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REVIEW ARTICLE

# Stability Engineering of Antibody Single-chain Fv Fragments

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The application of single-chain Fv fragments (scFv) in medicine and biotechnology places great demands on their stability. Only recently has attention been given to the production of highly stable scFvs, and in a number of examples it was found that such fragments indeed perform better during practical applications. The structural parameters influencing scFv stability are now beginning to be elucidated. This review summarizes progress in rational and evolutionary engineering methods, the structural implications of these results, as well as some examples where stability engineering has been successfully applied.



*Keywords:* protein stability; scFv fragments; recombinant antibodies; antibody engineering; protein folding

## Introduction

Recombinant antibodies are finding an ever increasing number of applications in biotechnology and medicine. A variety of antibody formats have been employed, which reflect differences in the production method, the need for mono- or multivalency as well as the intended use. The Fab fragment of an antibody is a structurally independent unit that contains the antigen-binding site; it is likely that its stability will not be influenced by the Fc part, as it is separated by the hinge region. The four domains of the Fab fragment ($V_H$, $C_H1$, $V_L$, $C_L$) interact through a large interface between the chains ($V_H/V_L$ and $C_H1/C_L$) and a small one between the variable and constant domains ($V_H/C_H1$ and $V_L/C_L$) of each chain. The antigen-binding ability of an antibody is usually conserved, even when only the $V_H$ and $V_L$ domains are used. These two domains can either be associated non-covalently (Fv fragment) (Skerra & Plückthun, 1988), connected by a peptide linker (scFv) (Bird *et al.*, 1988; Huston *et al.*, 1988), by a disulfide bond (dsFv) (Glockshuber *et al.*, 1990; Brinkmann *et al.*, 1993) or both (sc-dsFv) (Young *et al.*, 1995).

Abbreviations used: CD, circular dichroism; CDR, complementarity determining region; $C_H1$, first constant domain of the heavy chain of an antibody; $C_L$, constant domain of the light chain of an antibody; COS, CV1 monkey cells transformed with SV40 (simian virus 40) possessing a defective origin of replication; DMSO, dimethylsulfoxide; dsFv, disulfide-bridged Fv fragment of an antibody; DTT, dithiothreitol; ELISA, enzyme linked immunosorbent assay; Fv, antibody variable fragment consisting of $V_H$ and $V_L$; GdnHCl, guanidine hydrochloride; HuCAL, human combinatorial antibody library; $K_D$, equilibrium dissociation constant; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; PDB, protein database; PEG, polyethylene glycol; $p185^{HER2\text{-}ECD}$, extracellular domain of human epidermal growth factor receptor-2; RT-PCR, reverse transcriptase polymerase chain reaction; sc-dsFv, disulfide-bridged antibody single-chain fragment; scFv, antibody single-chain fragment; SIP, selectively infective phage technology; $V_H$, variable domain of the heavy chain of an antibody; $V_L$, variable domain of the light chain of an antibody.

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### *ScFv fragments: a popular antibody format and model system*

By far the most popular recombinant antibody format is the scFv fragment (Bird *et al.*, 1988; Huston *et al.*, 1988), and it is on this format we concentrate in this review. First, most data are available on scFvs, although it should be mentioned that other recombinant antibody fragments and even whole antibodies have also been studied regarding their stability (e.g. Shimizu *et al.*, 1992; Yasui *et al.*, 1994; Shimba *et al.*, 1995; Vermeer & Norde, 2000). Second, the role of the domains and the interface of scFv proteins for stability has been much easier to untangle than would have been feasible with other antibody fragments containing more domains and interfaces. Even though we will

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only treat the stability and stabilization of single domains and scFv fragments in detail in this review, we believe that many of the general principles as well as the identified stabilizing mutations will also apply to the other formats and whole antibodies.

As outlined above, scFv fragments contain the complete antigen-binding site of an antibody. If no reorientation of the two domains occurs, they should have the same monomeric binding affinity as the parental monoclonal antibody, as experimentally demonstrated in a number of cases (e.g. Bird *et al*., 1988; Glockshuber *et al*., 1990; Huston *et al*., 1991). Moreover, antibody single-chain fragments can be conveniently expressed in a variety of hosts (reviewed by Verma *et al*., 1998), including bacteria (reviewed by Plückthun *et al*., 1996), yeast (reviewed by Fischer *et al*., 1999), and plants (reviewed by Smith, 1996; Shimada *et al*., 1999). Because of these advantages, scFv fragments have found broad applications in medicine (reviewed by Huston *et al*., 1993) and have also great potential in biotechnology (Harris, 1999). Single-chain fragments and other recombinant antibody constructs have, for example, made their way to a number of promising pre-clinical and clinical trials in cancer therapy (reviewed by Hudson, 1999), either as diagnostic or therapeutic agents. In addition, multiple applications of scFv fragments in the non-pharmaceutical sector, such as in the food, cosmetic or environmental industries can be envisaged (Harris, 1999). The unique and highly specific antigen-binding ability might, for example, be exploited to block specific enzymes (e.g. enzymes that cause food spoilage), bacteria (e.g. in toothpaste or mouthwashes) or to detect environmental factors present in very low concentrations (as biosensors).

### *Demands on scFv stability*

One common requirement for almost all of these medical and non-medical applications mentioned above is that the scFv fragment is highly stable. A therapeutic or diagnostic tumor-binding scFv fragment employed in a human patient needs to retain its activity for several hours or days after injection at 37 °C in human serum without precipitating or being degraded, in order to localize and enrich at the specific site, e.g. tumor tissue. Even more adverse environments, such as non-polar solvents, surfactants, proteases or higher temperatures may challenge the molecules in some of non-pharmaceutical applications (Dooley *et al*., 1998; Harris 1999). Unfortunately, many scFv fragments are not stable for longer periods under such conditions. Even when provided with superb antigen-binding properties, they often fail to show convincing effects during such practical applications, because they tend to denature and aggregate under the conditions faced in practice (e.g. by Reiter *et al*., 1994b; Willuda *et al*., 1999). We will show, however, that this is not an intrinsic drawback of the scFv format, but rather a property of particular sequences, which can be corrected by engineering.

