An intrinsically stable antibody scFv fragment can tolerate the loss of both disulfide bonds and fold correctly

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Abstract A fully functional cysteine-free derivative of the intrinsically stable anti-HER2 scFv fragment hu4D5–8 was generated by replacing the disulfide forming cysteine residues in V_H and V_L with the amino acid combination valine-alanine in both domains. The antigen binding properties, determined by ELISA and BIAcore measurements, were not affected by removal of the disulfide bonds. The thermodynamic stability of the disulfide-containing scFv of 8.1 kcal/mol is decreased upon complete reduction of both disulfides to 2.7 kcal/mol, while that of the valine-alanine variant is somewhat higher (about 3.8 kcal/mol). Our results suggest that, in principle, a disulfide-free fully functional derivative of any scFv can be obtained, as long as the corresponding disulfide-containing scFv has a high enough thermodynamic stability.

Key words: Disulfide bond; ScFv fragment; Intrabody; Antibody engineering; Protein stability

1. Introduction

Antibody domains carry an internal disulfide bond, which connects both β-sheets of the β-sandwich structure and is strictly conserved in evolution ([1–4]), witnessing its important contribution to the stability of the folded domain. Goto and Hamaguchi [5] have first experimentally demonstrated the strongly stabilizing effect by measuring the energy loss observed when refolding reduced C_L domains. Genetic removal of the disulfide bonds in the variable domains of the Fv, scFv or Fab fragment of the antibody McPC603 yielded no functional protein, suggesting a severe loss of stability in all variants tested, as well as in the reduced wild-type protein [6].

However, there are several antibodies in the database, which lack the conserved disulfide bridge in one of the domains. One of them, for which functionality has been proven, is the levan binding antibody ABPC48 ([7,8]), which has cysteine H92 in the heavy chain changed to tyrosine by somatic mutation. This antibody has been further studied in a scFv format [3], and restoration of the V_H disulfide bond resulted in a scFv fragment with above average stability [3]. The remaining disulfide bridge in the light chain of the A48 scFv could also be removed and a functional, completely cysteine-free antibody was obtained. However, this was only possible by introducing several additional mutations during an evolutionary approach, in which DNA shuffling [9] and selection for functionality by phage panning were alternated [10]. Some of the additional mutations created during DNA shuffling showed a globally stabilizing effect (A. Wörn et al., manuscript submitted).

It was also possible to remove the disulfide bond from the V_L domain REIv-κ, after introducing a globally stabilizing mutation ([11,12]). The structure of the disulfide-free REIv-κ V_L domain has been solved and was found to be essentially identical with the disulfide-containing REIv-κ domain [13]. Taken together, these results suggest that the disulfide bonds can be removed only from very stable antibody variable domains.

There is great interest in obtaining disulfide-free functional antibodies, because they could be expressed as intracellular antibodies (intrabodies) in the reducing environment of the cytoplasm ([14–16]). Many interesting applications can be envisaged, where antibodies would have to 'titrate out' cellular proteins. Intrabody studies carried out until now have been performed with antibodies still carrying the conserved cysteines. Because of the strongly reducing environment of the cytoplasm [17] it must be assumed that the disulfides do not form under these conditions. Indeed, this has been shown for one scFv fragment [18]. Nevertheless, the antibodies seemed to bind their antigen intracellularly, at least in the published experiments (for representative examples, see [19–21]). However, it cannot be excluded that only a small fraction of these intrabodies will be folded correctly. Thus, even better results may be achievable and a wider variety of antibodies may be functional when using antibodies optimized to fold in a disulfide-free format. In the case of the disulfide-free A48 scFv it could be shown by ELISA of crude cell extracts that the stabilized disulfide-free derivative was indeed more functional under reducing cytoplasmic conditions than a control A48wt scFv still carrying the cysteines in the heavy chain [10].

