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Selection for Improved Protein Stability by Phage Display

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A library of mutants of a single-chain Fv fragment (scFv) was generated by a combination of directed and random mutagenesis, using oligonucleotides randomized at defined positions and two rounds of DNA shuffling. The library was based on the already well folding and stable scFv fragment 4D5Flu. In order to further improve this framework and test the efficiency of various selection strategies, phage display selection was carried out under different selective pressures for higher thermodynamic stability. Incubation of the display phages at elevated temperatures was compared to exposure of the phages to high concentrations of guanidinium chloride. Temperature stress-guided selection yielded the most stable scFv mutant after two rounds of mutagenesis and selection, due to the irreversibility of the unfolding process. It possessed only two mutations (His(L27d)Asn and Phe(L55)Val) and showed a thermodynamic stability improved by roughly 4 kcal/mol, threefold better expression yields in *Escherichia coli* as well as a 20-fold better binding constant than the 4D5Flu wild-type. The selection results obtained in this study delineate the advantages, disadvantages and limitations of different stability stress selection methods in phage display.

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Introduction

Recombinant antibody fragments produced in microorganisms have a great potential as specific targeting reagents and may replace traditional animal immunization. This is a technology based entirely on microbial biotechnology, which is amenable to high throughput generation of reagents. There has been growing interest especially in the single-chain Fv (scFv) format of antibodies, particularly for *in vivo* diagnostic and therapeutic applications, due to its small size. Nevertheless, for overtaking traditional monoclonal technology, scFv fragments need to fulfil four important requirements: (i) they need to bind strongly and specifically to the target; (ii) they

must be stable at the body temperature of 37 °C for a long time as well as during the storage and incubation steps of analytical applications; (iii) they must be available in large amounts in a cheap production system, such as *Escherichia coli*; and (iv) for therapeutic applications, they should also resemble human antibodies so that they are not attacked by the patient's immune system.

ScFv fragments have been derived from existing monoclonal antibodies or from natural (Vaughan *et al.*, 1998), semisynthetic (Griffiths & Duncan, 1998) or fully synthetic (A. Knappik *et al.*, unpublished results) libraries (reviewed by Winter *et al.*, 1994). Natural libraries can be derived from human blood or tissue samples, while semisynthetic and synthetic are often constructed by introducing randomized CDR-cassettes in human Fv frameworks. ScFv fragments specific for a given target have to be obtained from these libraries by various selection techniques, most commonly by phage display (reviewed by Griffiths & Duncan, 1998; Hoogenboom *et al.*, 1998). The advantages of the scFv selection in a recombinant system are that the complete procedure occurs without the need for animal experiments and that the selected scFv

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Abbreviations used: scFv, single-chain Fv antibody fragment; V_H, heavy chain of an antibody Fv fragment; V_L, light chain of an antibody Fv fragment; ELISA, enzyme-linked immunosorbent assay; BSA-FITC, fluorescein-isothiocyanate coupled to bovine serum albumin; GdmCl, guanidinium chloride; g3p, gene-3-protein of filamentous phage; w.t., wild-type.

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fragment is already in a format suitable for production in a bacterial host.

Nevertheless, the use of phage display does not automatically guarantee selection either for superb folding properties and thus high production yields, or for high stability. Very poorly folding molecules can be displayed, and whether they will be selected depends entirely on whether the library contains any "better" molecules. Furthermore, the enrichment of superb molecules over poorly folding ones proceeds with relatively low enrichment factors (Jung & Plückthun, 1997), if no particular selective pressure is exerted for stability.

Different phage display strategies have been used to select for higher stability. One strategy consisted of the initial reduction of the protein stability by protein engineering and selection under normal phage display conditions (Braisted & Wells, 1996; Proba *et al.*, 1998; Ruan *et al.*, 1998), partly with the subsequent reintroduction of stabilizing structural elements in the selected molecules. Another approach was the selection for protease resistance as a property of native, thermodynamically stable proteins (Kristensen & Winter, 1998; Sieber *et al.*, 1998). However, in the present study, we wished to investigate how a protein of interest can be selected for higher stability directly, without previous destabilization, and under constant selection pressure for functionality.

The framework of the humanized 4D5 anti-HER2 antibody has been successfully used for CDR-grafting in several cases (Carter *et al.*, 1992; Rodrigues *et al.*, 1992; Jung & Plückthun, 1997; Willuda *et al.*, 1999). This framework carries all important properties that a scFv for a medical application should have: it is humanized, stable at $37\,^{\circ}$C and well folding during production in *E. coli*, and we show here that it behaves well in a phage display library setting. We wanted to use this favorable framework as a starting point in a model study to investigate whether it can be improved even further with respect to folding and stability. For this purpose, we randomized a scFv called 4D5Flu (Jung & Plückthun, 1997) that consists of the 4D5 framework with grafted CDRs from the fluorescein-binding antibody 4-4-20 (Bedzyk *et al.*, 1992) and selected the resulting library for stability. As the selection method, we chose phage display. In particular, we investigated different strategies to apply selection pressures for the improvement of folding and stability. For randomization, we chose DNA shuffling (Stemmer, 1994b), as well as oligonucleotides encoding mutations at defined positions. Further improved scFv fragments could indeed be obtained, and the merit of different selection strategies could be compared.

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† http://imgt.cnusc.fr:8104/
‡ http://www.mrc-cpe.cam.ac.uk/imt-doc/
§ http://www.ibt.unam.mx/paginas/almagro/
V_mice.html#DAMvh

Results

Library construction

In previous studies (Jung & Plückthun, 1997), we had found that the framework of the antibody hu4D5 (Carter *et al.*, 1992), itself the product of humanization and derived from the human V_H germline IGHV 3-66 (IMGT nomenclature, Marie-Paule Lefranc†), or VH 3-18 (Vbase nomenclature, Ian Tomlinson‡), locus DP 3-66 (DP-86) and the kappa light-chain variable domain derived from germline IGKV 1-39 (IMGT), VK 1-1 (Vbase), locus DP O12, could accept the loops of the poorly folding anti-fluorescein antibody 4-4-20 (Bedzyk *et al.*, 1992; Nieba *et al.*, 1997) with the murine V_H germline V(H)22.1 and V_L kappa germline V-1A/gene K5.1 (murine germline database ABG§), and result in a molecule with superior folding properties and without any loss of affinity. In order to optimize this framework further, we have now combined both insight from a series of defined mutagenesis experiments (Knappik & Plückthun, 1995; Forsberg *et al.*, 1997; Nieba *et al.*, 1997; L. Nieba & A.P., unpublished results) with further random mutations and the power of evolutionary methods. The scFv fragment 4D5Flu was subjected to DNA shuffling and random mutagenesis at a few defined positions, as explained in the following paragraphs.

The folding yield of the original 4-4-20 scFv could be significantly improved by substituting solvent-exposed hydrophobic residues by polar residues (Nieba *et al.*, 1997). Particularly the residues of the former interface between the variable and the constant domains in the Fab fragment (V/C interface), exposed to solvent in the scFv fragment, frequently form large hydrophobic patches. Nevertheless, in the V_L domain, lateral interactions between hydrophobic surface residues contribute to stability, and the disruption of these patches led to decreased stability of the domain (L. Nieba & A.P., unpublished results). In contrast, the substitution of the less contiguous hydrophobic V/C interface residues to polar or charged ones in the V_H domain decreased the aggregation of the scFv fragment in folding without adversely affecting the stability (Nieba *et al.*, 1997). Therefore, the V/C interface residues have to find a difficult balance between sufficient lateral interactions, yet without generating any hydrophobic patches, to optimize the compromise between stability and folding yield. The V/C interface composition in an scFv fragment is therefore an ideal target for an evolutionary approach.

In the scFv hu4D5, and therefore also in the 4D5Flu graft, most of the successful substitutions are already present, contributing to the good folding behavior of this antibody fragment. For this reason, 4D5Flu variants with single point mutations towards serine in positions H11, H89 or H108, respectively, were randomly combined with the 4D5Flu w.t. using the DNA shuffling method

(Stemmer, 1994b) (see Materials and Methods). In addition, residues H6 (Glu/Gln) and H9 (Gly/Pro/Ala/Arg) were randomized by using an oligonucleotide added to the DNase digest during DNA shuffling to cover the natural variants of framework I conformations. The substitution of the buried Glu side-chain in the framework position H6 by an uncharged Gln had been reported to improve stability and production of certain antibody fragments (Kipriyanov *et al.*, 1997; de Haard *et al.*, 1998; Langedijk *et al.*, 1998). Moreover, H6 and H9 define different framework classes (A.H. *et al.*, unpublished results; Saul & Poljak, 1993). In position H49, Gly was introduced as an alternative to the natural Ala, since Gly is more compatible with the positive Phi torsion angle usually found in this position. The residues introduced by this randomization strategy were found in unselected sequences, indicating that the introduction of the mutations did occur at the DNA level, but with the exception of GlyH49, they were not found after selection in the 4D5Flu sequences. The position of the randomized amino acid residues in the sequence and structure model of the 4D5Flu are shown in Figure 1(a), and the reasons for targeting these amino acid residues are summarized in Table 1.

The initial library was created by a combination of targeted and error-prone randomization. A mini library of mutants L11S, V89S and L108S was created by PCR assembly and cloning, followed by DNA shuffling in the presence of five oligonucleotides with the randomized codons mentioned above (see Materials and Methods, and Table 1). This procedure allowed the targeted randomization of the analyzed positions in a combinatorial fashion. At the same time we used *Taq* polymerase in this experiment to allow additional mutations to appear in order to find new leads for enhanced folding and stability, which may not have been identified yet by rational modeling.

