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Journal of Molecular Catalysis B: Enzymatic 28 (2004) 173–179
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Stabilization of antibody V_H-domains by proteolytic selection

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Received 27 September 2003; received in revised form 1 December 2003; accepted 20 December 2003

Abstract

Single variable domains of antibodies represent the smallest antigen binding fragments but are less stable than when associated with their cognate variable domains. Here we have attempted to improve the thermodynamic stability of a model heavy chain variable domain (V_H) by "proteolytic selection" a method whereby the protease-resistance of the displayed protein is coupled to the infectivity of a filamentous bacteriophage. The gene encoding the heavy chain variable domain was taken from the anti-lysozyme antibody HyHEL-10, mutated at random by error-prone PCR, and displayed on filamentous bacteriophage by fusion between the domains of the phage p3 protein. As the entire p3 protein is required for phage infectivity, treatment of the phage library with trypsin at an elevated temperature (which leads to cleavage of p3 fusions with unfolded variable domains) selects for infectious phages bearing the more stable variable domains. After several rounds of selection, a mutant (S65G/T70S/D99N) was obtained with improved stability ($T_m = 58.5\,^{\circ}C$ and $\Delta G_{25\,^{\circ}C} = 6.3$ kcal/mol compared to 51.6 °C and 4.2 kcal/mol for the parent domain). These mutations are conservative and the mutant domain retains the ability to pair with its cognate light chain variable domain in an Fv fragment and to mediate binding to lysozyme. Our results show that the thermodynamic stability of antibody single domains can be improved by "proteolytic selection" and this may represent a step towards making useful antibody single domains for biotechnological application.

Keywords: Antibody variable region; Antibody single domains; Antigen binding; Phage display; Thermostability

1. Introduction

Antibody binding sites consist of associated heavy and light chain variable domains, but it has also been discovered that antibody heavy chain variable domains can bind to antigen with good affinities in the absence of a light chain [1]. This represents the smallest antigen binding fragment and is of biotechnological interest as it should permit the development of novel antibody formats for therapy and diagnostics. However, these domains are less stable thermodynamically than when paired with their cognate light chains [2], and are prone to aggregate and stick to the agarose–dextran matrix during size-exclusion chromatography [1,3–7]. In principle, the properties of these single domains might be attributed to the properties of the native state, for example, "stickiness" of the exposed light chain interface; indeed the introduction of hydrophilic residues in the interface can improve their properties [3,4]. Here, we have considered an alternative view; that the properties of the single domains might also be dependent on the properties of the unfolded state, for example its tendency to aggregate. In this case the properties of the single domains might be improved by stabilizing the native state [8].

Attempts have been made by other groups to develop methods for the isolation of stable antibody fragments. For example, a library of heavy and light chain variable domains were displayed on the tip of filamentous phage as single chain Fv fragments (scFv) and selected by binding to antigen under stringent conditions [9]. This approach did result in the selection of antibody fragments with improved thermodynamic stabilities but these properties were inextricably linked to those of the phage in binding to solid phase antigen. Here, we have explored a different strategy; we have taken advantage of a protease-based selection method (proteolytic

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doi:10.1016/j.molcatb.2003.12.018

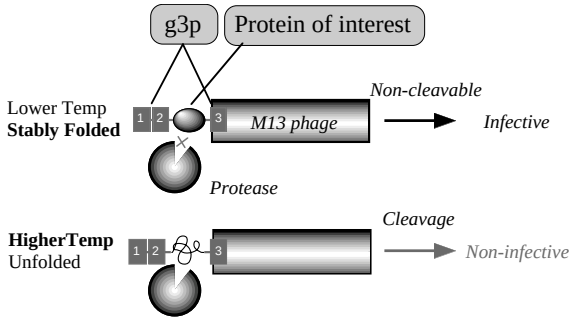

Fig. 1. Schematic drawing of the selection. The three boxes (1–3) show the three domains of g3p. The selection can be performed in homogeneous solution.

selection) in homogeneous solution [10,11] that utilizes the infective function of filamentous phage gene 3 protein (g3p).

