC peaks underwent progressive shifts or broadening with increased concentrations of thrombin, indicating specific interactions between the two protein molecules (Figure 5).

Discussion

Our work shows that the properties of a typical human V_H domain (based on engineering consensus framework residues of the human antibody BT32/A6) can be transformed by two single mutations in each of FR1 and FR4. We thereby conferred on a human V_H favorable properties typically associated with camelid V_Hs, namely, good solubility and high thermal refolding efficiency. V_Hs with these properties are desirable in immunotherapeutics as homing agents to target disease molecules as well as in contexts involving transient denaturing temperatures. The gain in V_H solubility was neither at the expense of expression yield, as both mutated V_Hs maintained a high level expression of the parent V_H, nor at the expense of stability, as was shown by $T_{m}$ measurements. In fact, the mutated V_Hs had slightly higher $T_{m}$s than the wild-type V_H.

This is in sharp contrast to a previous example where V_H solubilization by camelization was accompanied by a drastic decrease in $T_{m}$ (Davies and Riechmann, 1996a).
Fig. 4. Anti-thrombin activities of the thrombin-binding V_H obtained from the BT32/A6.L1 library. (a) The CDR3 sequences of anti-thrombin V_Hs are shown with the randomized region underlined. (b) The anti-thrombin activity of PEPC2 V_H assayed by inhibition of fibrinogen clotting catalyzed by thrombin.

Fig. 5. Titration of 15N-PEPC2 with PPACK-thrombin monitored by NMR 1H–15N HSQC experiments. NMR spectrum in black, 0.08 mM 15N-PEPC2 in PBS, pH 6.5; in red, the same sample after addition of 4.4 μM PPACK-thrombin (molar concentration ratio of 18:1). Cross-peaks that underwent progressive shifts or broadening upon thrombin titration are boxed.
Our strategy may provide an alternative to camelization in instances in which camelization fails (Martin et al., 1997; Voordijk et al., 2000), and also provide stable VH scaffolds for synthetic protein libraries. Synthetic libraries constructed by randomizing one CDR are better suited as a source of peptideidiotypics than camel VH libraries (Davies and Riechmann, 1996b). Indeed, VH hits specific for human thrombin showed characteristic binding sequences common among many natural thrombin-binding proteins.

In the case of BT32/A6, BT32A6.L1 and BT32A6.L2, higher refolding efficiency appears to correlate with slight increases in \( T_m \), but this correlation does not hold true for the llama VH which has the lowest \( T_m \) but has by far the highest refolding efficiency. This, as mentioned previously, is in agreement with the finding that the VHs are characterized by high thermal refolding efficiencies but not always by high \( T_m \)s (Ewert et al., 2002). The mutations in BT32A6.L1 and BT32A6.L2 must have a stabilizing effect on the VH fold resulting in somewhat improved \( T_m \)s. However, the dramatic increases in refolding efficiencies of the mutated VHs relative to BT32A6 suggest that, more importantly, the mutations must stabilize folding intermediates which lead to the native structure. It seems that for the llama VH used in this study, by contrast, the residues resulting in high thermal refolding do not confer high \( T_m \)s. Based on the finding that the mutated BT32/ A6.ERI with camidel residues 44E, 45R and 47I had only a modest improvement in thermal refolding over BT32/A6 it may conclude that the camidel residues at the above-mentioned positions do not play a significant role in thermal refolding of VHs. On the other hand, this may be a rather simplistic view of the situation since the camidel residues can improve the thermal refolding properties of some VHs depending on the sequence context.

In conclusion, there appear to be several strategies for improving solubility and/or refolding characteristics. For example, mutations introduced into the CDRs have proved effective in increasing solubility (Jespers et al., 2004a) and/or refolding (Jespers et al., 2004b). Our work has shown that several FR residues (E6A, S74A, R83K, A84P and L108Q), identified earlier from a set of llama VHs (Tancah et al., 2002), can confer higher thermal refolding efficiencies on human VHs, and that a library comprising only two of these mutations (E6A and L108Q) can lead to the isolation of VH domains with excellent thermal refolding properties and antigen-binding activities.

Acknowledgments

We thank Doris Bilous for oligonucleotide synthesis, Anna Cunningham and Joe Michniewicz for DNA sequencing and Tomoko Hirama for biocore assistance. Ginette Dubuc. This is National Research Council of Canada publication 42510.

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Received July 26, 2006; accepted August 10, 2006

Soluble and refoldable human VHs