For the DNA shuffling, we found that there was an optimum total amount of 3-25 pmol for the DNA fragments and oligonucleotides to be incorporated in the shuffling reaction, and the oligonucleotides had to be present in up to 20-fold molar excess over the DNase I-generated fragments. The library sizes were between 1.1×10^7 and 5.8×10^7, which resulted in a total of 1.3×10^8 members in all four libraries together, created by different molar ratios of spiking oligonucleotides to DNase-generated DNA fragments. The theoretical size of the library with all combinations of oligonucleotide-introduced random mutations was 1.5×10^6, so that each mutant should be represented approximately 100 times in the experimental library. In practice, however, this overrepresentation is reduced due to the PCR procedure. First, additional random mutations are introduced by the *Taq* polymerase used for the assembly PCRs and by the assembly process itself, generating about six mutations per scFv gene, on average (detected range 1-13), which should lead to about
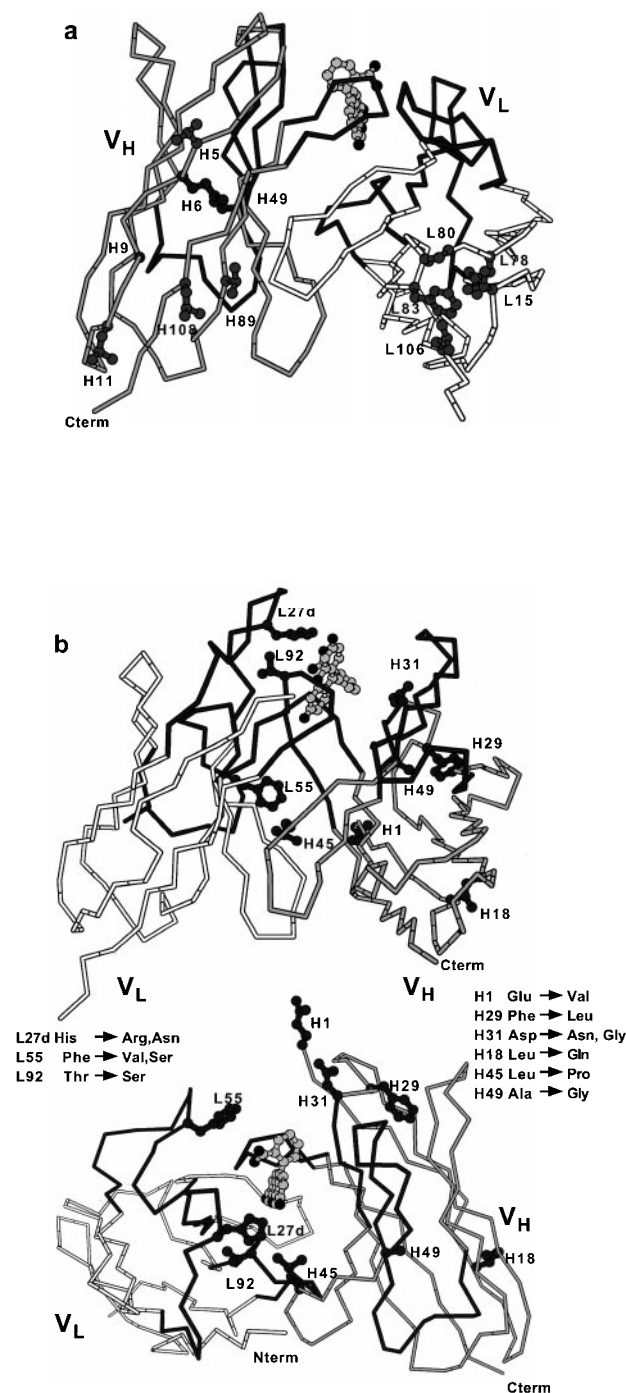
Figure 1. Model of 4D5Flu showing the position of (a) the purposely randomized and (b) the selected mutations. The model was created using the structures of the parent Fv fragments 4-4-20 and 4D5 (for details, see the text). (a) Residues in the 4D5Flu model randomized by targeted randomization. H5, H11, H89, H108, L15, L78, L80 and L83, solvent-exposed hydrophobic residues; H6 and H9, framework I conformation; H49, positive φ torsion angle (for details, see Table 1). (b) Some of the amino acid positions frequently found altered after six panning rounds in the second DNA shuffling round. Positions of mutations in clones that were further biophysically characterized.

Table 1. Amino acid positions randomized in the 4D5Flu phage display library for optimization of *in vivo* folding and thermodynamic stability

Position	w.t. amino acid	Intended mutations[a]	Allowed mutations in randomization[b]	Rationale
V_L 15	V	S,T,L,P	V,S,T,L,P,M	Residues at former V/C interface
V_L 78	L	T,V,A	L,T,V,A,P,M	and involved in core packing:
V_L 80	P	S,A	P,S,A	increase hydrophilicity and optimize
V_L 83	F	S,T,A	F,S,T,A,I,V	core-packing (Forsberg *et al.*, 1997;
V_L 106	L	S,T	L,S,T	Nieba *et al.*, 1997)
V_H 5	V	Q	V,Q,L,E	Q is highly conserved/exposed
V_H 6	E	Q	E,Q	Subgroup change: charged residue
V_H 9	G	A,P	G,A,P,R	buried in core
V_H 11	L	S,D,N	L,S,D,N,F,Y,P,H,I,T,V,A	Increase hydrophilicity
V_H 89	V	S		at former V/C
V_H 108	L	S		interface
V_H 49	A	G	A,G	Fairly conserved G with positive ϕ-angle

[a] Mutations intended for the reasons given in the column Rationale.
[b] The mutations were encoded on oligonucleotides by randomized bases. The oligonucleotides were introduced into the DNA shuffling experiment, shuffling the 4D5Flu w.t. and the previously constructed single point mutants L(H11)S, V(H89)S, L(H108)S.

one to two amino acid exchanges per gene, on average. Second, in the standard commercial oligonucleotide synthesis, the base mixture at the randomized positions may be biased to various degrees. Additionally, there is the possibility of bias in the incorporation frequency of the five oligonucleotides. Sequencing results of the unselected library, however, suggested a sufficient random distribution at all positions, and a negligibly low percentage of genes with base-pair deletions outside the synthetic oligonucleotides, while base-pair deletions within the synthetic oligonucleotides reduced the library size by 20%.

Choice of selection conditions

The appropriate selection conditions for the 4D5Flu-based library were initially established by investigating the stability and binding limits of phages displaying the 4D5Flu w.t. scFv fragment (Figure 2). This was achieved by incubating the phages either at different elevated temperatures or different concentrations of guanidinium chloride (GdmCl) for a defined time and subsequently assaying the integrity of both the displayed scFv fragment and the phage itself, as a function of the incubation temperature or GdmCl concentration. The functionality of the scFv was tested by phage ELISA on FITC-BSA (fluorescein-isothiocyanate coupled to bovine serum albumin), while the integrity of the phage was tested by measurement of reinfection titers. It was found that under both stress conditions the stability of the scFv 4D5Flu was limiting (Figure 2).

The reinfection titers are not affected significantly by GdmCl, as they drop by only approximately one order of magnitude after an overnight incubation of phages in GdmCl between 0 and 6 M GdmCl (Figure 2(b)), consistent with previous determinations (Figini *et al.*, 1994). The ELISA signal on a FITC-BSA coated surface, however, drops sharply between 2 M and 2.5 M GdmCl, reflecting the stability limit of 4D5Flu (Figure 2(b)). Since binding of the scFv 4D5Flu to fluorescein is itself affected by GdmCl (Table 2, Figure 2(c)), ELISAs and panning can be performed only after diluting the incubation mixture to one-tenth of the GdmCl concentration during the binding procedure. The drastic decrease of the signal between 2 and 2.5 M GdmCl (incubation concentration) (Figure 2(b)) is nevertheless a stability and not a binding effect, suggesting partial irreversibility in GdmCl-induced denaturation, as native 4D5Flu phages show a gradual decrease in ELISA signal between 0.1 and 1 M GdmCl present in the ELISA well, not reflecting this transition, which should occur between 0.2 and 0.25 M, if it existed (Figure 2(c)). With the phage library pools giving a higher ELISA signal than the w.t., it could later be confirmed that the transition in the ELISA signal around 2.5 M GdmCl was indeed a stability effect and not a binding effect, as this observed transition takes place at the same denaturant concentration in the undiluted incubation mixture, independent of the subsequent dilution chosen for the ELISA (see below and Figure 3(b)).

Temperature stress is more harmful to the phage itself, as increasing temperature continuously decreases reinfection titers with a sudden drop of reinfection by several orders of magnitude between 60 °C and 65 °C (Figure 2(a)). The ELISA signals of 4D5Flu phages, however, reflect a transition already between 50 °C and 55 °C, which has to be attributed to the stability of the scFv fragment rather than to that of the phage (Figure 2(a)). Therefore, we can conclude that by both types of denaturing conditions the stability of a library of 4D5Flu point mutants can be challenged, before the stability limits of the phage itself are reached. For a 4D5Flu-based library we chose as starting selection pressure 50 °C or 2 M GdmCl, respectively, as these conditions still yield sufficient

activity of w.t. scFv for ELISA binding or for phage capture in the panning procedure (Figure 2(a) and (b)).

Library selection

The library selection conditions are represented in Figure 4. In order to retain a maximum of diversity and to select merely for functionality in the initial rounds, thereby eliminating sequences with deletions or unfavorable mutations, the four libraries assembled from different ratios of fragment to oligonucleotide were kept separately, and no selection pressure in the form of GdmCl or temperature was employed during the first two rounds. Furthermore, the first phage proliferation step was conducted at 24 °C after induction of phage and scFv production. From the second round on, the phage proliferation temperature was set to 37 °C, thereby establishing a selection pressure towards correct folding at 37 °C, which is often already a critical temperature for the *in vivo* folding of several scFvs. The 4D5Flu w.t., however, shows sufficient *in vivo* folding at 37 °C (Jung & Plückthun, 1997), so that the w.t. and all putative ''better'' mutants do not have any problem with this selection barrier. From the third round on, the four libraries, which were assembled with different ratios of ''doping'' oligonucleotides and template, were combined and subdivided into three sublibraries that were subjected to different stress conditions: a control sublibrary that was not put under any stress in any round; a temperature-stressed sublibrary and a GdmCl-stressed sublibrary, which were exposed to the respective denaturing stress conditions prior to panning in every round (Figure 4).