In the present study we wanted to test the generality of the concept that global stabilization is the prerequisite for disulfide removal. The successful production of the disulfide-free A48 scFv is so far the only one that has been reported. How-

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Abbreviations: AP, alkaline phosphatase; CDR, complementarity determining region; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; FU, fraction unfolded; GdnHCl, guanidinium hydrochloride; hu, humanized; IMAC, immobilized metal ion affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; K_D, equilibrium dissociation constant; PBS, phosphate-buffered saline; PBST, PBS with 0.05% Tween-20; PCR, polymerase chain reaction; $p185^{HER2-ECD}$, extracellular domain of human epidermal growth factor receptor-2; scFv, antibody single-chain fragment; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TBST, TBS with 0.05% Tween-20; V_H, variable domain of the heavy chain; V_L, variable domain of the light chain; 4D5-SS$^+$, oxidized form of 4D5 scFv with cysteines; 4D5-SS^{red}, reduced dithiol form of 4D5 scFv with cysteines; 4D5-SS$^-$, cysteine-free variant of 4D5 scFv

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ever, the A48 may be an unusual antibody, since it appears that the unpaired Cys H22 can face outward [3]. We thus wanted to test whether the ability to obtain the mutated A48 scFv fragment in a completely disulfide-free, correctly folded form had to do with this structural peculiarity. To address this question we investigated whether another 'normal' but very stable scFv can be converted to a stable disulfide-free form.

We have chosen the hu4D5–8 framework [22] to create a cysteine-free scFv for several reasons. First, the hu4D5 scFv, which binds the extracellular domain of human epidermal growth factor receptor-2 ($p185^{HER2-ECD}$) [23], has repeatedly been used in successful CDR grafting experiments ([24,25]). Therefore, a potential disulfide-free hu4D5 framework should also provide a promising framework for grafting different specificities, which could then be tested in intrabody studies. Second, one of the grafts derived from the hu4D5–8 scFv was shown to have above average thermodynamic stability [24], as does the hu4D5–8 scFv itself (shown in this study). We therefore reasoned that, from an energetic point of view, it might be possible to remove the conserved disulfide bridges without having to introduce any further stabilizing mutations in this framework, and still obtain acceptable stability.

The resulting cysteine-free hu4D5–8 scFv fragment (termed 4D5-SS$^-$) and a control hu4D5–8 scFv in exactly the same format, but still carrying the disulfide bridges (termed 4D5-SS$^+$), were purified and analyzed to prove that the removal of the disulfide bonds has no influence on the functionality of the scFv fragment. This was demonstrated qualitatively by ELISA and quantitatively by BIAcore measurements [26]. In addition, the thermodynamic stabilities of the two scFv fragments and the dithiol form of the cysteine-containing variant were compared by urea induced equilibrium unfolding.

2. Materials and methods

2.1. Construction of expression vectors

V_H and V_L domains were PCR amplified separately from a vector encoding the hu4D5–8 scFv in a V_H-$(Gly_4Ser)_3$-V_L orientation [27]. Overlapping sequences encoding a non-repetitive linker ([28,10]) were added at the 5′ end of V_H and 3′ end of V_L during the PCR reaction. The two PCR products were gel purified and assembled in a further PCR reaction. The assembled PCR product, encoding the hu4D5–8 scFv in a V_L-linker-V_H orientation, was cloned into the *Eco*RV and *Eco*RI sites of the secretion vector pIG6 [29], carrying an N-terminal FLAG and C-terminal Myc- and His-tag sequences. In the case of the cysteine-free hu4D5 mutant, the primers used for PCR amplification of V_H and V_L encoded point mutations replacing Cys H22 by Val, Cys H92 by Ala, Cys L23 by Val and Cys L88 by Ala (numbering according to Kabat [30]).

2.2. Protein expression and purification

Both the disulfide-containing 4D5-SS$^+$ and the cysteine-free 4D5-SS$^-$ were expressed in the periplasm of *E. coli* JM83 (λ^-, *ara*, Δ(*lac, proAB*), *rpsL*, *thi*, Φ80, *dlacZ*ΔM15) [31]. Cells were grown in SB medium at 25°C and induced in the late logarithmic phase by addition of 1 mM IPTG. Six hours after induction at 25°C the cells were harvested. The cell pellet from 1 l of culture was suspended in 20 ml of 40 mM Tris-HCl pH 7.5, 150 mM NaCl. Cells were lysed using a French press.

For the 4D5-SS$^+$ scFv protein, the supernatant of the whole cell extract was applied to two columns coupled in-line [32]. An immobilized metal ion affinity chromatography (IMAC) column was used first (making use of the C-terminal His-tag of the scFv fragment) and the eluate at pH 9.0 was directly pumped onto a HQ-anion exchange column from which the 4D5-SS$^+$ could be eluted with a 0–500 mM NaCl gradient at pH 9.0. The peak fraction was dialyzed against 40 mM Tris pH 9.0, 150 mM NaCl.