A further application of scFv fragments with great demands on stability is the cytoplasmic expression in eukaryotic host cells. Intracellularly expressed antibodies, often called intrabodies (Biocca & Cattaneo, 1995), can be directed to a number of compartments (such as the cytoplasm, endoplasmic reticulum, Golgi apparatus, mitochondria, or lysosomes) by attaching the corresponding signal sequence to the fragment. These intrabodies are intended for binding and inactivating their target protein in the respective compartment. Many regulatory and proliferating viral proteins are active in the cytoplasm, once a cell has been infected. Therefore, cytoplasmically expressed intrabodies might in the future be used to treat viral infections and perhaps also to inhibit the action of specific oncogenes, possibly in a gene therapy setting (Marasco, 1995).

Under the reducing conditions of the cytoplasm (Gilbert, 1990) the conserved intradomain disulfide bond of antibody domains (Williams & Barclay, 1988) cannot form. Indeed, cytoplasmically expressed scFv fragments in COS cells, plants and bacteria were shown to carry free sulfhydryl groups (Biocca *et al*., 1995; Martineau *et al*., 1998; Tavladoraki *et al*., 1999). As the highly conserved intradomain disulfides are critical for the stability of scFv fragments (Proba *et al*., 1997; Glockshuber *et al*., 1992), only intrinsically very stable scFv fragments will be able to fold correctly in sufficient amounts to be active as cytoplasmic intrabodies. This is consistent with the finding that many cytoplasmic intrabodies show low expression levels and limited half-lives (Cattaneo & Biocca, 1999), and therefore only weak inhibitory effects. Moreover, many fragments selected under oxidizing conditions (for example, by phage display) fail to be active as intrabodies at all in the reducing cytoplasm (Visintin *et al*., 1999), probably because they are not stable enough. Others only prove to be active in the cytoplasm when expressed at lower temperature (20 °C), but not at 30 °C (Visintin *et al*., 1999), which would prevent many *in vivo* applications.

For the reasons mentioned above, the general interest in stability engineering as one specific aspect of antibody engineering in the broader sense (reviewed by Dall'Acqua & Carter, 1998) has increased over the last few years. It is a very important challenge for protein engineers to understand the factors influencing scFv stability in more detail, in order to be able to produce highly stable fragments, following either rational or evolutionary approaches. The significantly improved activity of scFv fragments with increased *in vitro* stability during extracellular and intracellular applications *in vivo* has been demonstrated in a number of examples (Benhar & Pastan, 1995; Willuda *et al*., 1999; Wörn *et al*., 2000) and further underlines the fact that stability engineering is worth the effort.

The challenges in engineering antibody scFv fragments for higher stability are not fundamentally different from those in stability engineering of other proteins (reviewed by Fágáin, 1995; Shaw & Bott, 1996; Rubingh, 1997; Forrer *et al.*, 1999), where also rational and evolutionary approaches have been used. However, in the case of antibody fragments, and scFvs in particular, several advantages are apparent, which may even make them useful as a model system for extracting general features about protein stability. Firstly, there are probably more crystal structures solved than for any other class of related topology (not counting the point mutants of some enzymes). Secondly, the domains do interact, but can also be expressed and studied individually, allowing a quantification of the contribution of the domain interactions to stability. Thirdly, the conserved disulfide bonds can be taken out and thus the domains can be studied as ''low-stability'' and ''high-stability'' versions.

The present article summarizes and critically reviews the literature about stability and stability engineering of scFv fragments. In the first section, the various definitions of the term ''stability'' in connection with scFvs are discussed as well as common ways of measuring scFv stabilities. We explicitly discuss the possibility and limitations of deriving free energies of folding from transition curves of scFv fragments. This section is followed by a summary of the known structural parameters influencing scFv stability, such as intrinsic domain stability, stability of the interface, contribution of intradomain disulfides and monomer-dimer equilibria. The central part of this article covers rational and evolutionary approaches for engineering stable scFv fragments. We will provide some guidelines to answer the question as to how to increase the stability of a chosen scFv fragment. Examples where scFv fragments with increased stability have shown improved effects for particular applications *in vivo* are also included. Finally, the interplay between scFv stability and expression behavior is reviewed.

## Measuring scFv stability

### *Experimental approaches*

The thermodynamic definition of stability requires the quantification of a freely reversible equilibrium. From such measurements one can, in principle, derive a free energy of folding (Pace, 1986, 1990). This is usually done by measuring the fraction of unfolded protein as a function of denaturant concentration (either urea or GdnHCl) and linearly extrapolating from the free energy of unfolding in the transition region to zero denaturant (Pace, 1986). Even though the merits of using other methods than linear extrapolation have been discussed (Pace, 1986), the linear method is most widely used and, as far as we are aware, all scFv literature has employed it.

Different biophysical properties of the protein have been used to follow denaturation of the scFv fragment. Circular dichroism (CD) (e.g. Yasui *et al.*, 1994) measures the amount of secondary structure directly, but has the disadvantage that the change in signal upon denaturation of a purely β-sheet protein such a scFv fragment is rather small and requires relatively high concentrations of protein (about 10 μM). This, in turn, may compromise the reversibility, because of aggregation in the transition region. Much less protein (sub-micromolar) is needed if scFv denaturation is followed by the intrinsic Trp fluorescence of the protein. This is usually done by measuring the fluorescence emission maximum (e.g. Wörn & Plückthun, 1999) or the fluorescence intensity at a given wavelength (e.g. Langedijk *et al.*, 1998; Jäger & Plückthun, 1999a,b). Practical aspects of preparing and performing equilibrium denaturation measurements are covered by Pace & Scholtz (1997).

The emission maximum signal is normally ''robust'' and not influenced by small changes in protein concentration. Moreover, all changes occurring during denaturation of a scFv (disruption of the interface, denaturation of $V_H$ and $V_L$) will cause a change of the fluorescence emission maximum into the same direction (red shift). One has to caution, however, that a free energy of folding can only be derived from emission maximum curves if the signal reflects the molar ratio between native and denatured protein. This will strictly only be the case if the quantum yield of native and denatured state are identical (Eftink, 1994).

A related method is to use the determination of the ''center of spectral mass'' as an empirical measure of the shift of the maximum (Royer, 1995). Occasionally, this may be easier and more precise to determine than the shift in fluorescence emission maximum, especially if the latter is very small.

