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# Helix-stabilized Fv (hsFv) Antibody Fragments: Substituting the Constant Domains of a Fab Fragment for a Heterodimeric Coiled-coil Domain

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Antibody Fv fragments would in principle be useful for a variety of biotechnological applications because of their small size and the possibility to produce them in relatively large amounts in recombinant form; however, their limited stability is a drawback. To solve this problem, both domains are usually fused *via* a peptide linker to form a single-chain Fv (scFv) fragment, but in some cases this leads to a dimerization. We present an alternative format for stabilizing antibody Fv fragments. The $C_H1$ and $C_L$ domain of the Fab fragment were replaced with a heterodimeric coiled coil (WinZip-A2B1), which had previously been selected using a protein-fragment complementation assay in *Escherichia coli*. This new antibody format was termed helix-stabilized Fv fragment (hsFv), and was compared to the corresponding Fv, Fab and single-chain Fv format. Bacterial growth and expression of the hsFv was significantly improved compared to the Fab fragment. The hsFv fragment formed a heterodimer of heavy and light chain with the expected molecular mass, also under conditions where the scFv fragment was predominantly dimeric. The hsFv fragment was significantly more stable than the Fv fragment, and nearly as stable as the scFv fragment under the conditions used (80 nM protein concentration). Thus, the format of a helix-stabilized Fv (hsFv) fragment can be a useful alternative to existing recombinant antibody formats, especially in cases where poor expression of Fab fragments or multimerization of scFv fragments is a problem.



*Keywords:* leucine zipper; antibody engineering; protein stability; protein design; protein engineering

## Introduction

Immunoglobulin Fv fragments are the smallest functional units of antibodies still containing the complete antigen-binding site. They are heterodimers of the heavy-chain variable domain ($V_H$) and the light-chain variable domain ($V_L$). Their relatively small size, the ability to produce them in functional form in the periplasm of *Escherichia coli*,[1] as well as their potential use in immunodiagnostics and immunotherapy makes them an interesting starting point for protein engineering.

However, Fv fragments are of limited stability due to dissociation of the two chains. Dissociation constants of $10^{-6}$ M to $10^{-9}$ M have been measured.[2–5] Therefore, different ways have been established to enforce the association of the two domains. In the Fab fragment, which in addition to the variable domains comprises the first constant domains of the light and heavy chain ($C_H1$ and $C_L$), the interaction between both chains is significantly improved. A further method to stabilize Fv fragments is the covalent linkage between both chains. This can be achieved by a flexible genetically encoded linker connecting $V_L$ and $V_H$, resulting in a so-called single-chain Fv fragment (scFv).[6–8] Alternatively, a covalent linkage can be achieved by the formation of a disulfide bridge between the two chains by introducing two engineered cysteine residues, resulting in a so-called

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Abbreviations used: dsFv, disulfide-stabilized Fv fragment; hsFv, helix-stabilized Fv fragment; scFv, single-chain Fv fragment.

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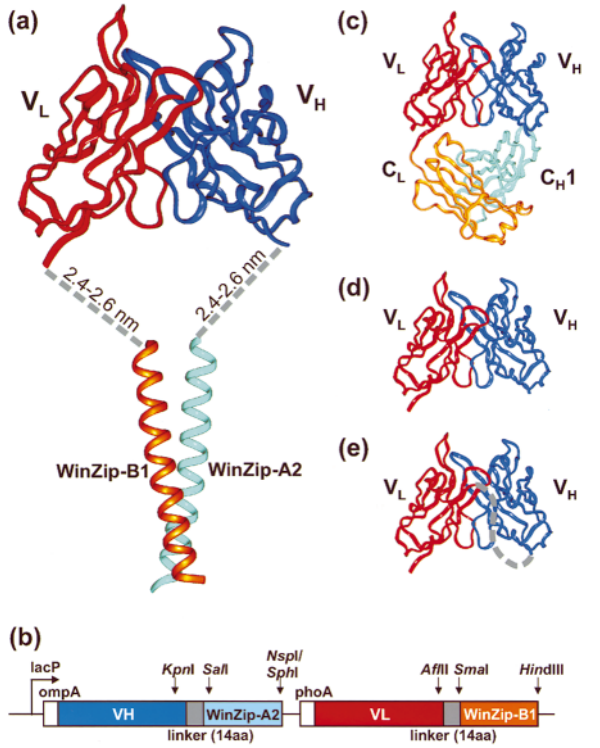