The g3p is composed of three domains D1–D3, interconnected to each other by glycine-rich linkers (Fig. 1). D3 is necessary for the anchoring of the protein to the tip of the phage particle, and D1–D2 are required for infection of the host. Phage are resistant to proteases, but if a protease sensitive cleavage site is interposed between D2 and D3, then addition of protease leads to loss of D1–D2 from the phage and infectivity. This provides a means of distinguishing folded and unfolded proteins as only native proteins are resistant to proteolysis. Thus, we interposed antibody V_H domains between D2 and D3, and added protease at an elevated temperature, but below the melting temperature of the domain. In this way, we expected to select for phage displaying V_H domains with slower rates of unfolding and higher thermodynamic stabilities.

2. Experimental

2.1. Materials

Plasmid pK1 and helper phage KM13 were as described [10]. pCANTAB5E-HyHEL-10 and pERfect were kindly provided by Drs. Philipp Holliger and Ian Tomlinson, respectively.

2.2. Plasmid construction

The phagemid pKU that is suitable for proteolytic selection of antibody fragments was made from pK1 as follows. The restriction site *Kpn*I in the cloning site between D2 and D3 were altered to *Not*I using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with primers Kpn2NotU (5′-CAAGCTGGAAATAAAGGCGGCCGCTTCCGAGGGTG GTTCC-3′) and Kpn2NotD (5′-GGAACCACCCTCGGAAGCGGCCGCCTTTAT TTCCAGCTTG-3′). The upstream *Sfi*I site was changed for its reading frame by substituting *Hin*dIII–*Sfi*I fragment encoding D1–D2 region with PCR fragment amplified with primers LMB3 (5′-CAGGAAACAGCTATGAC-3′) and D2SfiFor2 (5′-GGCGGCCGGCTGGGCCG-CGCCAGCATTGACAGG-3′) with VCSM13 ssDNA as a template. pCANTAB5E-HyHEL-10 was digested with *Sfi*I and *Not*I, and the 0.7 kb fragment encoding HyHEL-10 scFv was inserted, designated pKU-HyHEL10. The plasmid was digested with *Pst*I and self-ligated to give pKU, encoding out of frame ORF. HyHEL-10 V_H fragment was PCR amplified with LMB3 and VHNotFor (5′-GATCGCGGCCGCCGCGGAGACGGTGACCAG-3′) as primers and pCANTAB5E-HyHEL-10 as a template. The fragment was digested with *Nco*I and *Not*I, and ligated with pKU digested with *Nco*I and *Not*I, to give pKU-V_H.

2.3. Library construction

HyHEL-10 V_H gene was randomly mutated with PCR mutagenesis employing unequal concentrations of dNTPs and $MnCl_2$ [12]. A 25 μl reaction mix containing 10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100, 0.01% (w/v) gelatin, 0.5 mM $MnCl_2$, 2.5 mM $MgCl_2$, 0.35 mM dATP, 0.40 mM dCTP, 0.20 mM dGTP, 1.35 mM dTTP, 20 pmol (800 nM) each of LSPAfor (5′-GTAAATTCAGAGACTGCGCTTTCC-3′) and LSPAback (5′-ATTTTCGGTCATAGCCCCCTTATTAG-3′) primers, 40 ng pKU-V_H plasmid and 2 U *Taq* polymerase (pH 9.0) was subjected to 30 cycles of 94 °C 1 min, 55 °C 1 min, and 72 °C 3 min. The amplified fragment was digested with *Nco*I and *Not*I, and ligated with 10 μg *Nco*I–*Not*I digested pKU at 16 °C for 4 h. Phenol/chloroform treated, ethanol precipitated DNA was dissolved in 10 μl water, and used for the electroporation of freshly prepared TG-1 competent cells. Cells were plated to a large square plate filled with 15 g/l Bacto-Agar, 8 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract, 100 μg/ml ampicillin (Ap) and 1% glucose (TYEAG), and kept at 30 °C for 16 h. Colonies were harvested and used for the rescue with helper phage KM13 at m.o.i. of 20.