The fluorescence intensity at a given wavelength can also be monitored to follow scFv denaturation. However, for many scFv fragments this is difficult because denaturation of $V_H$, $V_L$ and disruption of the interface can cause opposite changes in fluorescence intensity, mutually compensating each other. Moreover, intensity data are often more noisy because fluorescence intensity is very sensitive to protein aggregates possibly forming at intermediate denaturant concentrations, where protein solubility is often decreased (Jaenicke & Seckler, 1997).

Global-fit algorithms can be used in which the intensities at all wavelengths are simultaneously fitted to a model (Gualfetti *et al.*, 1999). In principle, with this approach all available data are used and models more complicated than two-state can be directly fitted and thereby tested. However, in practice, the data from denaturation experiments are often not sufficiently accurate to obtain a unique solution and unique set of parameters. Particularly, the enormous influence of the pre and post-transition baselines on the thermodynamic parameters derived make a multi-parameter fit

truly challenging with the typical data that can be obtained, and thus seemingly acceptable solutions may be obtained with a variety of models. Nevertheless, such global fits are very useful as tests for internal consistency (Jäger & Plückthun, 2001).

Another property that can, in principle, be used to measure stability is the rate of hydrogen/deuterium exchange of the protein at equilibrium. The group of highly buried protons of a protein, which is most slowly exchanging, can only exchange when the protein unfolds completely. From the measurement of this exchange rate, $\Delta G$ values can be extrapolated (Clarke *et al.*, 1997). Such measurements have to be carried out by NMR, however, as mass spectrometry is not well suited to untangle simultaneous exchange processes of fast and slowly exchanging protons. Although such equilibrium exchange measurements have been carried out with scFv fragments (Freund *et al.*, 1997; Williams *et al.*, 1997), this is far too laborious as a routine procedure.

### *Deriving free energies of folding*

No matter which biophysical property is followed, deriving free energies of folding obviously requires the system to be two-state, if two-state equations are used, with only the native and fully denatured state present in significant amounts at equilibrium (Pace, 1986). However, this prerequisite is often not fulfilled during equilibrium denaturation of those scFvs where equilibrium intermediates (e.g. with one native and one denatured domain) accumulate in large quantities (Wörn & Plückthun, 1998a, 1999). When a clear intermediate is visible, this is obvious (Figure 1). Even though such equilibrium unfolding curves can in principle be divided into two subsequent two-state transitions, the pre and post-transition baselines are often not long enough to be reliably fitted. Much more problematic, however, are those cases, possibly the majority of all scFv fragments, where no obvious plateau is visible, and the transition curve is simply broad.

The steepness of a transition curve is described by the $m$ value. $\Delta G$ values are calculated at different denaturant concentrations by determining the fraction of unfolded protein. The $\Delta G$ of unfolding at a given denaturant concentration, as explained above, is treated to be linearly dependent on the denaturant concentration (Pace, 1986, 1990). The $m$ value represents the slope of the straight line that results when the calculated $\Delta G$ values in the transition region are plotted against the corresponding denaturant concentration. Obviously, this slope has an enormous influence on the extrapolation from the transition region to zero denaturant, which is

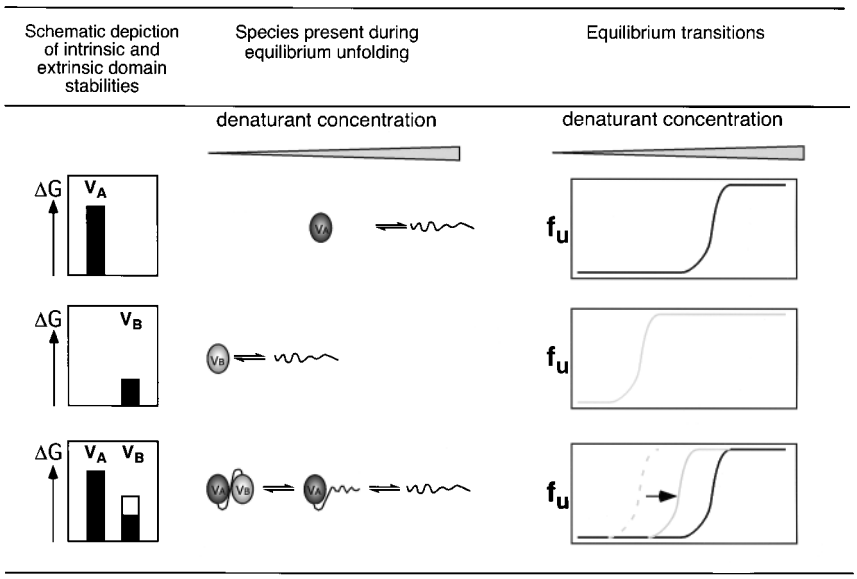


**Figure 1.** Effect of domain interactions on the stability of a domain. This is an illustration of the model of Brandts *et al.* (1989). $V_A$ and $V_B$ are the respective domains of the scFv fragment (either $V_H$ or $V_L$) and $V_A$ is defined as the intrinsically more stable domain. The unfolding curve of a stable domain $V_A$ is pictured, which undergoes a two-state unfolding. The second row shows the two-state unfolding of a less stable domain, $V_B$. In the bottom row, the unfolding of the same domains ($V_A$ and $V_B$) is shown when they form a linked dimer such as in a scFv fragment. In this case, a stable intermediate is formed, in which $V_B$ is unfolded and $V_A$ is still native, as the stability of the two domains is very different. Note that the transition of the less stable domain is shifted to higher denaturant concentrations, while that of the more stable domain remains as in isolation. When $V_A$ unfolds in the scFv fragment, no folded $V_B$ is left, $V_A$ denatures at the same denaturant concentration as in the absence of $V_B$. In the bar graph on the left, the intrinsic energy of each domain is indicated by the black bars, while the additional energy provided to $V_B$ by the presence of native $V_A$ in the context of the scFv is indicated by the white part of the bar (termed extrinsic energy). $f_u$, fraction unfolded; $\Delta G$, free energy of folding.

necessary to obtain the $\Delta G$ value in the absence of denaturants.