The temperature-sensitivity of the library was relatively high, so that in the first phage display round with an incubation temperature of 50 °C, which had been a tolerable temperature for the w.t. in the ELISA experiments, the recovery of phages of the temperature-stressed library from the panning tube was quite low (Table 3A). However, in the parallel control with an equal amount of 4D5Flu w.t. phage, the recovery after affinity selection was even lower so that the library still performs better under temperature stress than the w.t. scFv. In the subsequent rounds the phage recovery increased again for the temperature library, in the fifth round being one order of magnitude higher than for the control wild-type and more than one order of magnitude lower than for the control ''no stress'' library. This indicates that a population of scFvs has been selected with higher temperature stability than the wild-type.

The overnight incubation with 2 M GdmCl had only a small effect on the library: the numbers of library phages rescued from panning were only slightly lower in the GdmCl than in the no stress library aliquot, but 100 times higher than for the GdmCl-incubated wild-type control (Table 3A). Consequently, the GdmCl concentration was
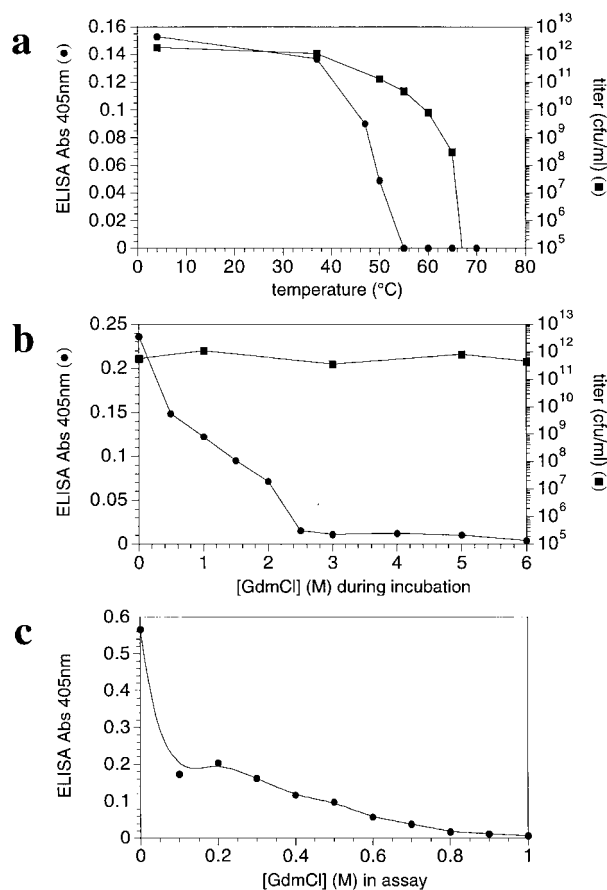
Figure 2. Antigen-binding ELISA signals and reinfection titers of 4D5Flu w.t. phages after denaturation under various conditions. ELISA signals were all completely inhibitable with 10 μM fluorescein, indicating specific binding. (a) Temperature-dependence of ELISA signals (●) and reinfection titers (■) of 4D5Flu w.t. phages. (b) Dependence of ELISA signals (●) and reinfection titers (■) of 4D5Flu w.t. phages after exposure to GdmCl at the indicated concentrations overnight at 10 °C. For the ELISA binding step, the phages were first diluted 1:10 into PBST. The ELISA-binding was carried out at 5 °C for 30 minutes. (c) Dependence of ELISA signals (●) of 4D5Flu w.t. phages on GdmCl concentrations present in the assay to measure the inhibition of the binding step itself.

increased in the fifth round to 2.25 M and in the sixth round to 2.5 and 3.0 M, respectively. After five or six rounds, the ELISA signals of the libraries were approximately tenfold higher than that of the w.t., suggesting a successful selection (Figure 3(a) and (b)). Sequencing of a small subset of selected clones indicated already a predominance of certain mutations for all libraries. On the other hand, the diversity within the pools was still considerable, and the number of mutations detected per clone was high, on average (ca four amino acid exchanges per sequence). Therefore, a second DNA shuffling round was carried out.

Table 2. Biochemical and biophysical properties of 4D5Flu w.t. and five mutants selected by phage display

Mutant	Library origin[a]	Mutations	Expression yield (mg/l A)	K_D (nM)[b]	K_D (in 0.3 M GdmCl)[c] (nM)[b]	Midpoint of GdmCl unfolding (M) (λ_{max}/I)[d]	ΔG of unfolding[e] (kcal/mol) (λ_{max}/I)[d]	T_M in DSC[f] (°C)
w.t.			0.6	34	180	2.4/2.3	14.0/11.6	62.3
50	*Pfu*/temperature	H(L27d)N/ F(L55)V	1.9	1.6	n.d.	2.8/2.7	17.7/14.4	66.2
3M	*Pfu*/GdmCl	H(L27d)R/ F(L55)V/ T(L92)S	0.7	1.2	8.8	2.6/2.5	15.1/12.4	n.d.
G1	*Taq*/GdmCl	H27(L27d)R/ F(L55)V/ L(H18)Q/ D(H31)G	0.5	3.9	n.d.	1.9/1.7	10.5/3.8	n.d.
G2	*Taq*/GdmCl	H(L27d)R/ F(L55)S/ T(L92)S/ E(H1)V/ D(H31)N/ L(H45)P	0.5	3.3	13	2.2/2.1	12.0/14.4	n.d.
T0	*Taq*/none	H(L27d)R/ F(L55)S/ F(H29)L/ A(H49)G	0.4	16	n.d.	2.3/2.1	13.0/7.3	n.d.

[a] The libraries from which the listed clones originated are defined by the polymerase with which the second generation was generated, and the type of selection pressure applied.

[b] The estimated error of K_D measurements by fluorescence titration is approximately a factor of 2, due to activity variations among sample preparations and the error inherent to acitivity determinations, both by curve fitting and activity titration.

[c] In this set of experiments, K_D was determined by fluorescence titration in the presence of 0.3 M GdmCl.

[d] The equilibrium unfolding was monitored by the change of the intrinsic protein fluorescence as a function of the GdmCl concentration. Transition data differ slightly when either the shift of the wavelength of the fluorescence maximum (λ_{max}) or the change in fluorescence intensity at 335 nm (I) are analyzed.

[e] ΔG values were estimated according to Pace (1990) assuming a two-state transition (Figure 6(a)).

[f] Differential scanning calorimetry.

Construction and selection of the second generation library

A second generation library was constructed in a second DNA shuffling round, using the selected clones from both temperature and GdmCl-exposed sublibraries (see Materials and Methods for details), and an excess of 4D5Flu w.t. for backcrossing to eliminate unproductive mutations. In this shuffling round, two separate sets of libraries were created. In one experiment, DNA shuffling was performed with *Pfu* proofreading polymerase for the first assembly PCR of the DNA fragments without primers, to merely recombine the previously selected mutations. In the other DNA shuffling experiment, *Taq* polymerase was used again to further randomize the library in order to find mutations that would further improve the 4D5Flu scFv.

The subsequent library selection was performed in six rounds of phage proliferation, temperature or GdmCl stress and panning (Figure 4). The GdmCl concentration for the GdmCl sublibrary was increased from 2.5 to 3.5 M in subsequent panning rounds, and the temperature stress from 50 °C to 60 °C. It had been possible to increase the selection pressure in each round with satisfying recovery rates from the panning steps (Table 3B).

After the sixth round, the ELISA signals of the phage pools showed a much higher signal than the w.t., which was confirmed after recloning of the pools in fresh phagemid vector and for single randomly chosen pool members (Figure 3(c) and (d)).