Phages were prepared as follows. Single colony of TG-1 cells having phagemids on TYEAG plate was picked and cultured in 2 ml 2 × TY, 100 μg/ml Ap, 1% glucose (2TYAG) in a disposable 14 ml tube (Falcon 2059, Becton Dickinson, Cowley, UK) at 37 °C with vigorous shaking. When the bacteria were grown to saturation, 100 μl of the culture was taken to inoculate 5 ml 2TYAG in a 250 ml flask. After 1 h at 37 °C with vigorous shaking, 9 μl of 5.5×10^{11} pfu/ml KM13 was added and stood for 30 min at 37 °C. Then 45 ml of 2 × *TY*, 100 μg/ml Ap, 50 μg/ml kanamycin was added (2TYAK) and shaken overnight at 30 °C. The culture was centrifuged with a benchtop centrifuge (Megafuge, Kendro, Bishop's Stortford, UK) at 4000 × *g* for 30 min at 4 °C, and the supernatant was recovered. Phages in the supernatant was purified by adding 0.2 vol 20% (w/v) polyethylene glycol/2.5 M NaCl, mixed and stood for 15 min at 25 °C. The turbid solution was centrifuged at 4000 × *g*, 4 °C for 30 min, and the pellet was re-suspended in 2 ml 10 mM Tris–HCl

pH 7.4, 1 mM EDTA (TE). Contaminating *E. coli* cells were removed with filtration through 0.45 μm nitrocellulose filter dish (Millipore).

2.4. Proteolytic selection

The phage solution (50 μl of 10^{13} cfu/ml) was mixed with 40 μl 2× digestion buffer (100 mM Tris–HCl, 2 mM $CaCl_2$, pH 7.4) and 10 μl of 100 μg/ml TPCK treated trypsin (Sigma, Poole, UK) in 2× digestion buffer. After incubation at 42–43 °C for 1 h, the reaction was stopped with 400 μl MTBSE (4% skim milk, 20 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, pH 7.4). After titration of the phages on TG-1, diluted phages of ∼10^6 cfu were infected to give the colonies used for the next round selection. Titer of the phage samples was measured by serial dilution of the samples in freshly grown TG-1 (OD_{600} ∼ 1.0) cells. Seven–ten microliters of dilutions were spotted on TYEAG plates, and the titer was calculated from the number of colonies and the dilution factor.

2.5. ELISA

For the ELISA to test the binding of phages to hen egg lysozyme (HEL), each well of microtiter plate (Falcon 3912, Becton Dickinson) was coated with 100 μl 5 mg/ml HEL in PBS at 4 °C for 16 h, and blocked with MPBS (4% skim milk/PBS) for 1 h at 25 °C. After washing four times with PBS-T (0.05% Tween 20/PBS), the plate was incubated with 100 μl phage prediluted with MPBS for 1 h at 25 °C with mild shaking. Each well was washed, incubated with 100 μl 1/5000-diluted HRP-anti M13 (Pharmacia, Uppsala, Sweden) for 1 h at 25 °C, washed, and developed with 80 μl of 100 μg/ml 3,3′,5,5′-tetramethylbenzidine (Sigma). Absorbance at 450 nm was measured with A_{650} as a control.

2.6. Protein expression

To prepare soluble V_H protein, 0.3 kb *Nco*I–*Not*I fragment encoding either wild-type or mutant V_H domain was subcloned to pERfect which has tetracycline operator/promoter at the upstream of *Nco*I site and Flag/6× His-tags at the downstream of *Not*I site. TG-1 harboring the recombinant plasmid was cultured in 2 × TY, 100 μg/ml ampicillin at 37 °C to OD_{600} ∼ 0.8 with vigorous shaking. Anhydrotetracycline (Acros Organics, Pittsburgh, PA) was added to 0.2 μg/ml to induce the expression, and shaking was continued for further 16 h at 30 °C. Cells were spun down and the supernatant was concentrated by ammonium sulfate precipitation at 66% saturation, followed by the purification with TALON metal affinity column (Clontech, Basingstoke, UK) and MonoQ anion exchange column on FPLC (Pharmacia). V_L protein was prepared in a similar manner starting from the same plasmid vector.

2.7. Circular dichroism (CD) analysis

CD analysis was performed at a V_H concentration of 20 μg/ml in 10 mM NaPi, 60 mM NaCl, pH 7.0 using Jasco J-720 spectropolarimeter with a PTC-348W1 thermocontroller connected to a DEC Venturis 575 computer. Wavelength scans were recorded at 20 and 90 °C. Thermodenaturation was measured by following the ellipticity at 222 nm in steps of 0.1 °C at a rate of 1 °C/min. Melting points T_m and the conformational stability ΔG were determined by curve fitting using Kaleida Graph 3 software (Synergy Software, Reading, PA).