The 4D5-SS$^-$ scFv was purified from the insoluble part of the whole cell extract. The pellet of the cell extract was suspended in 20 ml 40 mM Tris, pH 8.0, 6 M GdnHCl, and purified by denaturing IMAC chromatography. The peak fraction was refolded by 1:100 dilution into a refolding buffer (0.5 mM 6-amino-*n*-caproic acid, 0.8 M arginine, 0.5 mM benzamidine hydrochloride, 2 mM EDTA, 0.2 M Tris-HCl, pH 9.0). The refolding mixture was incubated at 4°C for 24–48 h. After concentration of the refolding mixture by a factor of about 5 using an Amicon model 8200 ultrafiltration cell, it was dialyzed extensively against 40 mM Tris pH 9.0, 150 mM NaCl to remove most of the arginine. The 4D5-SS$^-$ scFv fragment could then be further purified by Protein-A Sepharose (Pharmacia-Biotech) chromatography [33]. Bound scFv fragment was eluted in a 100 mM citrate buffer, pH 3.0. The peak fraction was collected in a flask containing 1 M Tris pH 9.0 to minimize the exposure time of the scFv fragment to low pH. Buffer exchange into 40 mM Tris pH 9.0, 150 mM NaCl was performed using a PD10 Sephadex G-25M gel filtration column from Pharmacia-Biotech.

2.3. ELISA measurements

The ELISA plate wells were coated with 50 μl of 200 nM $p185^{HER2-ECD}$ antigen (kindly provided by P. Carter, Genentech Inc.) in PBS buffer, pH 7.0, overnight at 4°C. Wells were blocked with 5% milk in PBST for 1 h at room temperature. The respective scFv fragments (about 300 ng or 10 pmol) were then allowed to bind in a volume of 100 μl in PBST buffer for 1 h at room temperature. After two washing steps with water, a 1:2 dilution of an *E. coli* extract containing the anti-His-tag-scFv-AP fusion [34] in high TBST, pH 7.0, was allowed to bind for 1 h at room temperature, and AP activity was detected as described [34]. Specificity of binding was tested by preincubation of scFv (100 nM) with 200 nM soluble $p185^{HER2-ECD}$ antigen for 10 min at room temperature, before adding the mixture to the ELISA wells. All measurements were performed in duplicate. An A48 scFv [3], also carrying a C-terminal His-tag, but with different antigen specificity, was used as a negative control.

2.4. Analytical gel filtration

Analytical gel filtrations were performed with the SMART-system (Pharmacia), using a Superdex 75 column. All measurements were carried out in 40 mM Tris-HCl, pH 9.0, 150 mM NaCl with 0.005% Tween-20. The respective scFv fragments were injected at 3 μM in a volume of 50 μl. The column was calibrated in the same buffer with alcohol-dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carboanhydrase (29 kDa) and cytochrome c (12.4 kDa) as molecular mass standards.

2.5. BIAcore measurements

The $p185^{HER2-ECD}$ antigen at 100 μg/ml in 20 mM citrate buffer, pH 4.0 was coupled to the sensor chip using an amine coupling kit (Pharmacia AB Biosensor, Uppsala, Sweden). The two hu4D5 scFv fragments were applied at 2, 5, 10, 15, 20 μg/ml to the chip at a flow rate of 30 μl/min at 20°C. The surface was regenerated by injection of 45 μl of 0.1 M glycine-HCl, pH 2.2, 0.5 M NaCl. Data were analyzed using the global fit in the BIAevaluation program version 3.0.

2.6. Urea denaturation measurements

Urea denaturation measurements were performed and analyzed as described before [10] with the following modifications: ScFv concentrations of 5 μg/ml (about 166 nM) were used, all measurements were carried out in 40 mM Tris-HCl, pH 9.0, 150 mM NaCl. The dithiol form of the cysteine-containing 4D5 (4D5-SS^{red}) and the respective control of the 4D5-SS$^-$ were measured in degassed buffer of 20 mM DTT, 40 mM Tris-HCl pH 7.0, 150 mM NaCl and prepared from completely denatured and reduced protein. The proteins were incubated for 4 h at room temperature in the latter buffer containing 8 M urea prior to the preparation of the different samples, to allow complete reduction of the disulfides in the cysteine-containing 4D5 scFv fragment. Measurements in the DTT buffer were performed at higher protein concentration (500 nM) to compensate for the absorption effect of the DTT. Free energies were fitted according to Pace [35], prior to normalization of the denaturation curve to the fraction of unfolded protein.