The suspicion that most scFv fragments do not show real two-state transitions is strengthened by the following observation. It has been noticed by comparing published data from 45 different proteins with known crystal structures and evaluated *m* values that the change in solvent-accessible surface area upon unfolding shows a strong correlation to the *m* value (Myers *et al.*, 1995). The solvent-accessible surface area itself can be determined from the crystal structure and even estimated fairly accurately from the molecular mass and the number of disulfide bonds of a globular protein. If we follow this approach and calculate typical *m* values for a protein of the size of a scFv, they should be around 5.3 kcal $mol^{-1}$ $M^{-1}$ in GdnHCl and around 2.6 kcal $mol^{-1}$ $M^{-1}$ in urea (taking into consideration the presence of two disulfide bonds) (Myers *et al.*, 1995). However, almost all *m* values reported for scFv fragments are significantly smaller by approximately 25-50 % (see e.g. Pantoliano *et al.*, 1991; Tan *et al.*, 1998; Tavladoraki *et al.*, 1999). Such *m* values, which are suspiciously smaller than expected, based on the theoretical calculations, are most likely due to a deviation from a two-state mechanism (Myers *et al.*, 1995; Pace, 1986). These low *m* values are unlikely to be a typical property for *all* scFv proteins, — even through such a deviation might conceivably be caused by some residual structure in the denatured state — since an *m* value of the expected size has been observed in at least a few cases (Jäger & Plückthun, 1999a; Jermutus *et al.*, 2001). If a denaturation curve represents a more complicated unfolding model than two-state, but is fitted anyway with an equation for a two-state model, an incorrect $\Delta G$ will be deduced.

Taken together, deriving conformational stabilities of scFv fragments from denaturant-induced transition curves, although theoretically feasible, is often problematic and may give misleading results. In such cases, a semiquantitative comparison of the denaturation midpoints may provide a rough idea about the *relative* stabilities, especially if related point mutants and transition curves with similar slopes are compared. Reliable *absolute* values of free energies can actually only be obtained if the two-state nature of a transition is confirmed experimentally. In denaturant-induced transitions this is rather difficult, but it is best done by monitoring the denaturation by different spectral probes, such as fluorescence, CD and NMR. Only if the transitions obtained from these different measurements coincide, the transition is consistent with a two-state model. And only in this case (or in the case of two clearly separated subsequent two-state transitions), the determination of $\Delta G$ is correct.

### *Other measures of stability*

A completely different problem of more practical relevance is the question of how fast a given scFv fragment denatures under a given set of conditions. For example, the rate of denaturation of a scFv fragment in the presence of various amounts of GdnHCl can be determined (Jäger & Plückthun, 1999a). This rate can be extrapolated to zero denaturant to obtain the spontaneous unfolding rate at room temperature. This extrapolation is also characterized by an *m* value, which is, however, usually much smaller than that from the equilibrium unfolding. The ratio of this kinetic *m* value to that obtained from equilibrium transitions allows one to derive information about the nature of the transition state of unfolding, as theoretically discussed by Tanford (1970).

Alternatively, the unfolding rates are measured directly under the conditions of interest. This is done by exposing a scFv fragment to elevated temperatures for several hours or days (e.g. Rajagopal *et al.*, 1997), human serum at 37 °C for prolonged time (e.g. Willuda *et al.*, 1999) or any other denaturing conditions. For example, the stability of scFv fragments after incubation in 50 % methanol, 50 % DMSO, 5 g/l Brij 35 (a detergent or surfactant), and 0.25 mg/ml pronase has been investigated (Dooley *et al.*, 1998). Moreover, the stability of scFv fragments against high shear forces and air-liquid interfaces has been tested by Harrison *et al.* (1998). This stress incubation is usually followed by some kind of functionality test (such as ELISA) or by the quantification of the amount of remaining monomeric species (for example, by gel filtration). Most of these harsh conditions, and thermal denaturation in particular, are usually accompanied by scFv precipitation and thus represent an irreversible reaction, dependent on protein concentration in addition to environmental parameters, such as pH or ionic strength. Therefore, such measurements do not allow one to derive a direct estimate of the thermodynamic protein stability, and they make the comparison of different proteins very dependent on details of the experiment. Thus the contribution of amino acid residues to the stability is more difficult to elucidate.

By testing for the resistance to the stress that a particular scFv fragment will experience during the intended application, such irreversible denaturation measurements do, however, allow an estimate on the potential of the scFv molecule in practice. Moreover, thermal aggregation properties (aggregation temperature and long-term aggregation at 37 °C) were shown to be well correlated with the stability against denaturants in equilibrium unfolding experiments for seven related point mutants of the scFv fragment A48 (Wörn & Plückthun, 1999). This correlation is believed to be due to the fact that aggregation usually starts from folding intermediates rather than from the completely denatured state (Wetzel *et al.*, 1990). The concentration of these aggregation-prone intermediates in a solution of scFv fragment put under slightly denaturing conditions is related to the equilibrium stability of the protein. Thus, stability against denaturants should also be a good predictor for the thermal stability of scFv fragments.

It has also been shown that amyloidogenic fibril formation of antibody $V_L$ domains *in vitro* is related to the thermodynamic stability of these domains, and that loss of stability is a major driving force in fibril formation (Hurle *et al.*, 1994; Raffen *et al.*, 1999). Moreover, aggregation temperature and thermodynamic stability are also correlated quite well, even though not perfectly, in different interleukin-1β mutants (Chrunyk & Wetzel, 1993). The coupling between thermodynamic stability and irreversible thermal denaturation or aggregation tendency may therefore be a common phenomenon, and it predicts that the intermediates in question, once formed, always aggregate at the same rate. This, however, implies that this correlation can only be good for similar molecules, such as point mutants.

The term ''stability'' has also been used more loosely to define the biological half-life of scFv fragments during *in vivo* applications in human patients (Hudson, 1999). This half-life is not only influenced by aggregation or proteolysis of the scFv protein, but also by the scFv fragment rapidly clearing from the blood, due to its small size. The pharmacokinetic clearance time is mainly influenced by the size of the molecule, and can thus be increased by forming defined scFv multimers (reviewed by Plückthun & Pack, 1997). Moreover, side-specific modification with polyethylene glycol has recently been shown to increase the *in vivo* half-life of Fab fragments without affecting the binding properties (Chapman *et al.*, 1999), a strategy that may also be applicable to scFv fragments. Unspecific attachment of PEG has already been proven to confer higher serum stability to antibody fragments, however, at the expense of a decreased antigen-binding affinity (Pedley *et al.*, 1994; Kitamura *et al.*, 1991). Alternatively, side-specific PEGylation of scFv fragments has been possible (Lee *et al.*, 1999) without affecting affinity by coupling polyethylene glycol to mannose residues of an *in vivo* glycosylated scFv fragment secreted from *Pichia pastoris*, and the resulting molecule displayed a tenfold increase in circulation time in mice (Wang *et al.*, 1998). Obviously, the biological *in vivo* half-life can only be measured experimentally in animals or human beings.

