Single antibody domains as small recognition units: design and in vitro antigen selection of camelized, human VH domains with improved protein stability

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Folding stabilities of camelized human antibody VH domains were studied through the determination of their melting points in thermodesnaturatation experiments. The melting point of a VH domain originating from a synthetic library of human VHs, which had been optimized for the use as small recognition units through the mimicking of camelid antibody heavy chains occurring naturally without light chain, was 56.6°C compared with 71.2°C of the original human VH. Its stability was improved (melting point 61.6°C) through three mutations to mimic camelid VHs even further: Val37 was replaced by phenylalanine and two cysteines were introduced at positions 33 and 100b. The resulting VH folded properly and formed a second intradomain disulphide between the extra cysteines. The new mutations were then built constitutively into a phage-display VH library, from which antigen-specific VHs were selected. Two were analysed for stability with melting points of 72.6 and 75.3°C. Thus secondary camelization enabled the isolation of VHs with improved folding stabilities exceeding even that of the original human VH. This indicates an effect on folding stability for some mutations specific in the light chain lacking camelid heavy chains. Keywords: camel/circular dichroism/immunoglobulin/protein folding/phage display

Introduction

The display of (poly)peptides on the surface of phage through fusion with a phage coat protein, typically protein 3 of the fd phage, has permitted the in vitro selection of recognition units through binding to ligand. The complexity of recognition units used ranged from small peptides (e.g. Parmley and Smith, 1988) to multimeric proteins such as antibody Fab fragments (e.g. Hoogenboom et al., 1991). Through phage technology antibody fragments have now proved to be not only in vivo but also in vitro a polypeptide framework, which is well suited for the generation of recognition units to target ligands with a high degree of affinity and specificity (Griffiths et al., 1994).

However, the considerable size of intact antibodies (155 kDa) impairs their performance when diffusion limits the recognition process as in the targeting of solid tumours. To reduce these problems, minimum-sized antibody fragments still functional with respect to antigen binding were designed. To date the most widely accepted is the so-called Fv fragment, a heterodimer of antibody heavy (VH) and light (VL) chain variable domains (together 25 kDa). The Fv harbours both sets of three hypervariable loops from VH and VL that usually form the antibody combining site (Davies et al., 1990).

Recently, efforts were made to reduce the size of the minimum size antigen binding fragment even further. These focused on the use of single VH domains, because early experiments had indicated that heavy chains can retain a significant portion of the original antigen affinity in the absence of light chain (Utsumi and Karush, 1964). More recently, antigen binding VH domains were recovered from immunized mice (Ward et al., 1989), and camel antibodies naturally lacking a light chain partner were described (Hamers-Casterman et al., 1993).

These observations led to the design of a size reduced VH (Pessi et al., 1993) lacking the third complementarity determining region (CDR) and the flanking framework regions (FR) and to the design of single, human VH domains optimized for solubility by mimicking camelid heavy chain sequences (Davies and Riechmann, 1994). Synthetic repertoires of both designs generated through randomization of hypervariable loops were displayed on phage and successfully selected for specific target recognition (Martin et al., 1994; Davies and Riechmann, 1995a).

The attraction of single antibody domains compared with the multidomain antibody fragments was not only their size but also a potentially higher stability. Association of VH and VL domains was found to be weak in the case of some Fv fragments (Glockshuber et al., 1990). Although the two domains can be linked covalently through the use of a domain linking peptide (Huston et al., 1988) or an interdomain disulphide bridge (Glockshuber et al., 1990; Reiter et al., 1994), the use of a single antibody domain, as long as it recognizes its target with high affinity and specificity, seemed conceptually more attractive.

To this end, we investigated the folding stability of a camelized, human VH domain, improved it through further camelization and constructed a new phage-display library based on these modifications. From this library, antigen-specific VH domains were selected and two of them analysed for folding stability. These experiments might also help in understanding the function of mutations specific for the light chain lacking, camelid heavy chains.