3. Results

3.1. Design of the cysteine replacements

We have replaced the conserved disulfide bridges in both the heavy and the light chain of the scFv fragment hu4D5–8 by the amino acid combination Val-Ala. This combination has been most frequently selected during phage panning from 25 possible combinations in the case of the disulfide-free A48 scFv V_L domain [10]. Moreover, Liu et al. [36] have performed a systematic study on different replacements of the disulfide bridge in bovine pancreatic trypsin inhibitor and also found the combination Val-Ala to be the least destabilizing one. A Val-Ala pair should have similar steric requirements as a disulfide bridge, and the hydrophobicity of these two amino acids is compatible with the hydrophobic environment in the interior of antibody domains. This is important, because the burial of charges and the creation of unsatisfied hydrogen bonds in a hydrophobic environment, even if sterically possible, would be energetically very unfavorable ([37–39], among others).

3.2. Protein expression and purification

The hu4D5 scFv fragment with the conserved disulfide bridges in V_L and V_H accumulated mainly in the soluble fraction, when secreted to the periplasm at 25°C (Fig. 1). Purification yielded about 10 mg of pure scFv protein per liter *E. coli* JM83. In contrast, the same scFv fragment lacking the two disulfide bonds could not be purified from the soluble fraction after identical expression conditions. Essentially all 4D5-SS$^-$ scFv protein was insoluble in the *E. coli* periplasm (Fig. 1). The 4D5-SS$^-$ protein was thus obtained from the insoluble fraction after IMAC purification under denaturing conditions, and the relatively pure IMAC fraction was subjected to in vitro refolding and Protein-A Sepharose chromatography [33] of the dialyzed refolding mixture. Approximately 1 mg of soluble, refolded cysteine-free scFv could be obtained from 1 l *E. coli*.

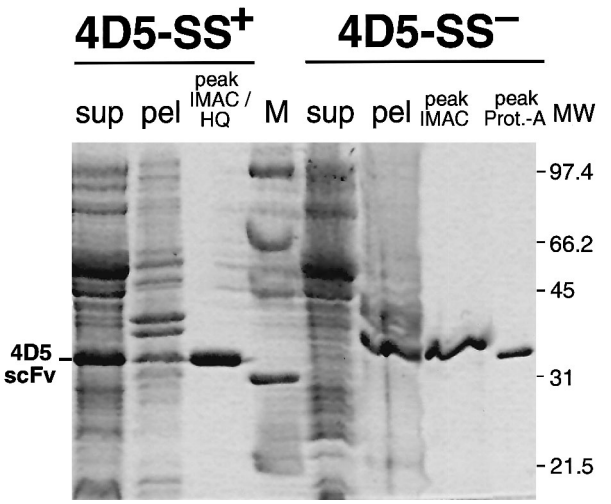

Fig. 1. 12% SDS gel documenting the periplasmic expression and purification of hu4D5 scFv with the conserved disulfide bridges (4D5-SS$^+$) and of the Val-Ala substitution mutant (4D5-SS$^-$) in V_H and V_L. Expression was performed at 25°C. 4D5-SS$^+$ was mainly in the supernatant (sup) of the cell extract, 4D5-SS$^-$ was mostly in the pellet (pel). The peak fraction of 4D5-SS$^+$, purified by IMAC and HQ-anion exchange chromatography is shown (peak IMAC/HQ). 4D5-SS$^-$ was purified by denaturing IMAC chromatography (peak IMAC), refolded in vitro and further purified by Protein-A Sepharose chromatography (peak Protein A). Note that the *pel* and *peak IMAC fraction* of 4D5-SS$^-$ contain GdnHCl, which disturbs the band shape.