2.8. SPR analysis

Antigen-binding activity of purified V_H domains was evaluated using SPR biosensor BIAcore 2000 (Biacore, Uppsala, Sweden) at 25 °C. 50 μg/ml HEL in 10 mM sodium acetate (pH 4.5) was injected to immobilize HEL (1720 RU) on CM5 sensor chip employing amine coupling reagents. Final 400 nM each of purified V_H and V_L fragments were applied as analyte at 20 μl/min with HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.05% Tween 20). The sensorgrams for HEL-immobilized and mock-immobilized flow cells were analyzed with BIAevaluation software 2.1 to derive kinetic constants.

3. Results

3.1. Display of antibody fragments between g3p D2 and D3

DNA fragments encoding either V_H, V_L or scFv of anti-hen egg lysozyme antibody HyHEL-10 were inserted into phagemid pKU and used to transfect bacteria (Fig. 2A). Phage displaying each fragment was prepared by the infection of the bacteria by protease-sensitive helper phage KM13 [10], and their antigen binding activities were checked by ELISA (Fig. 2B). Significant binding was seen for the scFv-phage but not the V_H- or V_L-phage. However, when V_H-phage was mixed with V_L-phage a binding signal was detected, and became even stronger when the V_H-phage was mixed with soluble V_L protein (data not shown). This indicates that these single V_H and V_L domains are displayed on phage but that the binding affinity to antigen is insufficient for detection unless paired with complementary variable domain (Fig. 3A) [13].

3.2. Effect of protease on infectivity

Phage displaying scFv, V_H or V_L were treated with 10 μg/ml trypsin for 3–20 min at 25 °C, and infectivity measured during the incubation (Fig. 3B). There was a drop in titer during first 3 min thought to be due to the rapid cleavage of the g3p of the helper phage KM13 (at the

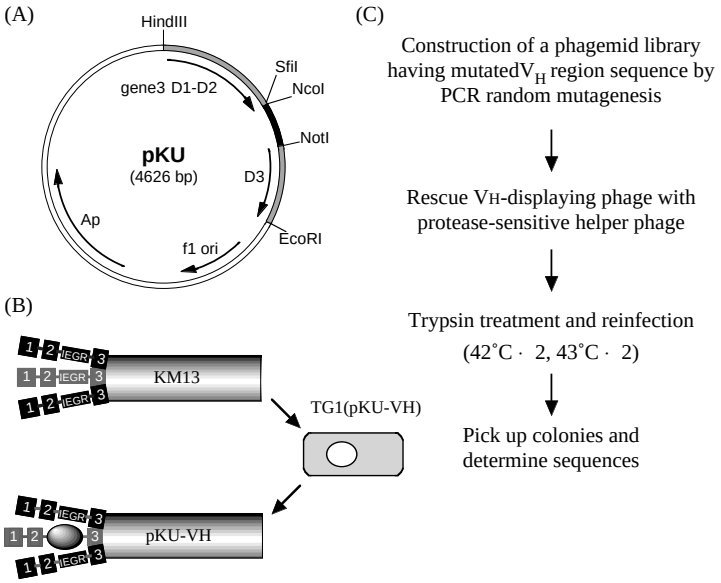

Fig. 2. (A) The structure of pKU. (B) Schematic structure of V_H-displaying phage rescued with protease-sensitive helper phage. The arginine in IEGR sequence between D2 and D3 is cleaved upon trypsin treatment, thus leave D1–D2–tagged V_H fragment (gray oval) displayed. (C) Selection diagram.