Materials and methods

VH mutants

Mutants of the VH-Ox21 (Davies and Riechmann, 1995a) and VH-Ox13 (Davies and Riechmann, 1994) genes were prepared through a polymerase chain reaction (PCR) with appropriate oligonucleotides utilizing the XhoI site at the beginning of CDR1 and the NotI site at the end of FR4. The resulting mutants were checked for the correct sequence by double-stranded sequencing of the plasmid DNA.

Library construction

To prepare the VH-CC library, the 3’ portion of the VH-Ox21 gene was amplified by PCR using the oligonucleotides H3CYSRAN [5’-GCC CCA ATA GTC AAA (SNN)₄ ACA

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using a Jasco J720 spectropolarimeter connected to a Vigelin performed at a VH concentration of 10 μg/ml in PBS (pH 7) free cysteine residues. Circular dichroism (CD) analysis was assay (Ellman, 1959; Riddles et al., 1983) was used to detect yet al, the presence and absence of -mercaptoethanol. The Ellman Protein analysis instructions (Diagen). Eluted VH was dialysed against PBS Hoffmann and Roeder, 1991) according to the manufacturer’s VH domains were purified from the bacterial periplasm period at 30°C with 1 mM isopropyl β-D-thiogalactopyranoside. G47I mutations. For protein expression bacterial cultures were grown overnight at 37°C in rich medium containing ampicillin and Pelham, 1986) and six histidine residues. The isoleucine myc peptide tag (recognized by the antibody 9E10; Munro Notl site, a Pstl site at the end of FR2 except for the mutations A33C and V37F and the silent incorporation of a Xhol site into the codons for residues 29–31. Ligated DNA was electrophoresed into bacteria of the Escherichia coli strain TG1 and transformants were analysed for correct insert size through PCR screening with the oligonucleotides FDPDRBACK and FDSEQ1 (Hoogenboom et al., 1991).

**Phage selection**

Phage originating from the library VH-CC was selected on 2-phenyloxazol-5-one-conjugated bovine serum albumin (BSA) as described (Davies and Riechmann, 1995a). Selection for lysozyme binders was performed by mixing 0.5 ml of lysozyme (1 mg/ml resin) conjugated to CNBr-activated Sepharose 4B (Pharmacia) with phage (10¹⁰ transfection units) in 1 ml of 3% BSA in phosphate-buffered saline (PBS) solution. After tumbling for 2 h at room temperature, the mixture was washed with 10 ml of PBS, 10 ml of PBS containing 0.1% Tween-20 and another 10 ml of PBS. Bound phage was eluted with 1 ml of triethylamine (0.1 M) and immediately neutralized. Reamplification of phage for the following rounds of selection and the subsequent ELISA were performed as described (Davies and Riechmann, 1995a).

**Protein preparation**

VH genes were subcloned as Xhol/Nol fragments into the vector pUC-VH-his for expression of soluble protein. This vector harbours within its pUC19-polylinker sites a pelB leader sequence, the 5’ portion of VH-Ox21 (including the Xhol site at the beginning of CDR1) up to a Pstl site at the end of FR2 (Davies and Riechmann, 1995a), stuffer DNA, a Nol site, a myc peptide tag (recognized by the antibody 9E10; Munro and Pelham, 1986) and six histidine residues. The isolateucine 47 version of VH-Oc6 was prepared through subcloning of its Pstl/Nol fragmnet into a pUC based vector identical with fd-cys to fuse it with the gene 3 coat protein. Fd-cys is identical to fd-47G (Davies and Riechmann, 1995a) except for the mutations A33C and V37F and the silent incorporation of a Xhol site into the codons for residues 29–31. Ligated DNA was electrophoresed into bacteria of the Escherichia coli strain TG1 and transformants were analysed for correct insert size through PCR screening with the oligonucleotides FDPDRBACK and FDSEQ1 (Hoogenboom et al., 1991).