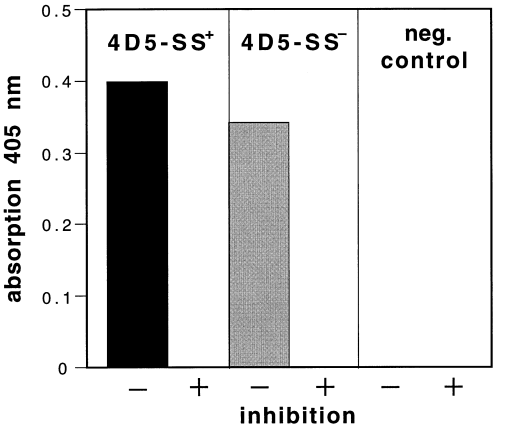

Fig. 2. Antigen binding ELISA of 4D5-SS$^+$, 4D5-SS$^-$ and a control scFv A48. ELISA wells were coated with 50 μl of 200 nM p185$^{HER2-ECD}$ antigen. Binding of 300 ng (about 10 pmol) scFv per well was performed in the absence (−) or presence (+) of 200 nM antigen. The 4D5-SS$^+$ and 4D5-SS$^-$ scFv fragments produced signals of similar intensities. No signal was produced by a control scFv fragment with His-tag, but a different antigen specificity (negative control). Binding could be inhibited with 200 nM p185$^{HER2-ECD}$ in all cases, proving the specificity of the binding reaction. Binding was detected with an α-His-tag-scFv-AP [34].

3.3. ELISA and BIAcore measurements

Both hu4D5 scFv fragments produced a signal in antigen binding ELISA, which was completely inhibitable by 200 nM antigen (Fig. 2). The intensities produced by equal amounts of hu4D5 scFv fragment with or without the disulfide bridges were similar. A control scFv (A48) carrying a His-tag, but directed against a different antigen (bacterial levan), did not give rise to any signal, further emphasizing the specificity of the hu4D5 scFv binding to p185$^{HER2-ECD}$. The K_D values determined by BIAcore were 121 pM for the 4D5-SS$^-$ scFv and 194 pM for the 4D5-SS$^+$ (data not shown).

3.4. Analytical gel filtration

Both the 4D5-SS$^-$ and the 4D5-SS$^+$ scFv fragment eluted in a sharp and symmetric peak from the Superdex-75 gel filtration column at an elution volume of 1.21 and 1.20 ml for 4D5-SS$^-$ and 4D5-SS$^+$, respectively. According to the molecular weight standards, this indicates a MW of about 29 000, and thus monomeric scFv for both molecules.

3.5. Urea denaturation measurements

The denaturation curve of the 4D5-SS$^+$ scFv had a midpoint at 4.8 M urea, that of 4D5-SS$^-$ scFv at 3 M urea (Fig. 3). The reduced dithiol form 4D5-SS^{red} of the cysteine-containing 4D5 scFv fragment, measured in 20 mM DTT, had its urea transition midpoint at 2.8 M urea, while the denaturation midpoint of the 4D5-SS$^-$ was shown to be not influenced by the presence of DTT, as one would expect from the absence of any cysteines. All scFv fragments showed a rather steep transition, suggesting that the unfolding follows a two-state model. Fitting of the denaturation curves [35] resulted in a stability of 8.1 kcal/mol for the 4D5-SS$^+$ (disulfides intact), about 3.8

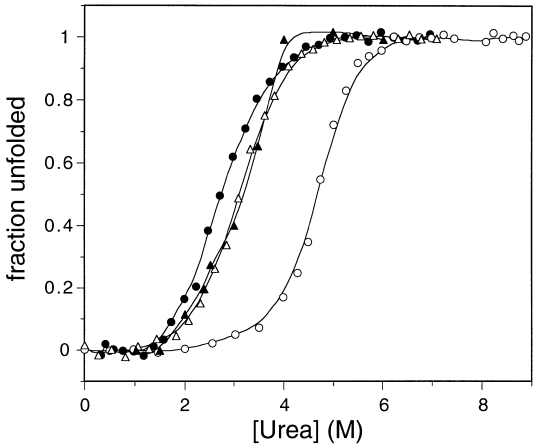

Fig. 3. Urea denaturation transitions of 4D5 scFv fragments with (4D5-SS$^+$) and without (4D5-SS$^-$) cysteines. The scFv 4D5-SS$^+$, carrying the conserved disulfide bridges, is shown in open circles (○). The corresponding dithiol form 4D5-SS^{red} is shown in filled circles (●). The scFv 4D5-SS$^-$, lacking all cysteines and thus the disulfides, is shown in the presence (▲) and in the absence (△) of DTT. The presence of DTT did not significantly affect the stability of the 4D5-SS$^-$, which contains no cysteines. In contrast, the reduced dithiol form 4D5-SS^{red} (●) of the cysteine-containing variant is significantly less stable than the oxidized form 4D5-SS$^+$ (○) and even less stable than the Val-Ala mutant (▲, △). Equilibrium unfolding in the absence of DTT (open symbols) was induced by incubating scFv solutions with increasing concentrations of urea. Measurements in the presence of DTT (filled symbols) were started from protein, which had been completely denatured and reduced with 20 mM DTT, pH 7.0, in 8 M urea for 4 h at room temperature. Denaturation transitions were measured by the shift in fluorescence emission maximum with changing urea concentration and are normalized to fraction unfolded (FU) protein.