**Figure 1.** Overview of recombinant antibody formats compared in this study. (a) Helix-stabilized Fv fragment (hsFv). In the final construct, the $V_H$ domain (blue) is connected via a flexible 14 amino acid linker to WinZip-A2 (cyan), while the $V_L$ domain (red) is connected via a similar linker to WinZip-B1 (orange) (see also Table 1). The broken lines (grey) symbolize the distance that should be covered by the flexible linkers. (b) Overview of the genes encoding the hsFv fragment. Important restrictions sites (see below) are indicated. (c) Fab fragment, consisting of the light chain, $V_L$ (red) and $C_L$ (orange), and the heavy chain, $V_H$ (blue) and $C_H1$ (cyan). (d) The Fv fragment, and (e) the single-chain Fv fragment (scFv) are given in the same color code for the variable domain. The broken grey line symbolizes the linker which connects $V_H$ with $V_L$. The constructs were modeled based on the PDB structure 1MCP.[29] The hsFv fragment is encoded in the vector pKA290_H11-A2B1. The starting point for this vector was the vector pHJ290huC,[48] which expresses a chimeric Fab fragment with the Fv domain from the murine antibody McPC603, and the constant part from the human antibody 4D5. $V_H$ and $V_L$ are preceded by a short FLAG tag.[49] In the first step, the three stabilizing mutations (''H11'') in the heavy chain[28] were introduced from the vector pHJ300H11[48] as a *Spe*I/*Hin*dIII fragment. In the next step, the constant domains were exchanged for the helices WinZip-A2 and WinZip-B1 forming a coiled-coil domain, via the unique restriction sites *Kpn*I/*Sph*I for WinZip-A2 and *Afl*II/*Hin*dIII for WinZip-B1, respectively. The genes for WinZip-A2 and WinZip-B1 were obtained by PCR from the resulting vectors of a library-*versus*-library selection using the vectors LibA-DHFR[1] and LibB-DHFR[2:I114A], respectively.[24,25] The amino acid sequence of the linker and the coiled-coil domain is given in Table 1. The scFv fragment of the antibody McPC603 with the three mutations in the heavy chain and an N-terminal FLAG-peptide[49] is encoded in the vector pLisc_SAFH11 (corresponding to pLisc_SF[48] with the H11 set of mutations[28]).

disulfide-stabilized Fv fragment (dsFv).[8,9] The last two strategies can also be combined.[10]

However, all these formats have advantages and disadvantages, and the method of choice depends highly on the application. The Fab fragment often expresses at significantly lower yield of functional protein compared with the Fv fragment,[11] even though the absolute yield depends strongly on the particular sequence of the antibody. In the dsFv, the additional cysteine residues usually dramatically decreases the expression yield in periplasmic expression, which usually requires that dsFv proteins are prepared by refolding.[8,9] Furthermore, the relative spatial orientation of the $V_H$ and $V_L$ domains is not constant for different antibodies, and can also vary upon antigen binding.[12–14] The precise orientation is therefore difficult to predict, and there may not be a single successful position to place the designed disulfide bridge for all antibodies. The scFv, although expressing comparably well in the periplasm of *E. coli*, has a tendency to form dimers or even higher aggregates,[15–20] depending on the linker length and on individual factors within the domains, which are still not well understood. It cannot be excluded that the linker might even directly or indirectly interfere with folding or antigen binding in some scFv fragments, which could be one contributing factor for reduced apparent affinities and/or reduced percentage of functional molecules of some scFv fragments compared with the corresponding Fab fragments[6,7,21,22] or Fv fragments.[23]