**Results**

**Camelizing I**

Camelized, human VH domains were previously isolated from a phage-display library through antigen-driven selection. These VHs bound their ligands with a reasonable affinity (dissociation constants between 100 nM and 1 μM) and good specificity (Davies and Riechmann, 1995a). The originating, synthetic library was prepared through introduction of CDR3 loops containing 12 randomized residues. Camelization of the human template VH-Ox13 (Figure 1; Davies and Riechmann, 1994) involved the replacement of residues in the former VL interface of the human VH with the amino acids overwhelmingly common in camelid antibody heavy chains, which occur naturally without light chain partner (Hamers-Casterman et al., 1993). Glycine 44 in the human VH was mutated to glutamic acid (G44E), leucine 45 to arginine (L45R) and tryptophan 47 to glycine (W47G). The effect was a reduction in non-specific binding of the former human VHs (Davies and Riechmann, 1995a).

During NMR analyses of the camelized VHs it became apparent that they were considerably less stable than the human VH before camelization. Amide protons, which were protected from D₂O exchange at 30°C in the human VH for several months, exchanged in the camelized VH in < 1 h (Davies and Riechmann, 1994). It was possible to increase the stability of the VH domains selected through antigen binding from the phage-display library later through mutation of residue 47 from glycine to isoleucine. However, constitutive introduction of isoleucine in the phage library resulted in the presence of...
reach of the side chain of residue 33, assuming some flexibility of two extra cysteine residues in addition to those at positions 44 and 100c in CDR3 (nine out of 17). This region is within position 22 and 92, which form the typical intradomain disulphide bond position 94 (alanine is present in 12 out of 17 sequences), position 101 (Figure 2; Chothia and Lesk, 1987). None of the 45 and 47 in camel VH domains have obviously evolved although two have instead a lysine residue at position 93. The side chain with the carboxyl group of an aspartic acid at salt bridge across the H3 loop through its positively charged naturally. We wondered, therefore, if there were other differences between camelid and human VHs, which might be able to compensate for the observed loss in stability due to the mutations at positions 44, 45 and 47.

Camelizing II
Muyldermans et al. (1994) pointed out several other differences between the light chain lacking camel VHs and members of the human VH3 subgroup. The VH3 subgroup is among both human and murine VH genes the one most similar to the camel VHs and was also the source of our camelized VH.

Residue 37 is located in the β-strand running alongside the β-strand harbouring residues 44, 45 and 47 (Figure 2). It is almost exclusively a valine (89 out of 92) among VH genes of the human VH3 subgroup (Kabat et al., 1991). In contrast, phenylalanine (12 out of 17) and tyrosine (four out of 17) are most common in the camelid VHs.

Another difference occurs at position 94. This residue is typically an arginine (31 out of 95) or lysine (28 out of 95) in human VH3 genes. In most antibodies it forms a surface salt bridge across the H3 loop through its positively charged side chain with the carboxyl group of an aspartic acid at position 101 (Figure 2; Chothia and Lesk, 1987). None of the 17 sequenced camel VHs has an arginine or lysine residue at position 94 (alanine is present in 12 out of 17 sequences), although two have instead a lysine residue at position 93.

A third difference of camel VHs is the common occurrence of two extra cysteine residues in addition to those at positions 22 and 92, which form the typical intradomain disulphide bridge within most VH domains. One of these additional cysteines is most frequently located at position 33 (seven out of 17), while a second is often located between residues 99 and 100c in CDR3 (nine out of 17). This region is within reach of the side chain of residue 33, assuming some flexibility within especially the long CDR3 loops (Figure 2). Therefore, the formation of a second intramolecular disulphide bond in some camel VH domains was proposed (Muyldermans et al., 1994).

A fourth prominent feature of the camelid heavy chains concerns the length of their CDR3 hypervariable loops. The 17 sequenced camelid VHs all have CDR3s between 10 and 24 residues in length with an average length of 17.5 residues. Human VH domains have an average CDR3 of 11.6 residues and mouse VHs of only 8.7 residues (Wu et al., 1993).