kcal/mol for the 4D5-SS$^-$ (Val-Ala protein) with or without DTT present, and 2.7 kcal/mol for the reduced, dithiol form 4D5-SS^{red}. The Val-Ala replacements in the 4D5-SS$^-$ gave therefore rise to a slightly more stable protein, compared with the dithiol form 4D5-SS^{red} of the cysteine-containing 4D5.

4. Discussion

The observed loss of stability between the 4D5-SS$^+$ and its dithiol form (about 5.4 kcal/mol) is of the same order of magnitude as the stability contribution of disulfide bond in the C_L domain of a type λ Bence-Jones protein (4 kcal/mol) [5], in the C_L domain of a type κ Bence-Jones protein (4.7 kcal/mol) [40] and in different mutants of the REIv-κ variable domain (4.5 kcal/mol) [12]. However, one has to take into account that, in contrast to the examples mentioned above, *two* disulfide bridges are reduced in the scFv fragment 4D5. Interestingly, the Val-Ala variant of the 4D5 scFv (4D5-SS$^-$) was found to be slightly more stable (by 1.1 kcal/mol) than the dithiol form of the cysteine-containing variant (4D5-SS^{red}). This suggests that by simply replacing the cysteines by the Val-Ala pair one may already obtain a slight stabilizing effect for the application as intrabodies, but this may not be enough by itself.

The hu4D5 scFv fragment, with its urea denaturation midpoint at 4.8 M, permits an estimate how stable a scFv fragment needs to be in order to allow removal of the disulfide bridges without the requirement of introducing further stabilizing mutations. With the midpoint of the denaturation curve being at 3 M urea, the 4D5-SS$^-$ has a similar thermodynamic stability as some scFv fragments, which carry the conserved disulfide bridges ([41,42]).

An important prerequisite for further studies concerning disulfide-free scFv fragments as potential intrabodies is that the disulfide-free scFv fragments need to have unchanged antigen binding properties. X-ray crystallographic studies of the REIv-κ domain suggested that, in the case of this single domain, removal of the disulfide bridge did not produce any obvious local change in the structure [13]. However, no structure of a V_H domain without disulfide bond has been reported yet.

For the hu4D5 scFv fragment, we could test the influence of the removed disulfide bridges directly by functional studies. The ELISA signals (Fig. 2) showed qualitatively that both the 4D5-SS$^+$ and the 4D5-SS$^-$ scFv bind specifically to the p185$^{HER2-ECD}$ antigen. The antigen dissociation constants determined by BIAcore were also in the same range (121 pM for the 4D5-SS$^-$, 194 pM for the 4D5-SS$^+$). This similarity of binding clearly shows that the same native structure is reached in the presence and absence of the disulfide bonds. These values are in general agreement with K_D values of different constructs derived from the hu4D5–8 antibody published previously (90 pM by RIA for the Fab [22], 290 pM by ELISA for the F(ab′)$_2$ [43]). The binding properties were not influenced by avidity contributions due to diabody formation [44] in the present study, because both scFv fragments were shown to be monomeric by analytical gel filtration.

The observed dramatic difference between the 4D5-SS$^+$ and the 4D5-SS$^-$ scFv fragments regarding their solubility in the *E. coli* periplasm implies that the conserved disulfide bridges in V_H and V_L are of critical importance for the correct in vivo folding in the environment of *E. coli* JM83 periplasm. As discussed previously [6], this may either be caused by the insufficient thermodynamic stability of the 4D5-SS$^-$ variant or by the fact that early disulfide formation may be kinetically required for correct folding in the periplasm. Early disulfide formation in the 4D5-SS$^+$ may prevent certain aggregation pathways that are accessible to the more extended, disulfide-free chain of the 4D5-SS$^-$. We showed previously, however, that the disulfide-free version of the scFv A48 can be functionally obtained from the cytoplasm [10]. The quantitative folding of the variants in the cytoplasm of different cells, which may provide different sets of molecular chaperones to disallow these aggregation pathways, still needs to be compared.