For most applications a defined oligomerization state is necessary, and a good yield of functional protein is desirable. We therefore developed a new format to stabilize Fv fragments which does not require any structural information of the Fv fragment, avoids the problem of multimerization, and is as well expressed as Fv or scFv fragments. This was achieved by combining an Fv fragment with a heterodimerization domain in a Fab fragment-like arrangement (Figure 1). Each chain of the antibody fragment is connected *via* a designed flexible linker to one chain of the heterodimerizing domain.

We utilized a coiled coil as heterodimerization domain, and therefore we named this stabilized Fv fragment ''helix-stabilized Fv fragment'' or briefly ''hsFv''. We chose a highly specific, heterodimeric coiled coil, named WinZip-A2B1 (Table 1), which

The single-chain Fv fragment is in the orientation $V_H$-$(G_4S)_3$-$V_L$. The Fv fragment with a FLAG-peptide before the heavy and the light chain and the same mutations in the heavy chain is encoded in the vector pHJ300H11. The vector coding for the corresponding Fab fragment carrying the same mutations in the heavy chain was obtained by combining the Fab vector pHJ290FF,[49] which expresses the wt-Fab fragment, with the vector pHJ300H11 via *Spe*I/*Hin*dIII, resulting in the vector pKA290_H11Fab. All vectors are based on pASK29[11] and pASK30.[30]

**Table 1.** Sequences of the linker and the coiled coil domain used for the hsFv fragment

| Antibody domain | Linker[a] | Coiled-coil domain[b,c] |
|---|---|---|
| $V_H$ | - S-GGTSGSTSGTGST | - T-**V**AQ**L**RER **V**KT**L**RAQ **N**YE**L**ESE **V**QR**L**REQ **V**AQ**L**-AS |
| $V_L$ | - A-GSSTGSSTGPGST | - S-**V**DE**L**QAE **V**DQ**L**QDE **N**YA**L**KTK **V**AQ**L**RKK **V**EK**L**-AS |
| Heptad position[d] | | **a**bc**d**efg **a**bc**d**efg **a**bc**d**efg **a**bc**d**efg **a**bc**d** |

[a] The first residue of the linker, which is formally the last amino acid of the variable domain (Ser149 in $V_H$; Ala150 in $V_L$, according to the consensus numbering[27]), is relatively flexible and therefore more likely to participate in the linker part.
[b] The last two amino acids of the linker (amino acids S, T), and the first amino acid of the coiled-coil domain (S or T, respectively) were designed to form an N-cap for the helix.
[c] The last two amino acids (AS) were included to increase solubility.
[d] The coiled-coil helix has a seven-residue structural repeat, with residues denoted by the same lower-case letter being in equivalent positions. Positions **a** and **d** (bold and underlined) are located in the interface between the helices.

we had previously selected from two coiled-coil libraries using an *in vivo* assay in *E. coli*.[24,25] We preferred this coiled coil over designed ones, because we had previously observed that some designed coiled coils, although being highly stable *in vitro*, are severely degraded during *in vivo* expression and purification (K.M.A., unpublished results). WinZip-A2B1 had been selected in the cytosol of *E. coli*, and is therefore known to be metabolically stable and functional in this environment. Furthermore, WinZip-A2B1 was selected to be highly heterospecific, since the selection system disfavored homodimerization.[24] Whether WinZip-A2B1 also functioned in a periplasmic expression system, i.e., whether it was transported to the periplasm and was resistant against proteases in this environment as well, had to be investigated in order to evaluate its general utility. Therefore, our approach not only demonstrates a new way to stabilize Fv fragments, but at the same time, also validates the general usefulness of WinZip-A2B1 as independent heterodimerization module. We have compared the novel hsFv with the corresponding Fv, scFv and Fab fragment regarding expression behavior, yield of functional protein, oligomerization state and stability.