## Parameters influencing the stability of scFv fragments

The overall stability of a two-domain protein such as a scFv fragment depends on the intrinsic stability of the $V_L$ and $V_H$ domains as well as on

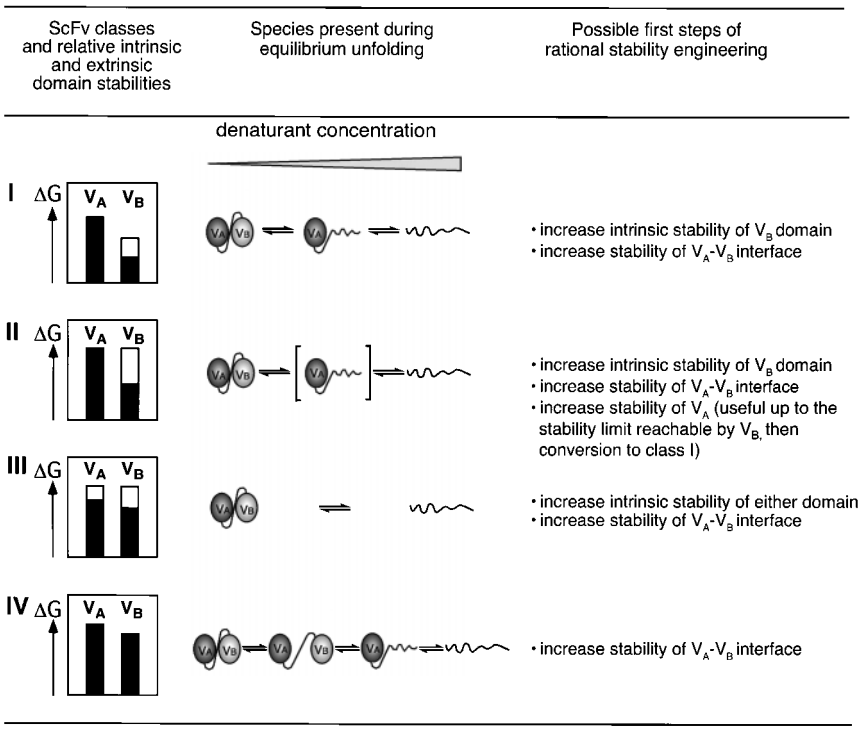


**Figure 2.** Possible equilibrium unfolding schemes of scFv fragments and resulting rational engineering strategies. Intrinsic domain stabilities are represented by filled bars, extrinsic contributions provided by the interface are represented by open bars (cf. Figure 1). $V_A$ and $V_B$ are the respective domains of the scFv fragment (either $V_H$ or $V_L$) and $V_A$ is defined as the intrinsically more stable domain. $\Delta G$ stands for the free energy of folding. ScFvs are classified by their equilibrium unfolding behavior, which is determined by the relative stabilities of $V_A$ and $V_B$ and the stability of the $V_A$-$V_B$ interface. Different parts of the molecule are limiting the overall scFv stability in fragments belonging to different classes. Rational stability engineering has to concentrate on the stability-limiting property of the fragment (adapted from Wörn & Plückthun, 1999).

the stability of the interface (Figures 1 and 2). In different scFv fragments, different properties can be limiting for the overall stability (Wörn & Plückthun, 1999). As a scFv loses its functionality with the first transition in a series of multiple transitions, it is essential to determine the stability-limiting portion of a scFv, as described by Wörn & Plückthun (1999), before performing rational stability engineering. This ordered strategy allows one to focus the engineering efforts to the stability-limiting property of the protein (Figure 2).

### *Disulfide bonds*

One critical hallmark influencing the intrinsic domain stability of both $V_H$ and $V_L$ is the intradomain disulfide bond, which is formed by the highly conserved Cys residues at framework positions H22/H92 and L23/L88, respectively (Williams & Barclay, 1988) (numbering according to Kabat *et al.*, 1991). The stabilizing contribution of intradomain disulfides in antibody domains has been calculated to be in the range of 4-6 kcal/mol (Goto & Hamaguchi, 1979; Goto *et al.*, 1987; Frisch *et al.*, 1996). The correct native structure can, in principle, also be reached in the absence of the disulfide(s), as shown by X-ray crystallography for the disulfide-free $REI_{v\text{-}\kappa}$ domain (Usón *et al.*, 1997) and by functional studies for disulfide-free variants of the levan-binding scFv A48 (Proba *et al.*, 1998) and the $p185^{HER2\text{-}ECD}$-binding 4D5 scFv fragments (Wörn & Plückthun, 1998b). However, many scFv fragments cannot fold to the native structure without disulfide bonds (e.g. Glockshuber *et al.*, 1992) because they are not stable enough to ''survive'' reduction or removal of the disulfide bonds energetically. Nevertheless, some exceptions have been reported. These proteins are either intrinsically stable enough (Wörn & Plückthun, 1998b; Tavladoraki *et al.*, 1999) or their overall stability had to be increased beforehand by rational engineering (as in case of the isolated $REI_{v\text{-}\kappa}$ domain; Frisch *et al.*, 1996) or by evolutionary approaches (Proba *et al.*, 1998; Martineau *et al.*, 1998).

Completely cysteine-free scFv fragments have been produced (Proba *et al.*, 1998; Wörn & Plückthun, 1998b). Val-Ala pairs were selected from 25 possible combinations as being the best substitution for the $V_L$ disulfide bond in the cysteine-free scFv fragment A48 (Proba *et al.*, 1998). Indeed, a cysteine-free variant of the scFv 4D5, where the two intradomain disulfide bonds had been replaced with Val-Ala pairs, was found to be slightly more stable than the reduced dithiol form of the cysteine-containing 4D5 (Wörn & Plückthun, 1998b). However, in a model system established in yeast, such a Val-Ala variant of a GCN4-binding scFv fragment turned out to be less active as cytoplasmic intrabody than the corresponding cysteine-containing variant (Wörn *et al.*, 2000). Thus, replacing the intradomain disulfides with Val-Ala pairs does at least not generally increase the performance of scFv fragments expressed under reducing conditions, and the structural reasons for this difference in behavior need to be studied further.