ScFv fragments expressed in the reducing environment of the cytoplasm in eukaryotic cells were shown to have reduced cysteine thiol groups [18], and the disulfide bridge indeed does not seem to be formed in the cytoplasm. Expression of 'normal' scFv fragments and derivatives, like the 4D5-SS$^-$ reported here, with replaced cysteine residues may lead to an answer of the question whether, under in vivo conditions, the Val-Ala combination as a replacement for the disulfide bridge is indeed superior to two reduced cysteine thiol groups in the interior of the antibody variable domain, provided sufficiently stable scFv fragments are compared.

The most important conclusion from this and previous studies for intrabody applications is, however, that a minimum stability is necessary for the scFv fragment to fold under reducing conditions, or to tolerate the removal of the disulfide

bond by Val-Ala pairs. We suggest that only few natural antibody fragments will have the required stability without further engineering. Thus, three approaches can be envisaged to arrive at a scFv fragment which does not require disulfide bonds. First, a given antibody can be stabilized by evolutionary approaches as previously shown in the case of the scFv ABPC48 [10]. Second, a very stable framework such as hu4D5 can be used as recipient for the CDRs of interest [45]. Third, a CDR library on such a framework as the one described here can be constructed to select directly the disulfide-free variants. In all three approaches, the use of Val-Ala replacement for the cysteines seems advantageous, because of its slightly increased stability, compared with the dithiol form of cysteine-containing scFv fragments. A rigorous, quantitative approach will be necessary to further develop the technology of intrabodies.