## Model system

For our studies we chose a mutant of the phosphorylcholine-binding antibody fragment McPC603, which was derived from a murine IgA antibody.[26] This mutant carries three mutations in the heavy chain (P47A, S71A, A72D, numbered according to the new AHo consensus scheme,[27] corresponding to sequentially numbered positions P40A, S63A, A64D), which result in better *in vivo* folding properties, and thus increased yield in functional expression.[28] This antibody is a particularly useful model system to elucidate the advantages of our new approach, because we had previously found that the corresponding single-chain Fv fragment can form dimers under certain conditions.[15]

## Design

The linker connecting each variable chain of the antibody with one helix of the heterodimerization domain should be flexible and sufficiently long so as not to put any strain on the $V_H$-$V_L$ assembly, but at the same time not too long so that the dimerization of the coiled coil might still be able to facilitate dimerization of $V_H$ and $V_L$ and thereby increase its stability. Using the structure of the Fab fragment of the antibody McPC603 (PDB code 1MCP[29]), we measured the distances between the C termini of $V_H$ (Ser149 (AHo numbering[27]), corresponding to Ser122 in sequential numbering[29]) and $V_L$ (Ala150 (AHo), corresponding to Ala115 (sequential)) to be 40.6 Å. In order to prevent a forced, sharp bend of the linker, an isosceles triangle was constructed with the bottom part of the Fv fragment (C termini) as base and pointing toward the N termini of the helices with an angle of about 45°. The resulting distances along this triangle from the C terminus of $V_H$ or $V_L$, respectively, and the N termini of the helices, were estimated to be 24 Å to 26 Å (Figure 1). The average distance of an amino acid in a peptide chain of extended conformation is 3.34 Å, in a peptide folded as a β-strand about 3.06 Å, and 0.70 Å in a peptide chain folded as α-helix. Thus, a minimum length of seven, eight or 32 amino acid residues would be required assuming a maximal extended conformation, a β-strand or an α-helix, respectively. To account for enough flexibility, we chose a 14 residue-long linker. To prevent genetic instabilities we avoided codon repeats in the linker, and chose a different sequence consisting of the amino acids Gly, Ser, Thr, Pro for both linkers (Table 1).

## Cell viability and protein expression

All constructs (Fv, Fab, hsFv, scFv) were expressed in the same vector, based on earlier versions[30] in the *E. coli* strain JM83 (Figure 1). The growth curves, measured at 26 °C (Figure 2), show no significant difference for all constructs before induction with 1 mM IPTG. However, cells expressing the Fab fragment grew considerably more slowly after induction. At 3.5 to four hours after induction, they reached a maximal $A_{550\ nm}$ of only 2.6 before starting to lyse as indicated by a decrease in optical density. This is the result of a toxic effect of the expressed product on bacterial physiology, whose exact mechanistic origin has not yet been elucidated. No significant difference in