Besides the obvious stabilizing effect of the intradomain disulfide bond, many single point mutations have been reported to increase the intrinsic stability of scFv domains. Some of these residues will be described in the section on domain stability.

### *Interface stability and the domain-swapping phenomenon*

Insufficient stability of the $V_H$-$V_L$ interface of scFv fragments has often been suggested as a main cause of irreversible scFv inactivation (Reiter *et al.*, 1994a), since transient opening of the interface, which would be allowed by the peptide linker, exposes hydrophobic patches that favor aggregation. Fv fragments have been reported to dissociate into $V_H$ and $V_L$ domains with $K_D$ values ranging from $10^{-9}$ to $10^{-6}$ M (reviewed by Plückthun, 1992), and this would allow dynamic and transient ''opening'' of the scFv assembly. The different strategies that have been followed to increase interface stability in scFv fragments are summarized below.

A different but related aspect influencing scFv stability is the monomer-dimer equilibrium (Figure 3). Understanding this aspect is important in order to be able to obtain homogeneous scFv preparations. ScFv fragments which favor dimerization can sometimes be desired to create heterodimeric or homodimeric dimers, which have been termed diabodies. They can be used to create bispecific fragments or homodimers with increased avidity and decreased renal clearance time (reviewed by Hudson, 1999; Colcher *et al.*, 1998). Many scFv fragments have been reported to form dimers to some extent, where the $V_H$ domain of one chain is paired with the $V_L$ domain of another chain and *vice versa* (Essig *et al.*, 1993; Holliger *et al.*, 1993; Whitlow *et al.*, 1993, 1994; Desplancq *et al.*, 1994) and the dynamics of this process have been investigated (Arndt *et al.*, 1998). The fraction of dimer formed is dependent on linker length (with short linkers between five and ten amino acid residues forming almost quantitatively dimer), but also on external parameters such as ionic strength, pH and the presence or absence of antigen (Arndt *et al.*, 1998). Also, different scFv fragments with intermediate linker lengths differ greatly in the amount of dimer formation; the molecular details of this phenomenon are not yet understood. The monomeric form of scFv with normal 15-20 residues linkers is believed to be thermodynamically most stable. However, depending on expression conditions, ionic strength, and pH the scFv dimer can initially be dominating and eventually remain trapped in this state in a protein-dependent manner (Arndt *et al.*, 1998). The role of the linker is discussed again below.

Intuitively, diabodies would appear at first to be ''more stable'' than the monomeric form of the molecule, yet experimental results point to a more

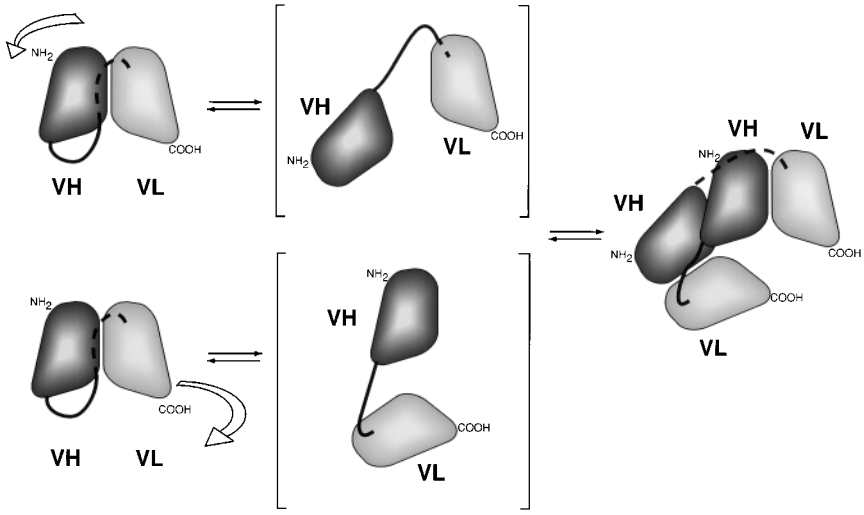


**Figure 3.** Structural relationship between a monomeric scFv and a diabody. Using the 3D structure of a diabody (PDB entry 1lmk), it is schematically shown how the monomeric form and the dimeric form may interconvert and are in equilibrium, even though the equilibration may be very slow (see the text). The monomeric form is favored by using long linkers (20 residues or more), while short linkers (five residues) make it sterically impossible to form monomers. By using intermediate linkers, mixed populations can be obtained during *in vivo* expression, depending on the variable domain sequences. The monomeric form appears to be the thermodynamically stable one in the concentration range examined (Arndt *et al.*, 1998), and the dimeric forms appear to be kinetically ''trapped''. For details, see the text.

complicated picture. The thermodynamic stability of the anti-digoxin scFv 26-10 has been examined with stepwise increasing linker lengths from zero to 25 residues (in units of ($Gly_4$-Ser)) by J. Huston *et al.* (personal communication; M.-S. Tai, H. Oppermann, W. F. Stafford & J. S. Huston, unpublished results). Saturating antigen served as a non-covalent linker, greatly reducing the difference in stability between monomer and dimer. However, in the absence of antigen, stability differences were quite marked. According to its transition curve in GdnHCl equilibrium unfolding, the normal scFv monomer, which was predominantly formed with linker lengths of 15, 20, and 25 amino acid residues, had denaturation midpoints at nearly twice the denaturant concentration as those of the diabodies, which formed with the 0, 5, and 10-mer linkers. In order to understand the basis for this difference between diabody and scFv stability, the corresponding unlinked 26-10 Fv fragment was also analyzed (J. Huston, personal communication). The midpoint of the transition curve of the Fv fragment was essentially the same as the midpoints of the 26-10 diabodies. For the scFv, a slight increase of stability was observed with increasing linker length, but they were all decidedly more stable than the diabodies, based on the transition midpoints. It might be noted that the $V_L$ domain of the Fv fragment was expressed as a fusion to a domain of protein A, but this fusion partner did not contribute to the transition curve of the Fv, but did improve bacterial expression of the free $V_L$ domain. The differences in stability between the Fv fragment, the scFv monomer and the less stable scFv dimer emphasize that diabodies are topologically more similar to unlinked Fv fragments than to scFv fragments. Although the chains of a diabody are associated by a tandem repeat of Fv contacts, their overall stability is at best only marginally higher than that of the Fv fragment comprising them. In contrast, the scFv is topologically a single polypeptide chain, where the domains are held at a high local concentration, recently estimated to be in the low millimolar range (Jäger & Plückthun, 1999a). Taken together, these experiments suggest that a diabody made from a weakly associated Fv fragment may also be poorly stable, and may thus be unsuitable for many applications *in vivo*.