The pools of selected scFv were cloned into an expression vector for soluble scFv fragments, and 10-14 clones were sequenced from each sublibrary (Figure 5). Large differences between the libraries became apparent according to the sort of stress exerted during selection. In the pools that were obtained by temperature selections, all selected phages from the *Pfu* polymerase-generated library had the identical sequence, and only two further variations of this species were found in the *Taq* polymerase-generated library. In contrast, in the GdmCl selected pools the diversity was much higher, although certain mutations predominate. The variability in the no stress control pool was even greater, showing the occurrence of selection by temperature, and somewhat less so by GdmCl. After a preliminary expression analysis of a small number of promising clones (data not shown), five clones with the best growth behavior and highest ELISA signals were chosen for detailed analysis of the scFv in comparison with the w.t. 4D5Flu with respect to *in vivo* folding and thermodynamic stability.
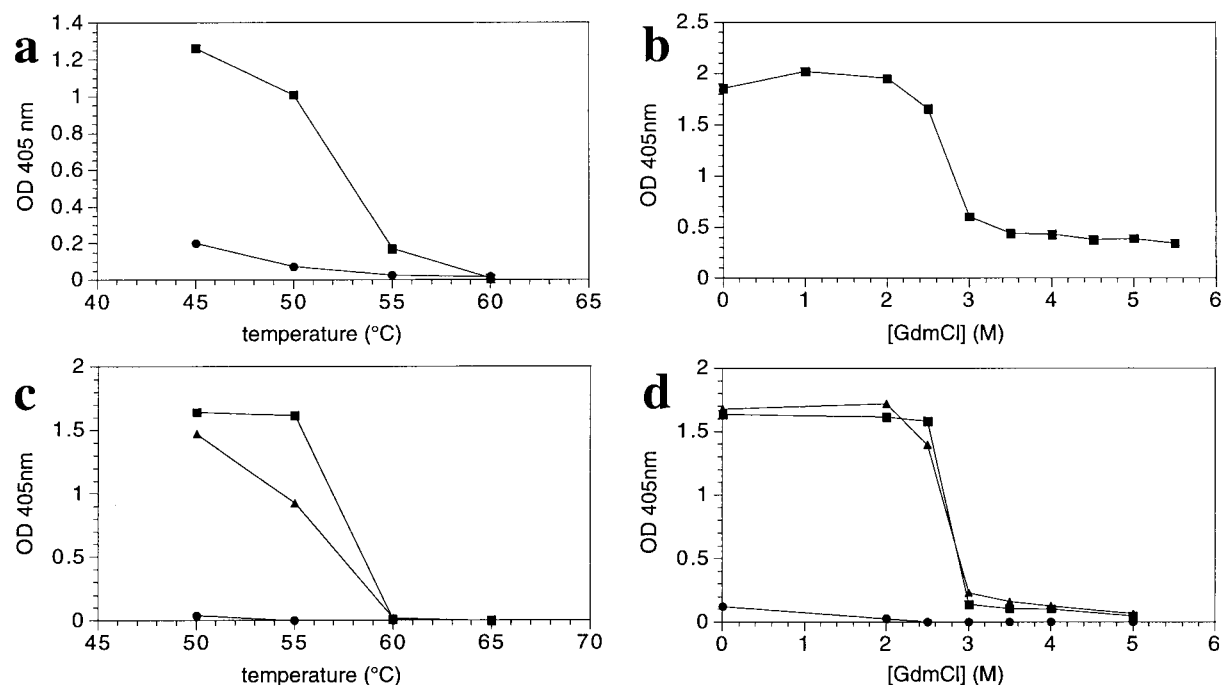
Figure 3. ELISA signals of selected pools after denaturation in comparison to w.t.: libraries generated with *Taq* (■), libraries generated with *Pfu* (▲), unmodified w.t. (●). For all experiments, inhibition with 10 μM fluorescein resulted in complete inhibition of the signals, indicating specific binding (data not shown). (a) Temperature-dependence of ELISA signals of pools selected after five rounds of phage proliferation, temperature stress and panning in the first DNA shuffling round. (b) GdmCl concentration-dependence of ELISA signals of pools selected after six rounds of phage proliferation, GdmCl stress and panning in the first DNA shuffling round. The phages were denatured overnight at 10 °C with the indicated GdmCl concentrations, and diluted 1:100 into PBST for the ELISA binding. The ELISA-binding was carried out at 5 °C for 30 minutes. (c) Temperature-dependence of ELISA signals of pools selected after six rounds of phage proliferation, temperature stress and panning in the second DNA shuffling round. (d) GdmCl concentration-dependence of ELISA signals of pools selected after six rounds of phage proliferation, GdmCl stress and panning in the second DNA shuffling round. The phages were denatured overnight at 10 °C with the indicated GdmCl concentrations, and diluted 1:10 into PBST for the ELISA binding. The ELISA-binding occurred at 5 °C for 30 minutes.
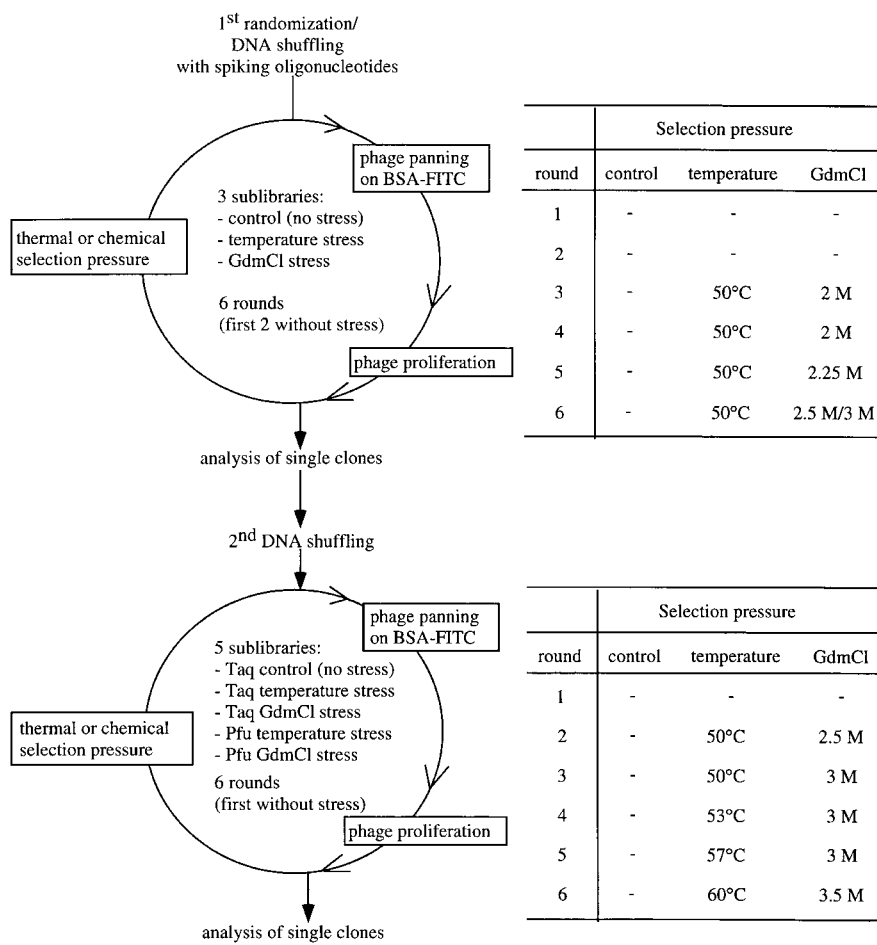
Analysis of selected scFvs

The selected 4D5Flu mutants chosen for biochemical and biophysical comparison with the w.t. are listed in Table 2, and the position of their mutations are illustrated by Figure 1(b). Clone 50 was the only member of the library pool generated by *Pfu* polymerase and subjected to temperature pressure. Clone 50 differs in only two mutations from the w.t., namely His(L27d)Asn in CDR-L1, which makes direct contact to the antigen in the parent structure of scFv 4-4-20 (Herron *et al.*, 1994; Whitlow *et al.*, 1995), and Phe(L55)Val at the end of CDR-L2, which is buried in the interface and has no apparent antigen interaction, but could have an influence on the V_L-V_H interface and the relative orientation of the two domains towards each other, thus indirectly affecting antigen binding. The other four clones chosen for analysis contain mutations at the same positions as clone 50 plus one to four additional mutations. Clone 3M is derived from the *Pfu*-generated library selected under GdmCl denaturation pressure, clones G1 and G2 are chosen from the *Taq*-generated pool

selected by GdmCl denaturation and clone 0T is a randomly chosen clone from the no stress control pool.

All five mutants and the w.t. scFv 4D5Flu could be easily produced by periplasmic expression in shake flask cultures and purified by the one hour, standard in-line two-column procedure (Plückthun *et al.*, 1996). All mutants gave yields comparable to the w.t. except for clone 50, which showed two to three times higher expression yields, suggesting a correlation of stability and *in vivo* folding yields in this case (Table 2). All mutants were monomeric, as determined by gel-filtration chromatography (data not shown), except for the w.t., which was partly dimeric under these conditions (ca 10 %).

The thermodynamic stability of all mutants and the w.t. was compared by GdmCl equilibrium denaturation curves, monitored by the shift of fluorescence maximum (Figure 6(a)). The denaturation of all species was found to be fully reversible, regarding both the midpoint of the transition as well as the fluorescence intensities in the native state (data not shown). All transitions were highly cooperative, indicating simultaneous unfolding of

1st randomization/
DNA shuffling
with spiking oligonucleotides

phage panning
on BSA-FITC

3 sublibraries:
- control (no stress)
- temperature stress
- GdmCl stress

thermal or chemical
selection pressure

6 rounds
(first 2 without stress)

phage proliferation

	Selection pressure		
round	control	temperature	GdmCl
1	-	-	-
2	-	-	-
3	-	50°C	2 M
4	-	50°C	2 M
5	-	50°C	2.25 M
6	-	50°C	2.5 M/3 M

analysis of single clones

2nd DNA shuffling

phage panning
on BSA-FITC

5 sublibraries:
- Taq control (no stress)
- Taq temperature stress
- Taq GdmCl stress
- Pfu temperature stress
- Pfu GdmCl stress

thermal or chemical
selection pressure

6 rounds
(first without stress)

phage proliferation

	Selection pressure		
round	control	temperature	GdmCl
1	-	-	-
2	-	50°C	2.5 M
3	-	50°C	3 M
4	-	53°C	3 M
5	-	57°C	3 M
6	-	60°C	3.5 M

analysis of single clones

Figure 4. Summary of the selection for increased stability of the randomized 4D5Flu phage library.

both domains (model 3 according to Wörn & Plückthun, 1999) so that ΔG values of unfolding could be estimated by curve fits describing a two-state model (Pace, 1990). The estimated ΔG values and the midpoints of transition are listed in Table 2, and the denaturation curves are shown in Figure 6(a). Two clones, clone 50 and 3M, both derived from the *Pfu* polymerase generated libraries, have a higher stability. The more stable one of them, clone 50, is the only selected sequence from the temperature selection. The other clones selected from the *Taq* polymerase generated library under GdmCl pressure (G1 and G2), or a control sequence selected from the *Taq* polymerase generated library without any stress (0T), lie in the same stability range or are even somewhat less stable than the w.t. (Figure 6(a)). The higher thermodynamic stability of clone 50 compared to the w.t. was confirmed by differential scanning calorimetry (Figure 6(b)). The heat capacity curves for both species were fully reversible up to 50 °C, but showed an irreversible transition with T_M values of 62.3 °C for the w.t. and 66.2 °C for clone 50 with severe aggregation and precipitation occurring after the transition.