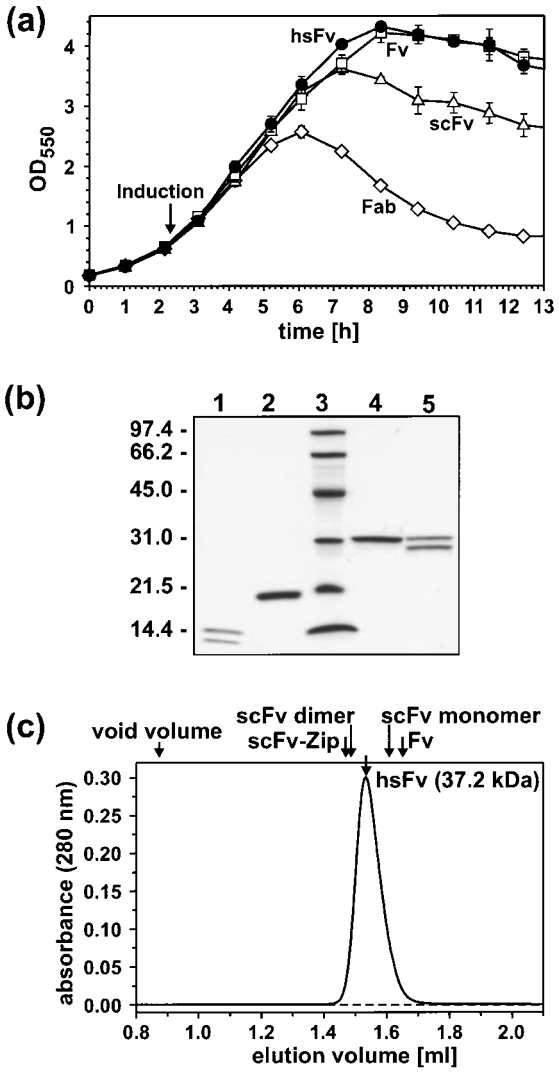


**Figure 2.** Characterization of the different antibody formats. (a) Growth curves in LB medium at 26 °C using the *E. coli* strain JM83 ($F^-$ *ara* (Δ*lac-proAB*) *rpsL* ($str^r$) [ϕ*80*d*lac* (*lacZ*)Δ*M15*] *thi*) harboring the various expression plasmids described in Figure 1. ●, hsFv fragment; □, Fv fragment; △, scFv fragment; ◇, Fab fragment. All growth experiments were carried out in LB broth in shake flasks, containing 0.1 mg/l ampicillin. Overnight cultures were grown at 30 °C. The main culture was inoculated to give an initial $A_{550\ nm}$ of 0.15 (typical dilution of 1:25-1:30) and grown at 26 °C. Cells were induced with a final concentration of 1 mM IPTG at an $A_{550\ nm}$ of 0.5 to 0.6, and the absorbance at 550 nm was measured every hour for 13 hours. (b) Coomassie blue-stained SDS-PAGE of purified antibody constructs. 1, Fv fragment; 2, hsFv fragment; 3, marker (97.4 kDa, 66.2 kDa, 45.0 kDa, 31.0 kDa, 21.5 kDa, 14.4 kDa); 4, scFv fragment; 5, Fab fragment. The expression and purification by affinity chromatography was performed as described previously.[15] The concentration was calculated from the absorbence at 280 nm.[50] The masses of the two chains of the hsFv, which run at the same height in SDS-PAGE, were verified by mass spectrometry and were found to be within the expected range of error (data not shown). (c) Size-exclusion chromatography of the hsFv fragment. The arrows indicate the elution volume for the corresponding scFv-Zip (66 kDa), scFv (dimeric form: 56 kDa; monomeric form: 28 kDa), and Fv fragment (27 kDa). All samples were loaded (30-50 μl) and run in BBS buffer (200 mM $H_3BO_3$, 160 mM NaCl (pH 8.9; NaOH)) with 20 mM phosphorylcholine on a Superose-12 column (PC3.2/30; 3.2 mm × 30 cm; Pharmacia, Sweden) at 20 °C.

growth was observed between the Fv fragment and the hsFv. Both reached a maximal $A_{550\ nm}$ of about 4.2 to 4.4 at six to 6.5 hours after induction. This indicates that the expression of the additional coiled-coil domain has no negative influence on the viability of the bacteria. In contrast, the scFv fragment imposed more stress on the cell, as those cells reached a maximal $A_{550\ nm}$ of only 3.6 already 4.5 to five hours after induction (Figure 2).

## Protein purification and yield

In order to purify only functional protein, and at the same time demonstrate antigen binding, we used a hapten-affinity purification, as described previously.[15,31] A washing step with 1 M NaCl was added to increase purity, and no impurities were observed in SDS-PAGE (Figure 3). All constructs could be affinity purified, and were thus functionally expressed. No degradation products was detectable, neither by SDS-PAGE nor by mass spectrometry (data not shown). The yield of functional protein from a two liter culture after four hours of induction was about 4 mg for the hsFv and scFv, but only 600 μg for the Fab fragment. The lower yield of functional protein from a Fab fragment compared with an Fv fragment has been described before, and even changing the disulfide pattern in the constant domains from the parental IgA type to the IgG1 type did not increase the amount of functional protein.[11] This points to a disadvantage of Fab fragments over other forms of stabilized Fv fragments. Nevertheless, the magnitude of expression decrease is dependent on the particular antibody.