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References

[1] Alzari, P.M., Lascombe, M.B. and Poljak, R.J. (1988) Annu. Rev. Immunol. 6, 555–580.
[2] Davies, D.R., Padlan, E.A. and Sherriff, S. (1990) Annu. Rev. Biochem. 59, 439–473.
[3] Proba, K., Honegger, A. and Plückthun, A. (1997) J. Mol. Biol. 265, 161–172.
[4] Padlan, E.A. (1996) Adv. Protein Chem. 49, 57–133.
[5] Goto, Y. and Hamaguchi, K. (1979) J. Biochem. 86, 1433–1441.
[6] Glockshuber, R., Schmidt, T. and Plückthun, A. (1992) Biochemistry 31, 1270–1279.
[7] Rudikoff, S. and Pumphrey, J.G. (1986) Proc. Natl. Acad. Sci. USA 83, 7875–7878.
[8] Auffray, C., Sikorav, J.L., Ollo, R. and Rougeon, F. (1981) Ann. Immunol. (Inst. Pasteur) 132D, 77–88.
[9] Stemmer, W.P.C. (1994) Nature 370, 389–391.
[10] Proba, K., Wörn, A., Honegger, A. and Plückthun, A. (1998) J. Mol. Biol. 275, 245–253.
[11] Frisch, C., Kolmar, H. and Fritz, H.J. (1994) Biol. Chem. Hoppe-Seyler 375, 353–356.
[12] Frisch, C., Kolmar, H., Schmidt, A., Kleemann, G., Reinhardt, A., Pohl, E., Usón, I., Schneider, T.R. and Fritz, H.J. (1996) Fold. Des. 1, 431–440.
[13] Usón, I., Bes, M.T., Sheldrick, G.M., Schneider, T.R., Hartsch, T. and Fritz, H.J. (1997) Fold. Des. 2, 357–361.
[14] Richardson, J.H. and Marasco, W.A. (1995) Trends Biotechnol. 13, 306–310.
[15] Biocca, S. and Cattaneo, A. (1995) Trends Cell Biol. 5, 248–252.
[16] Chen, S.Y., Bagley, J. and Marasco, W.A. (1994) Human Gene Ther. 5, 595–601.
[17] Gilbert, H.F. (1990) Adv. Enzymol. Relat. Areas Mol. Biol. 63, 69–172.
[18] Biocca, S., Ruberti, F., Tafani, M., Pierandrei-Amaldi, P. and Cattaneo, A. (1995) Biotechnology 13, 1110–1115.
[19] Mhashilkar, A.M., Biswas, D.K., La Vecchio, J., Pardee, A.B. and Marasco, W.A. (1997) J. Virol. 71, 6486–6494.
[20] Levin, R., Mhashilkar, A.M., Dorfman, T., Bukovsky, A., Zani, C., Bagley, J., Hinkula, J., Niedrig, M., Albert, J., Wahren, B., Göttlinger, H.G. and Marasco, W.A. (1997) Mol. Med. 3, 96–110.
[21] Biocca, S., Pierandrei-Amaldi, P., Campioni, N. and Cattaneo, A. (1994) Biotechnology 12, 396–399.
[22] Kelley, R.F., O'Connell, M.P., Carter, P., Presta, L., Eigenbrot, C., Covarrubias, M., Snedecor, B., Bourell, J.H. and Vetterlein, D. (1992) Biochemistry 31, 5434–5441.
[23] Fendly, B.M., Kotts, C., Vetterlein, D., Lewis, G.D., Winget, M., Carver, M.E., Watson, S.R., Sarup, J., Saks, S., Ullrich, A. and Shepard, H.M. (1990) J. Biol. Response Modif. 9, 449–455.
[24] Jung, S. and Plückthun, A. (1997) Protein Eng. 10, 959–966.
[25] Rodrigues, M.L., Shalaby, M.R., Werther, W., Presta, L. and Carter, P. (1992) Int. J. Cancer (Suppl.) 7, 45–50.
[26] Karlsson, R., Michaelsson, A. and Mattsson, L. (1991) J. Immunol. Methods 145, 229–240.
[27] Knappik, A. (1995) PhD Thesis, Biochemisches Institut, Universität Zürich, Switzerland.
[28] Tang, Y., Jiang, N., Parakh, C. and Hilvert, D. (1996) J. Biol. Chem. 271, 15682–15686.
[29] Ge, L., Knappik, A., Pack, P., Freund, C. and Plückthun, A. (1995) in: C.A.K. Borrebaeck (Ed.), Antibody Engineering, 2nd Edn., Oxford University Press, Oxford, pp. 229–266.
[30] Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S. and Foeller, C. (1991) NIH Publication No. 91-3242, National Institute of Health, Bethesda, MD, USA.
[31] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[32] Plückthun, A., Krebber, A., Horn, U., Knüpfer, U., Wenderoth, R., Nieba, L., Proba, K. and Riesenberg, D. (1997) in: J. Mc Cafferty, H.R. Hoogenboom and D.J. Chiswell (Eds.), Antibody Engineering, A Practical Approach, Oxford University Press, Oxford, pp. 203–252.
[33] Åkerström, B., Nilson, B.H.K., Hoogenboom, H.R. and Björck, L. (1994) J. Immunol. Methods 177, 151–163.
[34] Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R. and Plückthun, A. (1997) BioTechniques 22, 140–149.
[35] Pace, C.N. (1990) Trends Biotechnol. 8, 93–98.
[36] Liu, Y., Breslauer, K. and Anderson, S. (1997) Biochemistry 36, 5323–5335.
[37] Lim, W.A. and Sauer, R.T. (1989) Nature 339, 31–36.
[38] Serrano, L., Kellis, J.T.J., Cann, P., Matouschek, A. and Fersht, A.R. (1992) J. Mol. Biol. 224, 783–804.
[39] Ladbury, J.E., Wynn, R., Thomson, J.A. and Sturtevant, J.M. (1995) Biochemistry 34, 2148–2152.
[40] Goto, Y., Tsunenaga, M., Kawata, Y. and Hamaguchi, K. (1987) J. Biochem. 101, 319–329.
[41] Knappik, A., Krebber, C. and Plückthun, A. (1993) Biotechnology 11, 77–83.
[42] Pantoliano, M.W., Bird, R.E., Johnson, S., Asel, E.D., Dodd, S.W., Wood, J.F. and Hardman, K.D. (1991) Biochemistry 30, 10117–10125.
[43] Carter, P., Kelley, R.F., Rodrigues, M.L., Snedecor, B., Covarrubias, M., Velligan, M.D., Wong, W.L.T., Rowland, A.M., Kotts, C.E., Carver, M.E., Yang, M., Bourell, J.H., Shepard, H.M. and Henner, D. (1992) Biotechnology 10, 163–167.
[44] Pack, P. and Plückthun, A. (1992) Biochemistry 31, 1579–1584.
[45] Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S. and Winter, G. (1986) Nature 321, 522–525.