The K_D measurements with the antigen fluorescein by fluorescence titration finally show that all selected mutants, except for the clone 0T from the no stress control library, are better binders than the w.t. by approximately one order of magnitude (Table 2). The K_D values measured in the presence of 0.3 M GdmCl for the w.t. and clones 3M and G2 are one order of magnitude worse compared to the measurements in the absence of GdmCl. Nevertheless, the ranking of affinities remains the same as in the absence of GdmCl, suggesting that GdmCl weakens the binding energy of these clones by the same amount.

Discussion

The aim of this study was to evaluate the effect of various selection pressures to evolve improved scFv molecules by phage display. As a model system we chose the scFv 4D5Flu (Jung & Plückthun, 1997), a framework with known advantageous *in vivo* folding and thermodynamic properties. Nevertheless, recent studies of a tumor-specific antibody clearly showed that even this framework can be significantly improved by engineering, which is crucial for *in vivo* applications of scFv fragments in diagnostics and therapy (Willuda *et al.*, 1999). To find a general solution and to evaluate the best selection strategies, we subjected
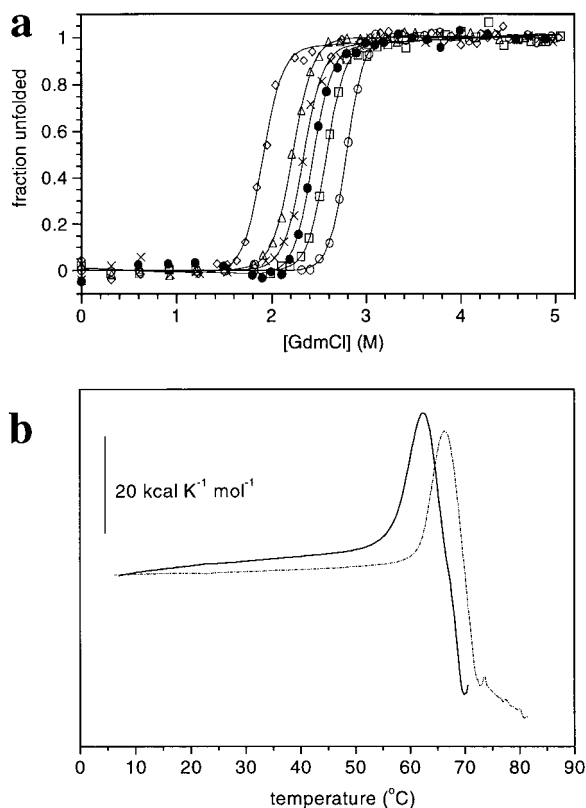
Table 3. Selection results of all panning rounds

A. *Selection results of all panning rounds of the first shuffling generation: output/input ratios of phage titers*

Round	Sublibrary[a]	No stress[b] (control) Library	w.t.	GdmCl incubation[c] Library	w.t.	[GdmCl][e] (M)	Temperature incubation[d] Library	w.t.	Temperature[f] (°C)
1	A	7.7×10^{-7}							
1	B	8.4×10^{-8}							
1	C	2.7×10^{-7}							
1	D	2.1×10^{-7}							
2	A	1.7×10^{-6}							
2	B	1.9×10^{-7}							
2	C	5.9×10^{-7}							
2	D	9.0×10^{-7}							
3		4.4×10^{-7}	1.7×10^{-8}	6.6×10^{-8}	4.0×10^{-10}	2	8.2×10^{-10}	3.1×10^{-10}	50
4		9.7×10^{-6}	1.9×10^{-6}	5.8×10^{-7}	3.5×10^{-9}	2	2.1×10^{-8}	3.8×10^{-9}	50
5		7.3×10^{-7}	1.1×10^{-8}	1.8×10^{-7}	1.6×10^{-9}	2.25	2.1×10^{-8}	1.1×10^{-9}	50
6		3.8×10^{-6}	2.1×10^{-8}	5.8×10^{-7}	5.5×10^{-10}	2.5			
6				3.5×10^{-8}	3.1×10^{-8}	3			

B. *Selection results of all panning rounds of the second shuffling generation: output/input ratios of phage titers*

Round	No stress[b] (control) *Taq* library	w.t.	GdmCl incubation[c] *Pfu* library	*Taq* library	w.t.	[GdmCl][e] (M)	Temperature incubation[d] *Pfu* library	*Taq* library	w.t.	Temperature[f] (°C)
1	8.1×10^{-7}	n.d.	8.1×10^{-8}			No stress	1.0×10^{-7}			No stress
2	n.d.	n.d.	1.5×10^{-9}	1.3×10^{-8}	1.7×10^{-8}	2.5	5.7×10^{-8}	8.3×10^{-10}	9.2×10^{-11}	50
3	n.d.	n.d.	5.7×10^{-8}	5.9×10^{-8}	n.d.	3	1.9×10^{-6}	2.6×10^{-6}	n.d.	50
4	8.5×10^{-8}	n.d.	4.0×10^{-9}	7.2×10^{-10}	3.6×10^{-10}	3	9.6×10^{-8}	2.7×10^{-9}	2.3×10^{-10}	53
5	2.8×10^{-6}	1.5×10^{-8}	1.2×10^{-9}	3.9×10^{-10}	3.6×10^{-10}	3	5.2×10^{-9}	1.4×10^{-8}	2.3×10^{-10}	57
6	8.2×10^{-7}	1.4×10^{-7}	8.2×10^{-10}	2.3×10^{-8}	1.1×10^{-10}	3	2.9×10^{-8}	2.4×10^{-8}	6.5×10^{-11}	60

[a] The sublibrary is defined by the ratio of synthetic oligonucleotides to DNase fragments (A = 10:1, B = 1:1, C = 1:10, D = 1:100) used in the assembly PCR reactions to create the libraries.
[b] Library or w.t. that has not been subjected to unfolding conditions.
[c] Library or w.t. that has been subjected to the GdmCl concentration indicated.
[d] Library or w.t. that has been subjected to the temperature indicated.
[e] GdmCl concentration present during phage incubation at 10 °C overnight.
[f] Temperature of phage incubation for three hours.

Figure 5. Sequences of selected mutants of 4D5Flu. Mutant clones with names starting with T are derived from temperature selections, clones with names starting with G are derived from GdmCl selections, clones with names starting with 0 are derived from the no stress control selections. The polymerase with which the library was assembled is indicated in the names. The mutants were selected after six panning rounds in the second DNA shuffling round. The amino acid residues initially targeted for randomization are highlighted by the black background. The numbers in parentheses indicate how often a clone was found from the number of clones sequenced.

Figure 6. Comparison of thermodynamic stability of some finally selected mutants to the w.t. by (a) GdmCl equilibrium unfolding curves monitored by intrinsic protein fluorescence: w.t. (●), clone 50 (○), clone 3M (□), clone G1 (◇), clone G2 (△), clone 0T (×); (b) thermal unfolding monitored by differential scanning calorimetry (line, w.t.; dotted line, clone 50).

the grafted fluorescein binding scFv 4D5Flu to random mutagenesis at defined positions, as well as to additional full randomization by DNA shuffling, and we used phage display selection in combination with various selection pressures for protein stability.

In phage display, selection of thermodynamic stability, folding yield or resistance to aggregation are important selection criteria for the displayed molecule, in addition to affinity (Jung & Plückthun, 1997), since, in general, only natively displayed protein can be selected on the antigen in the panning procedure (Deng *et al.*, 1994; Gu *et al.*, 1995; Jackson *et al.*, 1995). The functionality is influenced by the yield of correctly folded, non-aggregated gene-3-protein-fusion (g3p-fusion) protein in the inner bacterial membrane, which can be incorporated into the phage, as well as by the stability of the displayed protein during the process of phage production, harvest and panning. Both characteristics are dependent only on the nature of the displayed protein itself in an otherwise defined bacterial system.

The yield of correctly folded soluble protein in periplasmic expression is highly dependent on the expression temperature. At higher temperatures, the partitioning of folding intermediates favors the pathway to aggregation, perhaps because they are more highly populated or because the aggregation reaction itself is faster. Furthermore, functional proteins accumulating during the expression may slowly denature again, thus lowering the final yield. Consequently, phage production at 37 °C already exerts a selection pressure towards reasonably well folding displayed proteins.

To exert further selection pressure towards higher thermodynamic stability, the display phages can be exposed to harsher conditions, as phages turn out to be much less sensitive to heat or denaturing agents than the bacteria producing them. For the choice of denaturing conditions, we found that the selection by high-temperature incubation of the phage is much more stringent than incubation in GdmCl. After two rounds of random mutagenesis, each followed by six or seven rounds of phage proliferation, selection pressure and panning, the libraries selected under temperature pressure contained basically only one sequence, whereas the final diversity of the final pools obtained with GdmCl denaturation pressure was much higher. Even more highly diverse pools were found in control libraries without stress incubation, indicating the effectiveness of both selection pressure variants.

Temperature stress has the advantage that the thermal denaturation of scFvs is irreversible, at least for isolated scFv molecules at neutral pH in solution (see below and Figure 6(b)). Therefore, the danger is small that the scFv refolds during the phage capture procedure in the panning tube, and thus the incubation temperature actually reflects the stability of the scFv. On the other hand, the disadvantage of thermal stress is that the denaturation temperature under irreversible conditions is not necessarily directly proportional to the free energy, and furthermore, the relatively low temperature tolerance of the phage sets a natural limit to the highest stability selectable by this system.

The selection pressure exerted by GdmCl does not have this general limitation because of a strong resistance of the phage against even very high concentrations of GdmCl, at least up to 6 M overnight at 10 °C. However, GdmCl-denaturation is reversible for most proteins. Therefore, in those cases where antigen binding is affected by GdmCl, which will almost invariably be the case, and hence dilution is required in the phage capture step, the GdmCl concentration used in the denaturing step may not reflect the actual stability limit, as the scFv may refold on the phage during the panning step. In this case, the selection criterion may be refolding rate and efficiency rather than the thermodynamic equilibrium.