## Oligomerization state

Single-chain Fv fragments have the potential disadvantage that under certain conditions they can form dimers or even higher aggregates, as shown by several groups.[15–20,32,33] This behavior depends not only on the linker length, but can be influenced by a variety of structural and environmental factors.[16–19] We previously investigated this phenomenon for the same single-chain antibody fragment as studied here.[15] The dimeric form of this scFv fragment is initially formed during periplasmic expression and can get trapped in the dimeric form by all factors that stabilize the $V_H$-$V_L$ interface, such as the presence of the antigen, high ionic strength and a pH below 7.5. The antigen is bound in a cavity between $V_H$ and $V_L$, and thereby stabilizes the interface. Consequently, destabiliza-

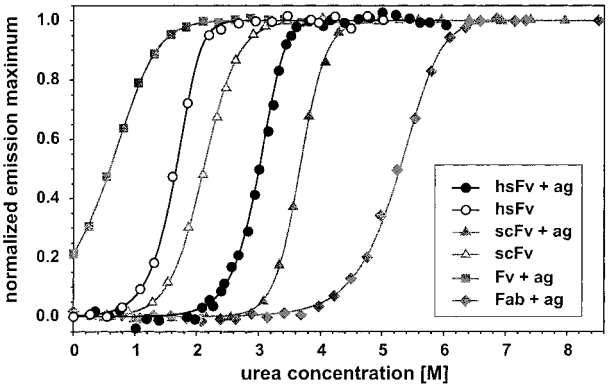


**Figure 3.** Urea denaturation curves of various antibody fragments in the presence (filled symbols) and in the absence (open symbols) of antigen. ●○, hsFv fragment; ■, Fv fragment; ▲△, scFv fragment; ◆, Fab fragment. Urea-induced denaturation was followed by the measurement of the intrinsic fluorescence emission spectra of the proteins. Protein/urea mixtures (2 ml) containing a final protein concentration of 80 nM, and denaturant concentrations ranging from 0 to 8 M urea were prepared from freshly purified protein (in BBS buffer) and a urea stock solution (9 M, BBS buffer). Measurements were performed in the presence or absence of the antigen (5 mM phosphorylcholine). Samples without antigen were obtained by preparative gel filtration on a Superdex 75 column (HiLoad 16/60 prep grade, Pharmacia, Sweden) at 4 °C in BBS buffer. The exact urea concentration of each sample was determined by measuring the refractive index. After an overnight incubation at 10 °C, fluorescence emission spectra were measured at 10 °C from 320 nm to 390 nm; excitation was at 280 nm (step size 1 nm, integration time 0.2 seconds, averaging of six measurements). All measurements were carried out under gentle magnetic stirring, using a PTI Alpha Scan (Photon Technologies, Inc.) or a Hitachi F-4500 spectrofluorimeter. Denaturation curves were obtained by plotting the normalized wavelength shift of maximal intensity *versus* the urea concentration. As the two-state nature of the transitions is not clear for the various fragments, we have not derived free energies. For comparison, the dissociation constant of the coiled-coil domain WinZip-A2B1 was estimated to be 400 pM at similar conditions as used in this experiment (K.M.A. *et al.*, unpublished results).

tion of the interface, for example by removing the antigen, allows equilibration to the thermodynamically favored monomeric form. Elongation of the linker from 15 to 25 amino acid residues reduced the amount of initially isolated dimer to about 60 %.[15]