## Strategies to improve scFv stability

Different routes have been followed to obtain scFv fragments with increased stability. The possible strategies can be divided into rational and evolutionary approaches (Figure 4). For rational approaches structure-based knowledge or sequence statistics are used to predict stabilizing mutations that are then introduced into a given scFv fragment by site-directed mutagenesis or, alternatively, into a framework which serves as the recipient of a CDR library. Strictly speaking, the use of mutations from sequence comparisons is not any more ''rational'' than taking them from a directed evolution experiment. We therefore define the term ''rational'' as meaning the use of any hypothesis-based mutations, which are introduced by site-directed mutagenesis. The predictability of such mutations is, however, still limited, even though the database of mutations empirically found to be

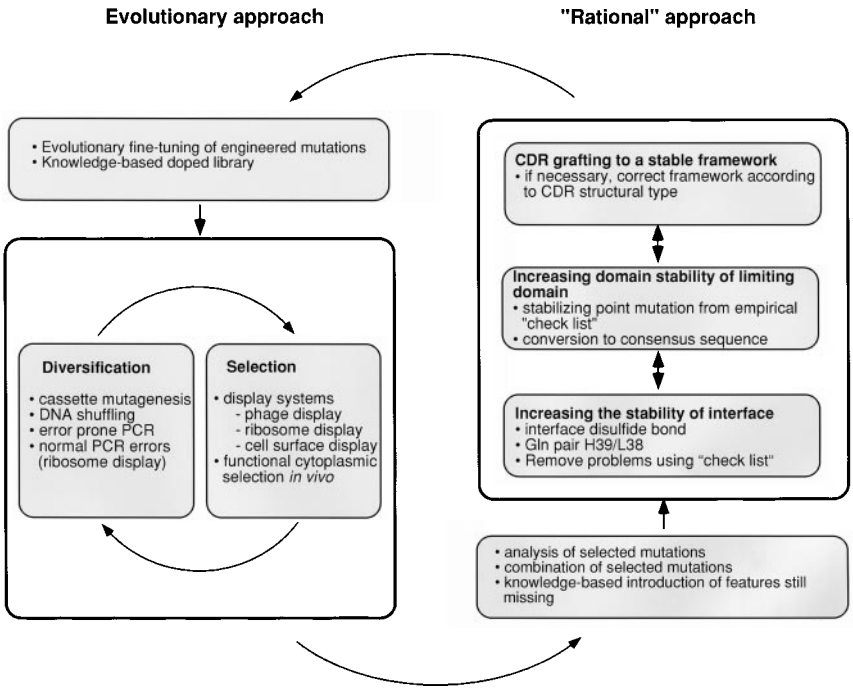


**Figure 4.** The interfacing of rational and evolutionary stability engineering. The Figure illustrates how rationally designed variants can be subjected to evolutionary fine tuning, and, conversely, how selected clones from a library evolution can be further improved by combining the discovered mutations and adding previously identified mutations. Importantly, the optimization project can start at any point, depending on the starting molecules. For details, see the text.

useful is increasing rapidly. As antibody stability depends on both framework and CDR contributions, rational engineering of stabilizing point mutations in the CDR loops would also be desirable but can be complicated, since the concomitant change in antigen-binding affinity is difficult to predict.

Alternatively, evolutionary strategies can be employed. Here, a randomized library is generated from a starting scFv fragment (which usually would have been pre-selected for its ability to bind the target antigen). The initial scFv can, for example, be diversified by error-prone PCR (Fromant *et al.*, 1995) or DNA shuffling (Stemmer, 1994). The library produced in this way is, in a next step, subjected to some kind of selection pressure, which tries to put emphasis on the selection of fragments with increased stability. All common selection strategies such as phage panning (Winter *et al.*, 1994), selectively infective phage technology (SIP) (Krebber *et al.*, 1997), ribosome display (Hanes & Plückthun, 1997; He & Taussig, 1997), bacterial display (Francisco *et al.*, 1993), yeast display (Boder & Wittrup, 1997) or any *in vivo* assay based on scFv functionality, select in a method-specific way for a compromise of different scFv properties (including affinity, stability, toxicity, expression rate, or dimerization tendency). The challenge regarding these evolutionary approaches, when used for stability engineering, is thus to find selection conditions that put a particular pressure on protein stability (Figure 5).

It is, of course, also possible to combine evolutionary and rational approaches in an arbitrary order to increase scFv stability even further, and this may currently be the best method to solve the problem. A particular scFv fragment that has been selected from a library under conditions putting focussed pressure on scFv stability can still be checked for the presence or absence of known potentially stabilizing framework residues. These residues, if not already present, can then be introduced into molecules selected from the library (''stability fine-tuning''). Due to the restriction in library size the optimal solution may not have been in the pool, or the most stable scFv may have been counter-selected because of compromises in antigen-binding affinity or other properties of the molecule. Conversely, it is worth considering ''doping'' a starting library with known potentially stabilizing mutations before performing selection rounds, in order to be sure that these mutations are represented in the initial library (Proba *et al.*, 1998; Jung *et al.*, 1999) (Figure 4).

Below, different rational and evolutionary approaches to increase scFv stability are discussed. They are also summarized in Figure 4, where the interfacing and possible work-flow between rational and evolutionary methods is indicated.