The reason why the temperature selection was more stringent than the GdmCl selection is probably connected to the fact that temperature-dena-

turation of soluble scFv leads to irreversible protein misfolding and perhaps micro-aggregation on the phage tip. This is paralleled by the DSC measurements with isolated scFv fragment, in which the denatured molecules are irreversibly removed from the equilibrium. In contrast, GdmCl denaturation of scFv fragments is usually largely reversible, due to the solubilizing effect of GdmCl. Some irreversibility of the GdmCl incubation, however, must cause the transition at 2.5 M GdmCl (Figures 2(b) and 3(b)). The irreversibility of the heat denaturation is crucial in this selection strategy, as the selective affinity capture step of functional and folded molecules is performed without selection pressure, i.e. at low concentrations of GdmCl (<1 M) and low temperatures (5-25 °C), in order to ensure correct binding. Therefore, chemically denatured scFv fragments may refold during the binding procedure at low concentrations of GdmCl, while the irreversibly heat-denatured scFv fragments remain unfolded and non-functional at low temperatures.

The stability limit of filamentous M13 phage lies at 60 °C (Figure 2(b)). It follows immediately that proteins that are still completely native and intact at this temperature are not improvable by temperature incubation. Our study indicates that the absolute T_M value itself may not be so crucial, with the T_M value of our starting molecule 4D5Flu being already higher than 60 °C. Rather, the differences in half-lives of the native states of the library members at elevated temperatures still below the T_M, and the temperature of where the transition starts, seem to be sufficient for temperature selectability. To drive stability selection further, one could develop a thermostable phage, as it has been done with T7 phages (Gupta et al., 1995), even though such an approach may lead to unwanted laboratory contaminations.

An alternative approach of selecting for high stability consists of an initial destabilization of the protein of interest by removing structural stability elements, such as a disulfide bond, thus rendering the protein of interest only marginally stable at room temperature. Then a randomized library of the destabilized protein can be conveniently selected for stabilizing mutations under normal phage display conditions. In this way, very stable molecules can be obtained, if the disulfide-bridge or another stabilizing element is reintroduced in the selected mutant. This strategy has been successfully demonstrated for a minimized protein A (Braisted & Wells, 1996; Starovasnik et al., 1997), a disulfide-free scFv fragment (Proba et al., 1998; Wörn & Plückthun, 1998) and the subtilisin prodomain (Ruan et al., 1998). This strategy and the strategy presented here may be alternatives or could be synergistically used to generate extremely stable proteins.

Recent studies (Kristensen & Winter, 1998; Sieber et al., 1998) have used another phage-based selection by separating folded from unfolded displayed molecules by protease cleavage and linking the protease-resistance to infectivity. However, for scFvs this approach may have limited applicability, as the linker and, depending on the specific antibody, the more flexible CDR regions are quite sensitive to protease cleavage anyway, and no selection pressure for keeping the functionality is exerted.

Under both selection pressures we found variants with improved affinity. This shows that affinity and stability selection are difficult to separate. The intrinsic problem of the selection design is the need for a selection of functionality, i.e. binding of the correctly folded molecules to an immobilized antigen. Thus, affinity is selected for at the same time as stability: the most stable and most tightly binding molecule was selected. In contrast, in the protease-based methods (Kristensen & Winter, 1998; Sieber et al., 1998), no selection pressure is exerted, which would guarantee that the displayed molecule remains functional.

The randomization strategy was based on a combination of targeted randomization and DNA shuffling to increase, on the one hand, the probability of accumulating beneficial mutations in a library of limited size and, on the other hand, have the chance of acquiring novel beneficial mutations. Replacement of hydrophobic surface residues by more hydrophilic ones has been reported in several studies to improve production yield (Forsberg et al., 1997; Nieba et al., 1997). This is due to improved kinetic partitioning between correct folding and aggregation of misfolded material, while the solubility of the native protein is not even affected (Nieba et al., 1997). The thermodynamic stability is not affected significantly by some of these mutations, either, and may even be decreased (Ohage et al., 1997). Compared to the 4-4-20 scFv fragment, the 4D5 scFv and the 4D5Flu scFv show few such hydrophobic V/C interface residues. To test for the effect of these residual hydrophobic side-chains on stability and folding efficiency, positions L15, L78, L80, L83, L106, H5 and H11 were allowed to occur in the original and a polar version by the design of the doping oligonucleotides, or by the DNA family shuffling with the hydrophilic point mutants (see Materials and Methods for details). In all randomized positions of this type, the hydrophobic residue present in the original 4D5 sequence was selected over the alternatives offered in the randomization procedure, consistent with a selection primarily for stability, rather than folding efficiency.

It has been shown that an accidental, PCR-primer-encoded replacement of germline Gln H6 by Glu can seriously impair the stability and/or folding efficiency of a scFv by combining incompatible framework classes (Kipriyanov et al., 1997; de Haard et al., 1998; Langedijk et al., 1998). On the other hand, some very stable V_H domains do contain a glutamate residue in this position (4D5, ABPC48), and it remains to be shown whether these antibodies would also benefit from a removal of the negative charge. Since in natural antibody

sequences, there is a clear correlation between the nature of the amino acid residue in H6 and the type of residue found in position H9 (A.H. *et al.*, unpublished results), both positions were randomized to cover the combinations found in nature. Again, the sequence of 4D5 (GluH6, GlyH9) was selected over the alternatives.

The main-chain φ-angle in position H49 is positive in almost all antibody structures, and the most frequent residue found in this position is glycine. A glycine residue replacing the alanine residue found in the original 4D5Flu survived ''unstressed'' selection, but was not strongly selected for, either. Unfortunately, we can currently not clearly distinguish whether none of the mutations was selected because they neither improved folding nor stability, or whether the mutations did indeed improve folding or stability, but could not compete by themselves in the selection with variants carrying affinity-improving mutations, which were randomly introduced using *Taq* polymerase in the construction of the initial library.

The selected 4D5Flu variant with improved folding and stability properties contains two mutations, Phe(L55)Val and His(L27d)Asn. These two mutations improve, at the same time, all three criteria that we selected for; stability, folding and affinity. From the model of 4D5Flu, we conclude that HisL27d, being in the middle of CDR-L1 and making direct antigen contact, is probably mainly responsible for affinity improvement, so that no conclusion for the framework can be drawn from this mutation. In contrast, PheL55 is semi-buried at the periphery of the V_L-V_H domain interface, so that it could either influence the interface stability by core packing effects or indirectly influence the V_L/V_H interface orientation, which could indirectly affect antigen affinity. In order to clarify the role of these two residues, single mutants will now have to be investigated in more detail. In any case, Phe55 seems to be a crucial residue that should be paid attention to in grafting experiments and CDR randomized libraries. The fact that residues L27d and L55, which were found to improve the behavior of the selected mutant, are CDR residues may be related to the extremely poor folding properties of the CDR donor 4-4-20. Hence, in the graft 4D5Flu, the CDRs were the sequence parts least optimized for folding and stability, and therefore potentially more amenable to further improvement than the framework regions derived from the already very favorable scFv hu4D5.

Additional mutations in 3M, G1, G2 and 0T partially counteract the positive effects on folding and stability and partly also on affinity. Leu(H18)Gln is semi-buried and may have a negative effect on stability (clone G1), Leu(H45)Pro is buried in the domain interface and may destabilize it (clone G2), while Phe(H29)Leu affects the conformation of

CDR-H1 and thus explains the lowered affinity to the antigen (clone 0T). These results suggest that the error rate in the generation of random libraries should be carefully controlled, as too many mutations can easily destroy the effect of single beneficial mutations, as seen in the analysis of this selection.

In conclusion, it is possible to select random libraries by simple phage display for improved stability and folding of the displayed protein in combination with affinity selection by stringent temperature pressure up to 60 °C. In contrast, exerting selection pressure on folding and stability by GdmCl is less stringent than temperature selection. With these guidelines at hand it should be possible in the future to select for the most stable molecules present in a phage-displayed library.
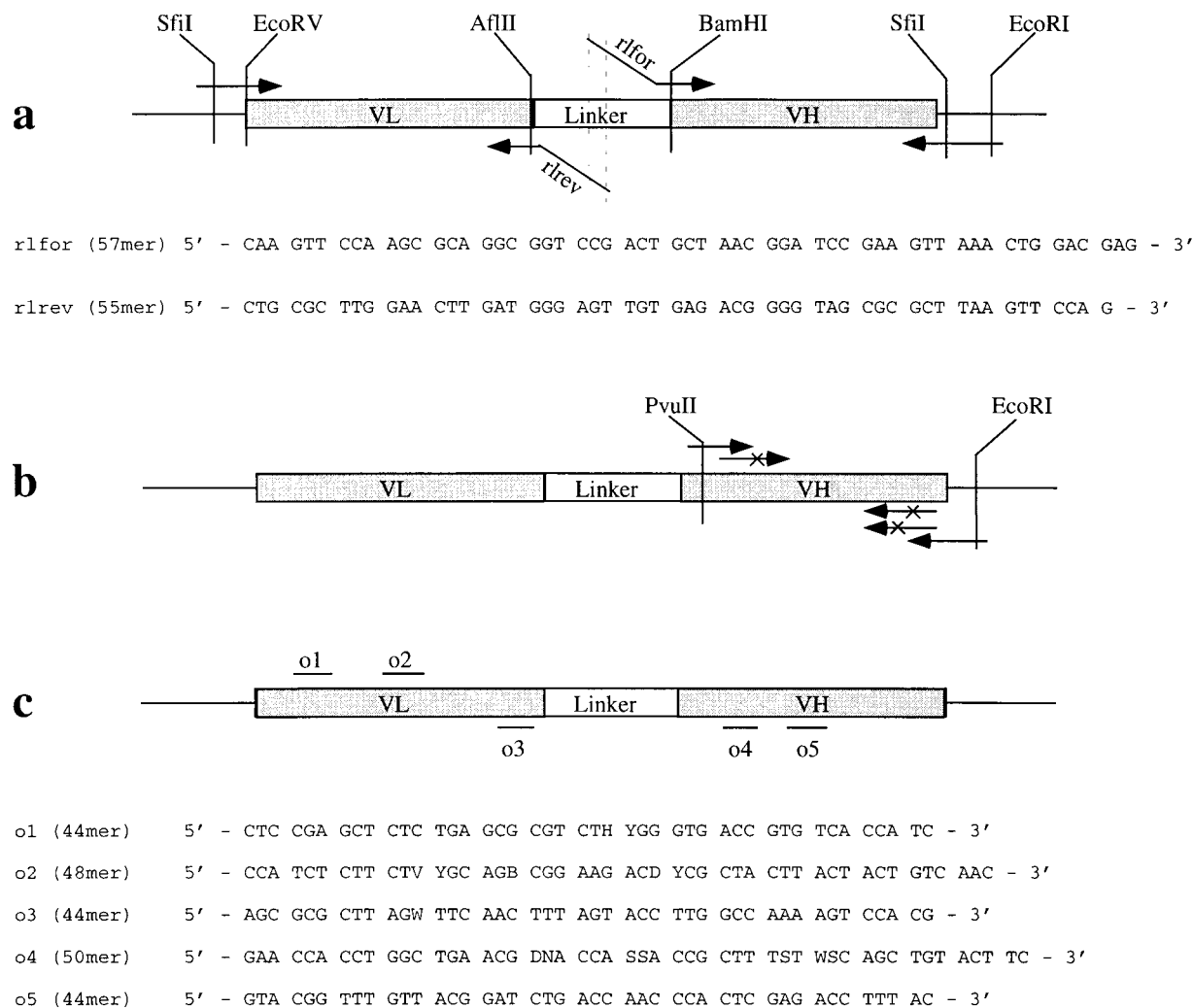
Materials and Methods