It might be conceivable that multimeric hsFv fragments are formed, if the helices and the antibody chains did not assemble, as depicted in Figure 1(a), but would each dimerize with different molecules. We therefore investigated the oligomerization state of the hsFv fragment by size-exclusion chromatography (Figure 2(c)). As reference proteins, the Fv fragment, the scFv in its monomeric and dimeric form,[15] as well as a dimeric mini-antibody, an assembly where each helix of a homodimeric coiled coil carries a whole scFv molecule connected by a hinge region (scFv-ZIP[34,35]) were used. The hsFv gave rise to a single sharp peak with an elution volume between the elution volumes of the dimeric and monomeric form of the scFv fragment (Figure 2(c)). This corresponds well to the expected molecule as depicted in Figure 2(a). No dissociation or aggregation was observed by size-exclusion chromatography. As expected, the elution volume was somewhat smaller than for the monomeric scFv. This is not only due to the slightly higher molecular mass (hsFv: 37.2 kDa, scFv: 28 kDa), but it had also been observed previously that the coiled-coil domain increases the apparent size, presumably due to its extended shape (compare scFv-dimer to scFv-ZIP in Figure 2(c)). This clearly demonstrates that under conditions where the single-chain Fv fragment of the same antibody is at least partially dimeric, a single species with the correct molecular mass is obtained in the case of the helix-stabilized Fv fragment, which is an important advantage over the single-chain Fv fragment.

## Stability

A further important point determining the usefulness of the various antibody formats is their stability (reviewed by Pack & Plückthun[35]). In the case of the antibody McPC603, the interface between $V_H$ and $V_L$ is not very stable in the absence of bound antigen. For the Fv fragment, the dissociation constant of $V_H$ and $V_L$ of a similar mutant as the one used here was estimated[37] to be $2 \times 10^{-7}$ M. Thus, any interface-stabilizing effect becomes immediately apparent. The stability of the hsFv fragment was compared with those of the Fv, Fab and scFv fragments in the presence and absence of the antigen phosphorylcholine (Figure 3). The Fv fragment was by far the least stable molecule. Even in the presence of antigen, it was already partially denatured in the absence of urea at a protein concentration of 80 nM; consistent with previous measurements.[37] As expected, the most stable fragment was the Fab fragment. Although the Fab fragment used here (derived from an IgA and thus having no H-L interchain disulfide bond) is also non-covalently associated, its dissociation constant is estimated to be much less than 80 nM.[38–40] The high degree of stability is most likely caused by the interaction between both chains, as well as by the orientation of the variable domains enforced by the constant domains. The single-chain Fv fragment and the helix-stabilized Fv fragment showed intermediate stability. The somewhat higher stability of the scFv fragment compared with the hsFv fragment at the chosen concentration of 80 nM can be explained by the fact that the hsFv consists of two separate chains, and the stability is concentration-dependent, whereas in the scFv both chains are covalently

linked and the stability is therefore concentration-independent. The effective local concentration of the domains in the scFv can be estimated to be in the range of 1 mM (assuming that the $(G_4S)_3$ linker spans up to 5 nm and the distances from the linker to the midpoint of the interface add a further 1.5 nm for each domain). This is consistent with measurements based on unfolding rates.[37]

The stability of the A2B1 coiled coil has been determined with synthetic peptides by CD spectroscopy (K.M.A. *et al.*, unpublished results). Coiled-coil helices undergo a cooperative transition to the monomeric random coil. Under the conditions at which the proteins were unfolded (10 °C, 80 nM dimer concentration), the coiled-coil peptide unfolds with a midpoint of about 2.4 M urea. The midpoint of unfolding is thus higher than the midpoint of unfolding of the Fv-part in the hsFv in the absence of antigen (1.6 M urea), but somewhat lower than in the presence of antigen (3.0 M urea). Most likely, the Fv domains and the coiled-coil domains stabilize each other, by bringing the fused domains to a higher local concentration within the heterodimeric molecule. As a consequence, the fusion protein is more stable than the average of its components. In the presence of antigen, the hsFv fragment is much more stable than the Fv fragment (Figure 3), even though the isolated coiled-coil peptide on its own denatures before the hsFv fragment does, while the hsFv fusion protein may unfold cooperatively. In the absence of the antigen, the stabilizing effect of the coiled coil on the variable domain unfolding is directly observable (Figure 3).