**VH-Ox21**
We decided to study the influence of these differences on the folding stability of a camelized VH with a long CDR3, which could accommodate all of the described modifications. **VH-Ox21** (Figure 2) has a CDR3 with 15 residues, the longest in our synthetic repertoire (Davies and Riechmann, 1995a). This length was still shorter than the average camel CDR3, but six out of 17 camel CDR3s had 15 or fewer residues (Muyldermans et al., 1994). The ‘wild type’ of the camelized VH will be referred to as VH-Ox21-AVRL, where the four final letters represent the amino acids at positions 33 (A), 37 (V), 94 (R) and 100b (L).

Folding stabilities of the VH domains were determined in thermodenaturation experiments by following their ellipticity at 235 nm between 20 and 90°C (Figure 3). At this wavelength the folded VH domains had a signal close to zero, which became negative upon denaturation (Figure 3). The spectra of the folded VHs had a negative band at around 225 nm with a sharp rise towards 210 nm and were typical for a polypeptide-containing sheet forming anti-parallel β-strands as the only element of secondary structure. The positive shoulder at around 235 nm (Figure 3) is caused by contributions from aromatic and disulphide chromophores (Perczel et al., 1992). A single transition of denaturation was observed between 20 and 90°C for all VHs analysed. Denaturation was reversible at the VH concentration used.

Initially the stability of VH-Ox21-AVRL was compared with that of the human VH (VH-Ox13), which had served as the template VH for the original camelization experiments (Davies and Riechmann, 1994). Camelizing had a strong effect on the stability of the VH as its melting point (Figure 4) was reduced from 71.2°C (VH-Ox13) to 56.6°C (VH-Ox21-AVRL). This result was expected because of the faster D2O exchange of amide protons observed in the NMR experiments with the camelized VH. However, the stability of VH-Ox21-AVRL was still superior to that of the isolated VH originating from the mouse antibody D1.3, whose Tm was found to be 43°C (Yasui et al., 1994).

**VH-Ox21 mutants**
A secondary camelization of VH-Ox21-AVRL was then performed through a stepwise introduction of new mutations. First residue 37 was mutated from valine to phenylalanine (V37F), resulting in VH-Ox21-AFRL. Its Tm was increased by 1.6°C to 58.2°C (Figure 4). Next, two additional cysteines were introduced in VH-Ox21-AVRL and VH-Ox21-AFRL at position 33 and 100b, which represented the two most frequently used positions for extra cysteines in the camel VHs. Purification yields for the resulting VH-Ox21-CVRC (0.3 mg from 1 l of bacterial culture) and VH-Ox21-CFRC (1 mg/l) were slightly lower than those for VH-Ox21-AVRL (1 mg/l) and VH-Ox21-AFRL (2 mg/l). However, virtually unchanged CD spectra indicated proper folding. The VHs (including those with the
additional cysteine residues) tested negative for free SH groups and also ran as monomeric polypeptides in SDS-PAGE analyses under both reducing and non-reducing conditions (Figure 5). This proved that VH-Ox21-CVRC and VH-Ox21-CFRC both contained two intradomain disulphide bridges. The $T_m$ of the VH-Ox21-CVRC was increased to 57.9°C and that of VH-Ox21-CFRC to 61.1°C (Figure 4). Thus the introduction of the extra disulphide bond was beneficial for the overall folding stability of both VHs. However, the effect was stronger in the case of the VH with the phenylalanine at position 37.

The difference in folding stability between the mutants was estimated in terms of energy from the denaturation curves and the melting points. Van’t Hoff’s enthalpy $\Delta H_{\text{obs}}$ of unfolding was approximated by curve fitting (Equation 22 in Agashe and Udgaonkar, 1995) from the denaturation curve of VH-Ox21-AVRL as 90 kcal/mol and the enthalpy $\Delta S$ (0.27 kcal/mol K) was determined as $\Delta H_{\text{obs}}/T_m$. The difference in free energy change of unfolding by thermodenaturation between VH-Ox21-AVRL and VH-Ox21-CFRC was then calculated from $\Delta G = \Delta H - T \Delta S$ as 1.2 kcal/mol. This gain in overall stability was less than, for example, the contribution to folding stability from a myethylene group in a fully buried residue of barnase (1.5 kcal/mol; Serrano et al., 1992).