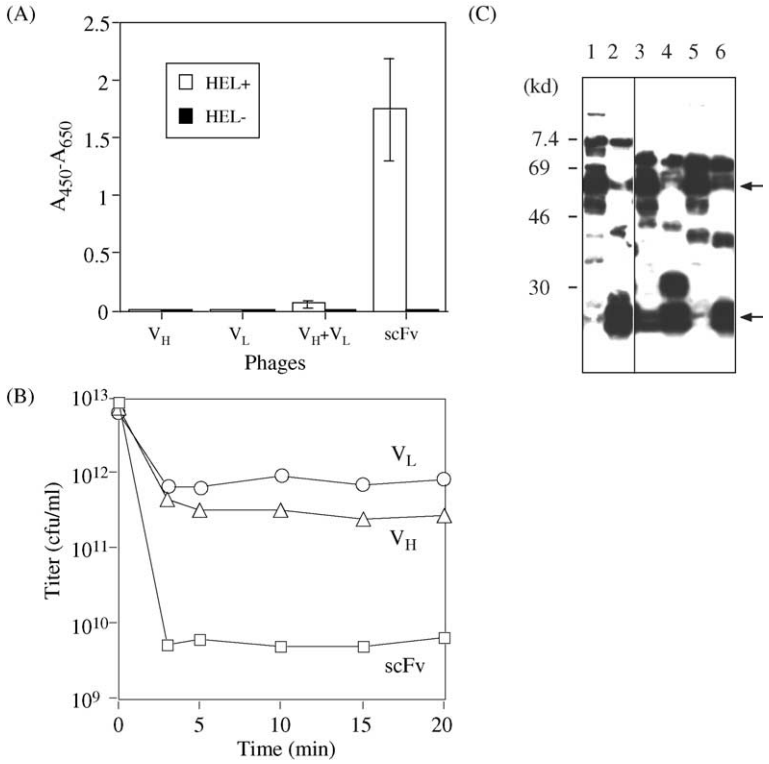

Fig. 3. Display of antibody domain(s) between g3p D2 and D3. (A) Comparison of ELISA signals derived from V_H-, V_L-, and scFv-displaying phages. $V_H + V_L$ shows the mixture of the two phages. (B) Time course of: (triangle) V_H-, (circle) V_L-, and (square) scFv-displaying phage titers during 10 μg/ml trypsin treatment at 25 °C. (C) Western blotting of phages treated with/without trypsin at 25 °C for 15 min serially probed with anti-g3p (Mobitec, Germany) and HRP conjugated anti-mouse antibody (Sigma). Phages displaying scFv (Lanes 1 and 2), V_H (Lanes 3 and 4), and V_L (Lanes 5 and 6) were analyzed either before (Lanes 1, 3 and 5) or after (Lanes 2, 4 and 6) trypsin treatment. Upper and lower arrows indicate the uncleaved and cleaved gp3, respectively, derived from protease-sensitive helper phage KM13.

protease-cleavable linker (IEGR) introduced between D2 and D3 in KM13) [10]. The drop was most notable for the scFv phage; this is thought to reflect the poor efficiency of display of this fragment and the correspondingly greater fraction of phage bearing only the helper g3p which is protease sensitive. Thereafter the titer remained constant for each of the phages, suggesting that under these conditions the displayed antibody fragments resist proteolytic cleavage. These interpretations are entirely consistent with our analysis of the cleavage products using Western blotting using an anti-g3p antibody (Fig. 3C). Thus, a strong band corresponding to the D1–D2–scFv–D3 fusion protein was evident on the Western blot both before (Fig. 3C, Lane 1) and after cleavage (Fig. 3C, Lane 2). By contrast the band for the unfused g3p from the helper phage was greatly diminished. The bands corresponding to the V_H and V_L fusions were also retained on cleavage (Fig. 3C, Lanes 4 and 6), and were much stronger than that of the scFv fusion.

3.3. Selection for a stable V_H fragment

Preliminary experiments revealed that at temperatures above about 30 °C the V_H-phage started to lose infectivity during the incubation with protease. We attempted to select for mutants that retained infectivity at elevated temperatures, and after some trial and error, we chose 42–43 °C. The DNA fragment encoding V_H domain was first randomized by mutagenic PCR with a mutation rate of 0.4%, cloned into the plasmid pKU, and used to transfect *E. coli* TG-1 cells. The diversity of the library (1.0×10^7 clones) would be expected to cover all possible single point and most of the likely double mutations [14]. The library was infected with the helper phage KM13, and the progeny phage (4.3×10^{10} cfu) incubated with trypsin at 42 °C for 30 min, leading to a loss of 10^4-fold in phage infectivity. After infection of fresh bacteria, several colonies were checked for the integrity of the insert DNA by PCR with V_H-flanking primers (LSPAfor and LSPAback) [10]. All of the clones contained inserts of the right size.