Plasmids and strains

The vector pAK100 was used for all phage display experiments (Krebber *et al.*, 1997). The scFv library was cloned between the two different *Sfi*I-sites, resulting in a scFv-g3p fusion in the suppressor strain XL1-Blue (Stratagene) used for phage display experiments. For small-scale expression experiments, the 4D5Flu w.t. and mutant scFv fragments were cloned *via* compatible *Sfi*I sites into the plasmid p416His (U. Horn, unpublished results) containing the ampicillin-resistance gene, a *lac* promotor and a His-tag fused to the scFv fragment. For large-scale expression, the mutant scFvs were cloned into the plasmid pIG6 (Ge *et al.*, 1995) by replacing the 4D5Flu w.t. between *EcoR*V at the N terminus and *Kpn*I in the FR-H4 region. For growth and expression studies of soluble scFvs the *E. coli* strain JM83 (Yanisch-Perron *et al.*, 1985) was used, while for large-scale production of the scFvs the *E. coli* strain SB536 (Bass *et al.*, 1996) was preferred.

Molecular Modeling

Residues on the surface, those contributing to the dimer interface and those contacting the antigen (Figure 5(c)) were identified by calculating the solvent-accessible surface for each residue in the liganded and unliganded 4D5Flu Fv as well as in the isolated domains using the program NACCESS (S. Hubbard & J. Thornton (1992†). The structure of the 4D5Flu graft was predicted by homology modeling using the Homology, Biopolymer and Discover modules of the program InsightII version 95 (Biosym/MSI, San Diego, CA). The V_H and V_L domains of 4D5 (PDB entry 1fvc (Eigenbrot *et al.*, 1993)) and of 4-4-20 (PDB entry 1flr (Whitlow *et al.*, 1995)) were aligned by a least-squares fit of the C^α-positions of residues L3-L7, L20-L24, L33-L39, L43-L49, L62-L66, L71-L75, L84-L90 and L97-L103 (V_L) and residues H3-H7, H20-H24, H35a-H40, H44-H50, H67-H71, H78-H82, H88-H94 and H102-H108 (V_H). Coordinates of the residues in the graft were taken from the 4D5 (framework) and the 4-4-20 (CDRs) templates. In addition to the CDRs, the residues determining the conformation of the outer loops (L66, H71 and H78) were retained in the 4D5Flu sequence. The resulting model was checked for potential steric conflicts before energy minimization.

† http://sjh.bi.umist.ac.uk/naccess.html

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rlfor (57mer) 5' - CAA GTT CCA AGC GCA GGC GGT CCG ACT GCT AAC GGA TCC GAA GTT AAA CTG GAC GAG - 3'

rlrev (55mer) 5' - CTG CGC TTG GAA CTT GAT GGG AGT TGT GAG ACG GGG TAG CGC GCT TAA GTT CCA G - 3'
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o1 (44mer)     5' - CTC CGA GCT CTC TGA GCG CGT CTH YGG GTG ACC GTG TCA CCA TC - 3'

o2 (48mer)     5' - CCA TCT CTT CTV YGC AGB CGG AAG ACD YCG CTA CTT ACT ACT GTC AAC - 3'

o3 (44mer)     5' - AGC GCG CTT AGW TTC AAC TTT AGT ACC TTG GCC AAA AGT CCA CG - 3'

o4 (50mer)     5' - GAA CCA CCT GGC TGA ACG DNA CCA SSA CCG CTT TST WSC AGC TGT ACT TC - 3'

o5 (44mer)     5' - GTA CGG TTT GTT ACG GAT CTG ACC AAC CCA CTC GAG ACC TTT AC - 3'
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Figure 7. Strategies for cloning and site-directed and random mutagenesis in the scFv 4D5Flu w.t. (a) Linker exchange; (b) site-directed PCR mutagenesis; (c) doping oligonucleotides for spiking the DNA shuffling.

Library design

The three 4D5Flu V_H mutants L11S, V89S and L108S were constructed by PCR mutagenesis of 4D5Flu exploiting the unique restriction sites *Pvu*II near the N terminus of V_H and *Eco*RI at the C terminus (Figure 7(b)). Two consecutive assembly PCR reactions with mutagenic primers and constant primers were carried out with Vent polymerase. Since the additional positions chosen for further randomization were positioned within five different regions of the scFv, they were encoded by mixed bases on five oligonucleotides (sequences shown in Figure 7(c)). To minimize 1 bp deletions caused by imperfect oligonucleotide synthesis, the oligonucleotides were less than 50 bp in length and were purified by polyacrylamide gel electrophoresis.

For reliable error-prone PCR and DNA shuffling, a non-repetitive linker with 20 amino acid residues was designed (Figure 7(a)). The linker was adapted from a selected linker sequence (Tang *et al.*, 1996) by converting all hydrophobic amino acid residues to more hydrophilic ones to ensure a high level of solubility of the scFv fragment. Furthermore, the chosen sequence contained no basic residue, to reduce the risk of protease cleavage of the linker. The DNA sequence was designed with the highest possible sequence diversity in order to avoid repetitive stretches within the linker and DNA sequence similarities between the linker and the scFv itself. The resulting sequence is shown in Figures 5 and 7(a). It has already been used successfully in another library setting (Proba *et al.*, 1998). The non-repetitive linker was introduced into the 4D5Flu w.t. by an assembly PCR using Vent polymerase (New England Biolabs) (Figure 7(a)) and the resulting scFv fragment was cloned in the *Sfi*I sites of the phagemid vector pAK100.

Library construction

DNA shuffling was carried out according to Stemmer (1994a) with the following modifications. For the DNase I digests, QIAEXII (QIAgen)-purified scFv fragment genes were used. In the first shuffling round, 600 ng of DNA of the 4D5Flu w.t. fragment and 100 ng of each of the three mutant 4D5Flu fragments were mixed in the DNase I digest, whereas in the second shuffling round 1 µg of DNA of 4D5Flu w.t. and of each library pool was digested separately. The digests were performed with 1 unit of RQ1 DNase (Promega) for 15 minutes at room temperature in 100 µl of 50 mM Tris

(pH 7.4), 1 mM MgCl$_2$. The reaction was stopped by adding EDTA to 20 mM final concentration and heating for 15 minutes to 65 °C. The DNase fragments with a size of 10-300 bp were purified from a 1 % TAE agarose gel by QIAEXII gel extraction.

The assembly PCR (without outer amplifying primers) was performed differently in the first and the second shuffling round. In the first shuffling round, the digested fragments were mixed with all five synthetic oligonucleotides containing the randomized bases, at molar ratios of 10:1, 1:1, 1:10 and 1:100. Different amounts of these fragment-oligonucleotide mixes were employed in different assembly PCR reactions using *Taq* polymerase (Gibco). For the absolute amount of the DNase-generated DNA fragments and the doping oligonucleotides in the first assembly PCR without primers, an optimum was found between 3 and 25 pmol. A ratio of DNA fragments to each oligonucleotide of 10:1 led to an average incorporation of one oligonucleotide per sequence, whereas ratios of 1:1 or higher in favor of the oligonucleotides led to the incorporation of four to five oligonucleotides per sequence. The libraries generated by different fragment to oligonucleotide ratios were kept separately for ligation, transformation and in the initial selection rounds.

In the second shuffling round, the assembly PCR without primers was used to create two different sorts of library: one library was assembled using *Taq* polymerase in order to introduce additional mutations, and the other with Turbo-*Pfu* polymerase (Stratagene) for simply recombining existing mutants, avoiding too many additional mutations. For the *Pfu*-generated libraries, the selected pools from temperature-stress and GdmCl-stress guided selections of the first generation library were kept separately, while these two pools were combined for the *Taq*-generated library. The cycling was carried out in 100 µl volume on an MWG Primus with ten cycles of 30 seconds at 92 °C, ramping down to 45 °C with 0.5 deg. C/second and 30 seconds annealing increasing the temperature by 1 deg. C in each cycle, starting at 45 °C in the first cycle, and elongation at 72 °C for ten seconds prolonging the elongation time by five seconds in each cycle. Then, 30 cycles followed with 30 seconds at 92 °C, 45 seconds at 55 °C and one minute at 72 °C. The resulting fragments were amplified with outer primers, containing specific *Sfi*I restriction sites, with *Taq* polymerase over 25 cycles with 30 seconds at 92 °C, 40 seconds at 68 °C and 40 seconds at 72 °C on an MWG Primus or a PTC-200 thermocycler.