Residue 94

Finally the mutation of residue 94 from arginine to alanine (R94A) was investigated. The positively charged side chain of the arginine forms in most human VH domains a salt bridge with the carboxyl group of the aspartic acid at position 101. In accordance with the presence of this salt bridge, the R94A mutation caused in the human VH-Ox13 a decrease in $T_m$ by 3.5°C, corresponding to a loss in energy of approximately 0.9 kcal. Similar values were also determined for the removal of surface salt bridges in barnase (Horovitz et al., 1990).

When the R94A mutation was introduced in VH-Ox21-AFRL and VH-Ox21-CFRC, different effects were observed. In the case of VH-Ox21-AFAL the mutation made a positive contribution to stability as $T_m$ was increased to 63.9°C. The same mutation in case of the VH with the additional disulphide bond, however, caused a decrease in $T_m$ from 61.1°C (VH-Ox21-CFRC) to 58.5°C (VH-Ox21-CFAC). This suggested that the formation of a salt bridge between the side chains of residues 94 and 101 might depend on the particular VH. Formation of a salt bridge including a small contribution to the overall protein stability can certainly not generally be excluded for camelized VH domains on the basis of these results.

**VH-CC library**

The effect of the designed mutations on the overall stability of VH-Ox21 was small (always < 2 kcal/mol). However, VH-Ox21 might not be best suited to display the full beneficial effect of these mutations, as it was selected from a repertoire in which all VHs had undergone only a primary camelization.
Also, as antigen-driven selection from phage-display libraries is not solely influenced by affinity but also by phage propagation related properties (Riechmann and Weill, 1993), the CDR3 in VH-Ox21 was more likely to be best suited for the folding of a VH without the secondary mutations. Hence it was encouraging to see beneficial effects (even though small) of the secondary camelization in the case of VH-Ox21 at all.

To establish whether camelized VH domains with other CDR3 sequences might benefit more from the secondary camelization, a new phage-display library (VH-CC) of camelized VHs was designed. The underlying framework of this new VH library contained the same camelizing mutations as the initial library, from which VH-Ox21 was selected: G44E, L45R and W47G. In addition, residue 37 was mutated to phenylalanine and residue 33 to cysteine. The VH-CC library was generated as before through introduction of a 15 residue long CDR3, in which 11 of the first 12 residues (95 to 100f, but not 100b) were randomized and the last three residues (F100g, D101, Y102) were kept unrandomized. Residue 100b was fixed as a cysteine in all VH domains of the new repertoire to allow the formation of the intradomain disulphide bond with residue 33. Residue 94 was kept as an arginine, because at least in case of VH-Ox21-CFRC, which contained a second intradomain disulphide, its presence contributed to the overall folding stability (Figure 4).

The VH-CC repertoire had $2 \times 10^6$ members and was comparable in size to that of the primary camelized VH library 12N (Davies and Riechmann, 1995a). The VH-CC library was selected through binding to phenyloxazolone conjugated BSA (Ox-BSA) or lysozyme for three rounds each. Phage from 95 clones each isolated after selection was tested in ELISAs for binding to Ox-BSA or lysozyme; 50% of the clones selected through Ox-BSA binding were positive for Ox-BSA [6% were weakly positive for 3-iodo-4-hydroxy-5-nitrophenylacetyl (NIP)-conjugated BSA on a control plate], and among those selected through lysozyme binding 90% tested positive for lysozyme (none was positive for NIP-BSA).