The determination of equilibrium unfolding curves demonstrates that the addition of the coiled-coil domain confers significant stability compared with the Fv fragment, and that this non-covalent interaction is able to achieve almost as high a stability as the covalently linked scFv fragment, even at a concentration as low as 80 nM.

## Conclusions and Perspectives

In summary, the helix-stabilized Fv fragment (hsFv) is a valuable alternative to presently used antibody formats. As shown by urea denaturation, the coiled-coil domain WinZip-A2B1 significantly stabilizes the heterodimerization of $V_H$ and $V_L$ in the Fv fragment, even without the formation of a covalent linkage between both domains. In contrast to the single-chain Fv fragment, which can form dimers under certain conditions, the hsFv fragment is a heterodimer of the expected molecular weight (37 kDa) with no further oligomerization or aggregation detectable. Furthermore, the rapid folding of the coiled-coil domain[41–43] is likely to bring the $V_H$ and $V_L$ domains already into the right orientation, and thus might facilitate their folding. The hsFv is well expressed, and leads to the same amount of functional protein as the corresponding single-chain Fv fragment, and no degradation was detected. In addition, the present study shows that the heterodimeric coiled-coil pair WinZip-A2B1, which has been selected in the cytosol of *E. coli*, is also well transported and stable in the periplasm of *E. coli*. In contrast, other naturally occurring as well as designed, coiled-coils, tested were prone to degradation during recombinant expression in *E. coli* (K.M.A. & P. Pack, unpublished results). Thus, the coiled coil WinZip-A2B1 is a very useful independent heterodimerization module working equally well in both compartments of *E. coli*.

The generation of dimeric and tetrameric mini-antibodies has been established by fusing scFv fragments to homodi- and homotetramerization domains.[34,35,44] Furthermore, bispecific mini-antibodies have been designed by fusing scFv fragments in single-chain constructs[45] or by fusing scFv fragments to heterodimerization domains, such as the antibody domains $C_H/C_L$[46] or the coiled-coil domains of the Jun and Fos proteins.[47] However, larger constructs usually express in smaller amounts. The heterodimeric coiled coil of Jun and Fos is of limited stability and is therefore often stabilized by the addition of a disulfide bridge.[47] WinZip-A2B1 was shown to be more stable and more specific than Jun/Fos (K.M.A. *et al.*, unpublished results),[24,25] and will, therefore, be better suited for recombinant expression and guiding specific association.

The format of the helix-stabilized Fv fragment has some interesting advantages over other antibody fragments. Some antibodies might require free N termini of the $V_H$ and $V_L$ domain for high-affinity binding, either of which is blocked in the scFv but free in the hsFv format. The tendency of some scFv fragments to form dimers (diabodies) is not desired in selections from libraries with phage display, where dimers would be selected because of an avidity effect, rather than a true high intrinsic affinity. Working with defined species is also essential in quantitative assays, which do not tolerate a mixture of monomers and dimers, and in biological assays, where dimeric scFv fragments might cross-link the antigen, and therefore provide unexpected responses. This can be avoided using the hsFv format instead. In addition, the hsFv format allows expression of functional molecules, which is often not possible after introducing additional disulfide bridges to form a dsFv fragment. Furthermore, WinZip-A2B1 is disulfide-free, and can thus potentially be used for cytoplasmic expression (''intrabodies''). While the stability of the hsFv format *in vivo* still needs to be established, the small size and the monomeric state can be advantageous where fast serum clearance is an issue. We therefore believe that the format of a helix-stabilized Fv fragment can be a useful format, complementing those of Fab fragments, single-chain and disulfide-stabilized Fv fragments.

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