The amplified fragments were purified from a 1 % TAE agarose gel by centrifugation in Spin columns (Suppelco), concentrated by precipitation in ethanol, and gel-purified again after an *Sfi*I-digestion by the same gel-extraction method. The fragments were desalted and concentrated over a Microcon-30 Spin column (Amicon) by three times diluting with UHP water and concentrating to a final volume of 10-20 µl: 60 ng of these fragments was ligated (T4 ligase, Boehringer, 16 °C for three days) into 300 ng of *Sfi*I-digested pAK100 vector that was previously purified on a 0.8 % TAE agarose gel, recovered by electroelution (Biotrap, Schleicher & Schuell) and desalted and concentrated on Microcon-30 Spin columns. The ligations were desalted and concentrated on Microcon-30 columns and transformed into XLI-Blue cells by electroporation (Eppendorf electroporator). The libraries were plated on 245 mm × 245 mm LB agar plates containing 25 µg/ml chloramphenicol (cam) and 1 % (w/v) glucose and incubated at 37 °C overnight. The resulting colonies were scraped from the plates with

SOC and diluted into 100 ml of 2 × YT containing 15 µg/ml tetracycline (tet) and 25 µg/ml chloramphenicol (cam) to an A_{550} of 0.1 and grown at 37 °C to an A_{550} of 0.8. They were then infected with M13 VCS helper phage (Stratagene), induced with 1 mM IPTG, and phages were produced overnight at 24 °C, adding kanamycin (kan) to a final concentration of 30 µg/ml three hours after induction. The phages were precipitated from the culture supernatant with 0.25 volume polyethylene glycol (PEG) solution (3.5 NaCl, 20 % PEG 6000) at 4 °C, and the phage pellets were redissolved in 1 ml of PBS (10 mM Na$_2$HPO$_4$/KH$_2$PO$_4$ (pH 7.2), 150 mM NaCl).

Library selection

For selection experiments, a 2 ml culture of XL1-Blue growing in the log-phase in 2 × YT medium containing 15 µg/ml tet and 1 % glucose at 37 °C was infected at an A_{550} of 0.5 with 10^2-10^7 cfu of scFv-library-displaying phages and diluted into 10 ml of 2 × YT medium containing 15 µg/ml tet and 1 % glucose. After one hour at 37 °C, cam was added to a final concentration of 25 µg/ml. After one additional hour at 37 °C, 10^{11} cfu VCS helper phage were added and the culture diluted into 50-100 ml of 2 × YT medium containing 25 µg/ml cam, 15 µg/ml tet and 1 mM IPTG. The culture was then grown overnight at 37 °C, and two hours after infection with helper phage, kan was added to a final concentration of 30 µg/ml. The cells were harvested and phage particles in the supernatant of the overnight culture were PEG precipitated (see above) and redissolved in 1 ml of PBS.

The phage libraries were then subjected to denaturing conditions to exert selection pressure on protein stability prior to panning or panned directly in the control experiments. For those experiments with a temperature denaturation step, the phage solution was incubated for three hours at the respective temperature. For GdmCl denaturation, the phages were incubated overnight at 10 °C in 2-3.5 M GdmCl in PBS. Immunotubes (Nunc) were coated with 20 µg/ml BSA-FITC in PBS overnight at 10 °C and blocked with 4 % (w/v) skimmed milk in PBST for two hours at room temperature. About 10^{11} phages in PBS containing 2 % skimmed milk were applied for 30 minutes at 4 °C, and the GdmCl-denatured phages were first diluted 1:10 into PBST. Tubes were washed ten times with PBST and ten times with PBS, before bound phages were eluted with 1 ml of 0.1 M glycine/HCl (pH 2.2) for 30 minutes at room temperature. The eluate was neutralized with 600 µl of 2 M Tris, and the phages were used for reinfection.

Analysis of single clones

After induction with 1 mM IPTG at an A_{550} of 0.5, the growth behavior of the mutants at 37 °C was monitored by measuring the A_{550}. Experiments were carried out with JM83 cells harboring p416His, which codes for the respective 4D5Flu mutant. The amount of functional scFv was assayed by ELISA on BSA-FITC after harvesting an aliquot of the cells three hours after induction, resuspending the cell pellet in PBS to an A_{550} of 20, disrupting the cells by French press and recovering the soluble material by centrifugation (20,000 *g*, ten minutes at 4 °C).

ELISA

To assay the amount of functional scFv either displayed on M13 phage or expressed in soluble form in the bacterial periplasm, ELISAs were carried out. For a phage ELISA, single colonies were grown separately at 37 °C in 2 ml of $2 \times$ YT medium containing 25 µg/ml cam and 15 µg/ml tet. At an A_{550} of 0.5, 4 ml of $2 \times$ YT medium containing 25 µg/ml cam, 15 µg/ml tet, 10^{10} cfu VCS helper phage (Stratagene) and 1.5 mM IPTG were added. The cultures were allowed to produce phages overnight at 37 °C. Phages from 4.8 ml of culture supernatant were precipitated with 0.25 volume polyethylene glycol (PEG) solution (3.5 M ammonium-acetate, 20 % PEG 6000) at 4 °C, and the phage pellets were redissolved in 300 µl of PBS (10 mM Na_2HPO_4/KH_2PO_4 (pH 7.2), 150 mM NaCl). ELISA plates (Nunc) were coated with 20 µg/ml fluorescein-isothiocyanate-coupled bovine serum albumin (BSA-FITC) in PBS at 4 °C overnight and blocked with 4 % skimmed milk in PBST (PBS with 0.05 % Tween20) for two hours at room temperature. A defined number of phages (measured by titer) per well was preincubated with 2 % skimmed milk in PBST in the absence and in the presence of 10 µM fluorescein for one hour at 4 °C and then applied to the blocked ELISA wells. For detection, an anti-M13 antiserum conjugated with horseradish peroxidase (Pharmacia) was used, the development was carried out with soluble BM blue POD-substrate (Boehringer Mannheim), and signals were read at 405 nm after stopping the reaction with 0.1 M HCl.

For ELISAs of periplasmically expressed soluble scFv, the protein concentrations were normalized to A_{550} with PBS, and dilutions in PBST either with or without one hour preincubation with 10 µM fluorescein at 4 °C were added to the precoated and preblocked ELISA wells (see above). For detection, an anti-Histag scFv fused to alkaline phosphatase (Lindner et al., 1997) was used, and the ELISA was developed with Na_2(4-nitrophenylphosphate)(H$_2$O)$_6$ (20 mg in 10 ml of 0.1 M glycine, 1 mM ZnCl$_2$, 1 mM MgCl$_2$, pH 10.4). The absorbance was read at 405 nm.

Production of scFv antibody fragments

The scFvs 4D5Flu w.t. and its mutants were expressed from the plasmid pIG6 in SB536 cells grown at 24 °C for four to five hours after induction with 1 mM IPTG and purified on a BioCad (Perseptive Biosystems) in HBS in a coupled two-column procedure (Plückthun et al., 1996). First, the scFv was eluted with 100 mM imidazole from an immobilized metal affinity chromatography (IMAC) column with a Ni-NTA matrix, and subsequently directly pumped to an HS cation-exchange column from which it was eluted with a NaCl gradient. The identity and purity of the scFvs was confirmed by Coomassie-stained SDS-PAGE, mass spectroscopy and gel permeation chromatography (Superdex 75, Pharmacia SMART system). The concentration and yield were determined photometrically using an extinction coefficient calculated according to Gill & von Hippel (1989).

Determination of antigen-binding constants

The K_D determination was carried out by fluorescence quenching of fluorescein with stepwise addition of purified scFv in HBS. The excitation wavelength was 485 nm. Five emission spectra per scFv concentration were recorded from 500 to 525 nm, and the emission intensities averaged at 511 nm were directly fitted in a three-parameter fit (Pedrazzi et al., 1997) or a two-parameter fit (Jung & Plückthun, 1997) using the concentration of active protein previously determined in an activity titration. The activity titration was accomplished similarly by stepwise addition of a fluorescein solution to a solution of the scFv at a concentration higher than the K_D. The equivalence point was determined by the intercept of the initial plateau with the rising straight line at high concentrations.

Determination of thermodynamic stabilities

The thermodynamic stability was determined by equilibrium denaturation with GdmCl. Samples (1.7 ml) containing 8 µg of scFv in HBS buffer (20 mM Hepes (pH 7.4), 150 mM NaCl) with different concentrations of GdmCl (0-5 M in 0.1 M steps) were prepared, incubated overnight at 10 °C and equilibrated to 20 °C for one to two hours prior to the measurements. The exact GdmCl concentration in each sample was determined by its refractive index. Excitation of the protein fluorescence was at 280 nm, and five fluorescence emission spectra of each sample were recorded from 325 nm to 360 nm at 20 °C, averaged, and the emission maximum determined by a polynomial fit. The shift of the emission maximum with increasing GdmCl concentration and the intensity change were used to calculate the fraction of unfolded scFv. The resulting curves were fitted according to Pace (1990).

Differential scanning calorimetry experiments were performed with a VP microcalorimeter (MicroCal Inc., Northampton) at a scan-rate of 1 degree/minute (Plotnikov et al., 1997; Weber-Bornhauser et al., 1998; Jelesarov & Bosshard, 1999). Protein samples were thoroughly dialyzed prior to measurements. The protein concentrations were 3 mg/ml in PBS (pH 7.0).

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