Six of the clones most strongly positive for either antigen were sequenced. Among the Ox-BSA specific clones VH-Oc6 (Figure 1) was found four times and among the lysozyme specific clones VH-Lc1 (Figure 1) was found five times. VH-Oc6 and VH-Lc1 were subcloned for soluble expression and corresponding VH proteins were purified, yielding 0.5 mg (VH-Oc6) or 0.8 mg (VH-Lc1) from 1 l of bacterial culture. Both VHs tested negative for free SH groups, run as monomeric VH on non-reducing SDS gels, and their CD spectra were similar to those of other VHs. Proper folding of the VHs was therefore assumed.

Both VHs were specific for their respective antigens in ELISAs (Figure 6). The dissociation constant for VH-Oc6 and its ligand glycylyphenyloxazolone was determined as 246 nM by fluorescence quenching. This result was comparable to that obtained with the best phenyloxazolone binder selected from the primary 12N library of camelized VH domains, for which a $K_d$ of 146 nM was measured. Binding of lysozyme to VH-Lc1 was too weak ($K_d$ between 1 and 10 µM) to be measured by fluorescence quenching.

However, the purpose of this selection was not to obtain phenyloxazolone or lysozyme-specific VH domains but to determine the folding stability of VHs selected from a repertoire built on a framework which had undergone the secondary camelization. Therefore, their melting points were determined again in thermodenaturation experiments. The $T_m$ of VH-Oc6 was found to be 72.6°C and that of VH-Lc1 was 75.3°C. The secondary camelization of the VH library has therefore made it possible to select antigen-specific VH domains, which were considerably more stable than VH-Ox21 even after this had undergone a secondary camelization itself. Additionally, both VH domains were even more stable than the original human VH-Ox13 ($T_m = 71.2°C$).

**Discussion**

**Stability**

It was shown previously how (primary) camelizing of a human VH domain in its former VL interface led to the design of single antibody VH domains suitable for antigen-driven selection from phage-display libraries. These experiments yielded small recognition units with high specificity for their ligands (Davies and Riechmann, 1995a). Here it was demonstrated that further (secondary) camelization of the human VH domains did not compromise antigen-driven phage selection but resulted in the selection of VH domains with superior folding stabilities. The improvement was due to three mutations in the VH template for the phage-display library. Residue 37 was mutated from valine to phenyalanine and two additional cysteine residues at positions 33 and 100b were introduced to form a second intradomain disulphide bond.

The stabilizing effect of the additional intradomain disulphide bond could easily be rationalized as a covalent linkage between two residues close in the tertiary structure (Figure 2) but distant in the primary sequence (Figure 1). The disulphide bond might be particularly important for the stabilization of local structures in the long CDR3 loops present both in the camelized VHs and in the natural, light chain lacking camelid VH domains. However, as far as antigen affinities were concerned, no improved selection result was obtained for the VH library after the secondary camelization.

The effect of the V37F mutation on stability was more difficult to interpret. In light chain associated VH domains, the side chain of residue valine 37 is buried among the neighbouring residues. It is located at the bottom of a hydrophobic pocket formed by residues 45, 47, 100b and 103 (Figure
2). In a complete antibody or Fv this pocket is filled by the side chain of a phenylalanine at position 98 in the VL (Chothia et al., 1985). Although the principal architecture of the VH was not affected by the camelizing (Riechmann and Davies, 1995), it is likely that the L45R and W47G mutations together with the absence of the VL-phenylalanine will have caused local structural changes in this region. As a result, a phenylalanine at position 37 in the VH seems now to create an energetically more favourable geometry in this region than a valine.

For the same reason, the V37F mutation might have evolved together with the mutations at positions 44, 45 and 47 in camelid VH domains. The mutations at positions 44, 45 and 47 were necessary to reduce the hydrophobicity of the former VL interface and the mutation of residue 37 might have corrected unfavourable geometries in the mutated VHs. Alternatively, a V37F mutation might have compromised VL association and consequently led to the occurrence of the other three mutations, resulting in a less hydrophobic, exposed VL interface. In any case, only the evolutionary appearance of all four mutations might have enabled camel VHs to form stable, well folded and non-sticky domains in the absence of a VL. VL association can contribute strongly to the stability of VH domains, as shown for the mouse D1.3 VH, whose $T_m$ increased from 43°C measured for the isolated VH to 62°C determined with the VH-VL complex (Yasui et al., 1994).