For a second round of selection the colonies were grown and infected with phage KM13 as above, and then treated with protease. After infection of fresh bacteria we now noted the appearance of colonies harboring phages lacking inserts, or with short inserts. The colonies were therefore pooled, and the bulk plasmid amplified with normal PCR using the V_H-flanking primers, and digested with the restriction enzymes *Nco*I and *Not*I. The full length fragments were excised from an agarose gel and recloned into the plasmid pKU for the next round of selection. After a total of two rounds of selection at 42 °C and two rounds at 43 °C for 30–120 min, 24 clones were taken and the insert V_H gene sequenced. Although 19 clones had the sequence of the parent, five clones were found with different sequences. Of the mutants, one had an amber codon (Q5amber), three had single mutations (F40L, D72Y, and S84T) and one had three mutations (S65G/T70S/D99N). When the individual clones were incubated with trypsin treatment at 37 or 42 °C for 15 min, the titer of the triple mutant was least affected (Fig. 4), suggesting that it is either displayed more efficiently or is more resistant to proteolysis. This clone was selected for further study.

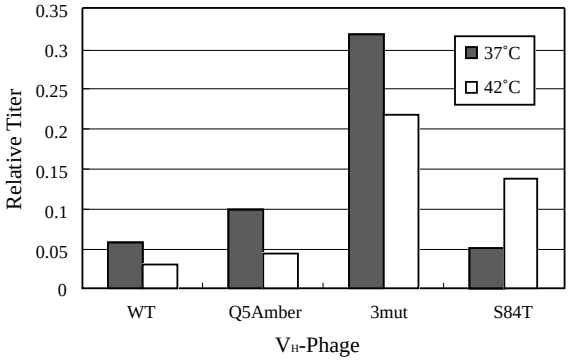

Fig. 4. Relative titers of selected clones after trypsin treatments at either 37 °C (black bar) or 42 °C (white bar). Phage solution (50 μl) of 10^{10} to 10^{11} cfu/ml was treated with 10 μg/ml trypsin in 100 μl digestion buffer at indicated temperature for 15 min, and titrated with TG-1. Titers are expressed as a ratio to that before trypsin treatment.

3.4. Thermostability of S65G/T70S/D99N mutant

The parent and triple mutant V_H domains were recloned into the expression vector pERfect, and soluble protein with C-terminal flag and 6× His-tags was expressed from TG-1 bacteria harboring the plasmid. After anhydrotetracycline induction, the culture supernatant were concentrated and the proteins purified using a metal chelating resin via 6× His-tag, followed by a Mono-Q anion exchange column, almost to homogeneity. The reversible thermal unfolding of each purified protein was monitored by circular dichroism (CD) at 222 nm, where a significant ellipticity change was observed when the temperature was raised from 20 to 90 °C (Fig. 5). As shown in Fig. 6A, the triple mutant V_H domain unfolds at a higher temperature than the parent, for similar data see [15]. Using a two-state model, the T_m of each protein was estimated by a curve-fitting program. As shown in Table 1, the T_m of the wild-type V_H was 324.6 K (51.6 °C) and that of the triple mutant 331.5 K (58.5 °C). The conformational stability ΔG of each protein was also calculated assuming constant ΔC_p (the difference in heat capacity between unfolded and folded conformation at constant pressure) of 12 cal/mol per residue [16]. The triple mutant proved more stable than parent by 2.1 kcal/mol (ΔG values 6.3 and 4.2 kcal/mol, respectively, for wild-type and mutant proteins). A double mutant (S65G/T70S) was derived from the triple mutant by back-crossing with parent. This double mutant and the mutant S84T (see above) were also expressed, purified, and subjected to thermal unfolding. The double mutant showed moderate increase in T_m of 54.7 °C, which is 3.1 °C higher than the parent, and with a stability of 5.4 kcal/mol (Fig. 6B). By contrast the S84T