The secondary camelization produced more stable VH domains with moderate antigen affinities, which were able to accommodate different CDR3 sequences. However, the isolation of VH domains with considerably higher antigen affinities might require larger VH repertoires, in which additional residues in other parts of the protein are randomized. For this purpose, we designed a VH domain with a lox-Cre site in the coding sequence for CDR2 (Davies and Riechmann, 1995b). This makes it possible to randomize CDR3 and CDR1 in separate repertoires, which can be recombined in bacteria with the Cre recombinase. It remains to be seen if the mutations described here will still produce the same stabilizing effect in these new VH domains.

Expression

Although secondary camelization created single antibody VH domains with significantly improved folding stabilities, no improvement in purification yields for the more stable VHs was observed. Thus, for both VH-Oc6 and VH-Lc1 less than 1 mg of protein was purified from 1 l of bacterial culture. These yields were similar to those of VH-Ox21-AVRL and other VH domains selected from the primary phage-display library. For the VH domains selected from the primary repertoire mutation of residue 47 from glycine to isoleucine (G47I) improved purification yields by a factor of ~10 (Davies and Riechmann, 1995b). This makes it possible to randomize CDR3 and CDR1 in separate repertoires, which can be recombined in bacteria with the Cre recombinase. It remains to be seen if the mutations described here will still produce the same stabilizing effect in these new VH domains.

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Table 1. Expression and purification yields of some camelized VH domains

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<tr>
<th>VH Domain</th>
<th>Expression</th>
<th>Purification Yield</th>
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<tr>
<td>VH-Oc6</td>
<td>+</td>
<td>7 mg/l</td>
</tr>
<tr>
<td>VH-Lc1</td>
<td>+</td>
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72.6 to 73.6°C. However, this indicated that the secondary camelization contributed to stability also in the ‘high expresser’ format. It has not yet been tested whether it is possible to use isoleucine 47 as a framework residue in the secondary phage-display library. However, in case of the primary library this led to the presence of too many ‘sticky’ VH domains, which obscured the selection result (Davies and Riechmann, 1995a).

Concerning the relation between stability and purification yields, it is worth noting that the $T_m$ of the isoleucine 47-containing version of VH-Ox21-AVRL (67.4°C) was 4.8°C lower than that of VH-Oc6 (72.6°C) containing a glycine at position 47. In contrast, the purification yield for the isoleucine version of VH-Ox21-AVRL (4 mg/l) was significantly higher than that for the more stable glycine 47 version of VH-Oc6 (0.5 mg/l). A possible explanation might be a stabilizing effect of the G47I mutation at an early stage of the folding pathway, which determines the ratio of correctly to wrongly folded protein. Protein misfolded at this stage would probably be removed from the thermodynamic equilibria of folding intermediates through aggregation and/or proteolysis. Then stabilizing effects at later stages of the correct folding pathway through either the V37F mutation or the second intradomain disulphide bond would not significantly increase the amount of correctly folded VH, as this was determined by the presence of either an isoleucine or a glycine at position 47 earlier.

Outlook

The results presented here help in understanding the effect of some of the mutations typical for camel VHs and led to the design of more stable camelized human antibody VH domains. However, more questions need to be answered before the functional role of all camel-specific VH mutations can be interpreted in respect of the choice of a framework best suited for the design of synthetic phage-display libraries of camelized VHs or even camel VH domains themselves. Thus it still remains unclear if and how these mutations affect the capability of VH domains to form good antigen binding sites. This question might be related to the R94A mutation found in most camel VH domains but not clearly beneficial for stability in the case of the camelized VHs. Detailed structural information about the antigen binding sites of camelized and camel VH domains are needed to analyse these questions further.

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References