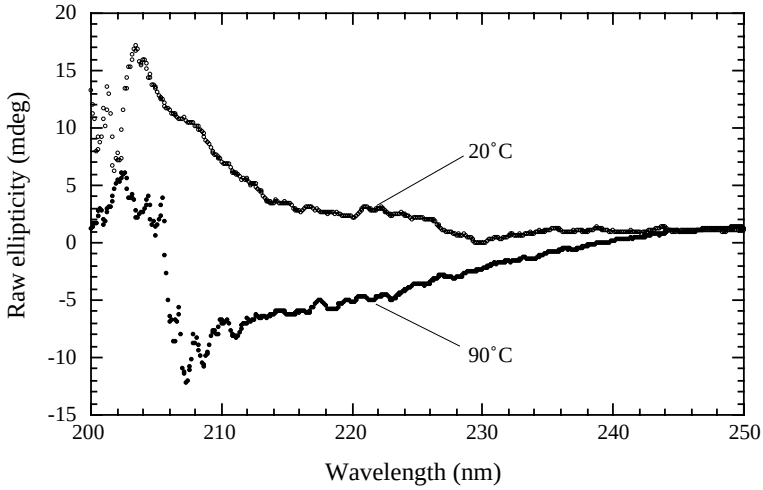

Fig. 5. CD spectra of native and denatured V_H (3mut) protein measured at 20 °C (open circle) and 90 °C (filled circle), respectively.

mutant (Fig. 6B) does not appear to be any more stable than the parent.

3.5. Antigen binding activity of mutants

To test whether the triple mutant V_H domain is able to pair with light chain, it was mixed with an equimolar amount of the V_L domain, and analyzed for binding to a HEL immobilized on a carboxymethyl dextran sensor chip. The triple mutant showed a reduced association rate and a faster dissociation rate for binding of lysozyme than the parent domain, leading to an eight-fold loss in apparent binding affinity (Table 2). In principle, this could be due to a reduced affinity in binding to lysozyme or a reduced affinity of pairing between the V_H and V_L domains. We believe that the former is much more likely, and can be attributed to the mutation D99N. The Asp99 residue lies within CDR3 and makes an ionic interaction with Lys 97 of lysozyme in the X-ray crystallographic structure of the complex HyHEL-10; this is lost in the triple mutant. As expected the double mutant, which retains Asp99, has a binding affinity close to the parent domain.

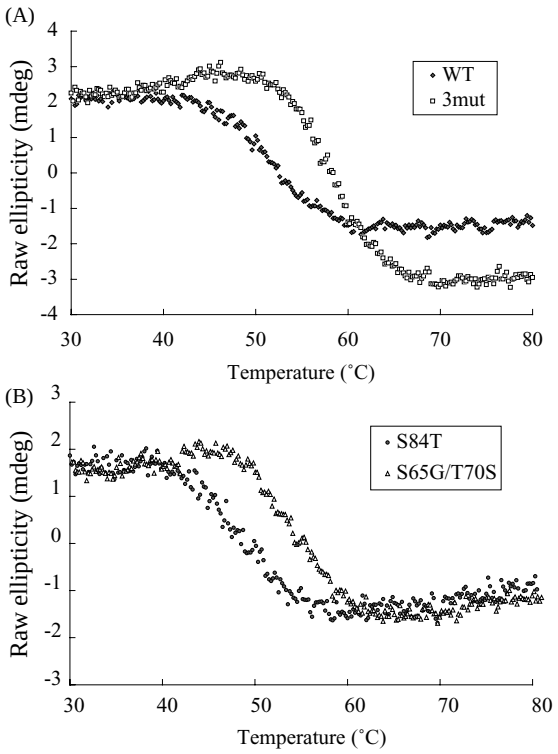

Fig. 6. Thermal denaturation curves obtained for: (A) the wild-type (filled diamond) and S65G/T70S/D99N (open square) V_H proteins; or (B) S84T (filled circle) and S65G/T70S (open triangle) V_H proteins.

Table 1
Parameters characterizing thermal unfolding of V_H fragments obtained with CD measurements

	T_m, °C	$\Delta G_{25\,°C}$[a] (kcal/mol)	$\Delta\Delta G_{25\,°C}$ (kcal/mol)
Wild-type	51.6	4.2	–
S65G/T70S	54.7	5.4	1.2
S65G/T70S/D99N	58.5	6.3	2.1

[a] The conformational stability ΔG at a temperature T was calculated by using the Gibbs–Helmholts equation $\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + \ln(T/T_m)]$, while inferring the midpoint of thermal unfolding (T_m) and the enthalpy change for unfolding (ΔH_m) at the T_m from the denaturation curve [24] and assuming for ΔC_p (the difference in heat capacity between unfolded and folded conformation at constant pressure) a value of 12 cal per residue [16].

Table 2
Kinetic analysis of Fv–HEL interaction with BIAcore 2000

	k_{on}[a] ($\times 10^4\ M^{-1}\ s^{-1}$)	k_{off}[a] ($\times 10^{-5}\ s^{-1}$)	K_a[a] ($\times 10^8\ M^{-1}$)
Wild-type	2.79 ± 0.68	6.61 ± 1.01	4.25 ± 1.12
S65G/T70S	3.10 ± 0.84	6.30 ± 1.23	5.04 ± 1.76
S65G/T70S/D99N	1.92 ± 0.36	38.0 ± 2.70	0.504 ± 0.075

[a] Averages of three independent measurements are shown.

4. Discussion

Here we have shown the improvement in the thermodynamic stability of a heavy chain variable domain by fusion within the p3 protein and rounds of proteolysis and infection. Alternative formats have been described for proteolytic selection, for example by fusion of the protein of interest between an N-terminal affinity tag and the p3 protein [17,18]; the folded protein is selected after proteolysis by binding of the phage to a solid phase through the affinity tag. The method we have described does not rely on binding to solid phase, and therefore may offer an advantage where the protein is sticky and there is a risk of enriching non-specific binders. There is a common problem for all these methods in the enrichment of deletion mutants during the selection, but we have shown here that it can be managed by re-cloning DNA fragments of the correct size at each round. An alternative possibility would be to use a common ligand to enrich the phage bearing the proteins, for example, protein A in the case of human heavy chain variable domains of the VH3 family.

Earlier work has reported the engineering of the stability of associated heavy and light chains together, linked as a single chain Fv fragment [9], whereas we have worked on the isolated domains. A potential complication of engineering heavy chain variable domain away from the context of its cognate light chain is that this may compromise its pairing with light chain. In this case, this does not appear to have happened; the triple mutant is still able to associate with its cognate light chain and to mediate binding to antigen.

In earlier work candidate residues were chosen for randomization from homologous proteins [11,19,20]. In our case, we did not chose the residues to mutate; the entire gene was mutated at random. As the frequency of mutations was low, and expected to give rise to all single and most double mutations, it is perhaps surprising that we succeeded in isolating a triple mutant. We suspect that this arose by recombination between single or double mutants, for example, by PCR-crossover during the selection process. However, we do not understand why the mutations of the triple mutant lead to an increase in stability. It is well known that changes in the densely packed core of the protein often create packing defects and are thus destabilizing [21], but recent reports suggest the importance of surface residues in stabilizing a protein domain [22,23]. All of the mutations of the mutant variable domain (residues 65, 70, and 99) are likely surface residues, based on the structure of HyHEL-10 (PDB 3HFM and 1C08 for Fab:HEL and Fv:HEL complexes, respectively). However, the changes (Ser to Gly, Thr to Ser, and Asp to Asn) are very conservative and their effects are likely to be subtle.

Comparison of the folding stability of the triple (S65G/T70S/D99N) with the double mutant (S65G/T70S) suggests that D99N contributes only 0.9 kcal/mol to stability and that the other two mutations together contribute 1.2 kcal/mol. This is entirely consistent with our view that the triple mutant has arisen by recombination of single or double mutants during the selection. Indeed we suggest that the combinatorial assembly of multiple small changes, each of which makes a modest contribution to stability, may represent a general strategy for improving the stability of antibody variable domains, and a step towards improving their biophysical properties for biotechnological application.

Acknowledgements

We are grateful to Philipp Holliger, Peter Jones and Lutz Riechmann for their comments and help, and Chris Johnson for CD measurement. HU was supported by Japanese Ramsay Fellowship during the stay in Centre for Protein Engineering, Medical Research Council, UK.

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