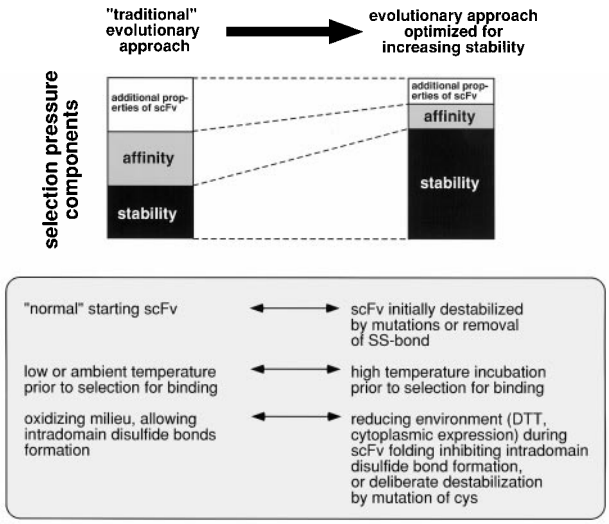


**Figure 5.** Directing evolutionary methods towards stability increase. All evolutionary methods select for combined, overall properties of scFvs, including stability, affinity and others (such as toxicity, folding efficiency or dimerization tendency, summarized as additional properties of scFv in the Figure). Therefore, a ''compromise solution'' is obtained. Different methods and selection conditions can, however, emphasize the contribution of these scFv properties to the overall selection pressure to a different extent. Some strategies are summarized which bias selection particularly towards selection for higher scFv stability. Details are given in the text.

## Rational approaches for stability engineering of scFvs

### *Increasing scFv stability by grafting CDRs onto a stable framework*

It is possible to transplant the antigen-binding loops from one antibody to a different antibody framework, a procedure called CDR loop grafting (Jones *et al.*, 1986). This grafting procedure was originally used to humanize murine antibodies to decrease their immunogenicity in human patients (reviewed by Winter & Harris, 1993). However, CDR grafting onto a superior framework has now also been successfully used in several examples to generate scFv fragments with improved stability and folding properties.

The CDRs from the fluorescein-binding 4-4-20 scFv, which yields almost no soluble protein in the *Escherichia coli* periplasm, have been transplanted to the stable and well-expressing 4D5 scFv fragment. The resulting 4D5Flu was shown to have significantly improved expression properties, unchanged binding affinity, and the midpoint in urea equilibrium unfolding was shifted from 4.1 M for the 4-4-20 to 6.4 M for the 4D5Flu scFv (Jung & Plückthun, 1997). The 4D5 framework (PDB file 1fvc) is itself the product of loop grafting the CDRs of the murine anti-HER2 antibody to a human framework for application in tumor therapy. It consists of a heavy chain variable domain essentially identical to the germline IGHV 3-66 (IMGT nomenclature) (Giudicelli *et al.*, 1997), or VH 3-18 (Vbase nomenclature; http://www.mrc-cpe.cam.ac.uk/imt-doc), locus DP 3-66 (DP-86), and the kappa light chain variable domain derived from germline IGKV 1-39 (IMGT nomenclature), or Vκ 1-1 (Vbase nomenclature), locus DP O12.

The 4D5 framework also served as acceptor in a loop grafting from the EGP-2-binding MOC31 scFv (Willuda *et al.*, 1999). The graft proved to have significantly increased serum stability at 37 °C *in vitro*, better expression behavior and unchanged antigen-binding properties compared with the original MOC31. However, it could be significantly improved further by introducing eight additional substitutions in the heavy chain, which were taken from the CDR donor and maintain the structural framework class (see below). Importantly, only the graft, but not the CDR donor MOC31, showed efficient tumor localization on lung tumor xenografts in mice, suggesting that high thermal stability of scFvs is decisive for tumor targeting *in vivo*.

In a cellular assay system which measures the inhibitory activity of cytoplasmically expressed anti-GCN4 scFv intrabodies in the budding yeast *Saccharomyces cerevisiae*, CDR grafting was also used to produce a fragment with increased *in vitro* stability and significantly improved *in vivo* activities, compared to the starting scFv (Wörn *et al.*, 2000). In this particular case, the CDRs from a λ-domain had to be grafted to a κ-domain, and thus two variants were made, either containing most of the κ-framework (''κ-graft'') or including some interface residues from the λ-loop donor (''λ-graft''), to allow for possible relative domain rearrangements. The λ-graft, generated by grafting the loops of the anti-GCN4 wild-type to the very stable ''hybrid scFv'' (Wörn & Plückthun, 1999), was more stable than the wild-type variant in GdnHCl denaturation. The affinity of the grafted variant to the GCN4 antigen was decreased by an order of magnitude from a $K_D$ of $\sim 4 \cdot 10^{-11}$ M to $\sim 4 \cdot 10^{-10}$ M. Nevertheless, the λ-graft showed considerably increased activity in inhibiting the transcription factor GCN4, measured by β-galactosidase reporter activity *in vivo*, since a higher fraction of the stabilized graft variant was able to fold into the correct native structure under the reducing conditions of the cytoplasm. This was also reflected in a dramatically improved solubility of the λ-graft in the yeast cytoplasm, compared with the loop donor. A destabilized point mutant of the anti-GCN4 wild-type with unchanged antigen-binding properties, on the other hand, showed a decreased inhibitory effect as intrabody. Thus, a direct correlation between *in vitro* stability and *in vivo* performance of the different anti-GCN4 intrabodies as cytoplasmic inhibitors was observed.

Stability engineering by loop grafting has also been performed for an unlinked Fv fragment. A synthetic Fv framework consisting of a $V_\kappa$ domain,

engineered from McPC603 for stability using the consensus sequence approach (see below), and the $V_H$ domain of the antibody B1-8, which naturally contains most residues from the murine Kabat consensus sequence, served as acceptor in a CDR grafting experiment for the intracellular production of a catalytic antibody called Ica-Fv20 (Ohage *et al.*, 1999). The grafted Fv fragment could be expressed in soluble form in the cytoplasm of *E. coli*, but an *in vivo* esterolytic activity of this fragment could not be assessed.

If the 3D structures of framework acceptor and donor are unknown, CDR grafting requires careful homology modeling of both of these structures, prior to the modeling of the actual graft. Frequently, additional changes in the acceptor framework region are necessary, in addition to the changes in the CDRs (as was done in all cases reported here), in order to prevent obvious clashes in the graft model structure, to maintain critical contact residues of the CDRs or to avoid a switch between framework classes. When a suitable acceptor is required for a given loop donor, it would be very useful if one could rely on a set of very stable, well-expressing scFv frameworks, similar to those used in above examples, but belonging to different structural subclasses. With the increasing number of stability-engineered scFvs, such a set of stable and yet diverse frameworks, which would improve the ''hit rate'' of successful grafting experiments, is now emerging and is likely to become available within the next years. The synthetic consensus sequence concept of the HuCAL (Knappik *et al.*, 2000; see below) is based on this premise.

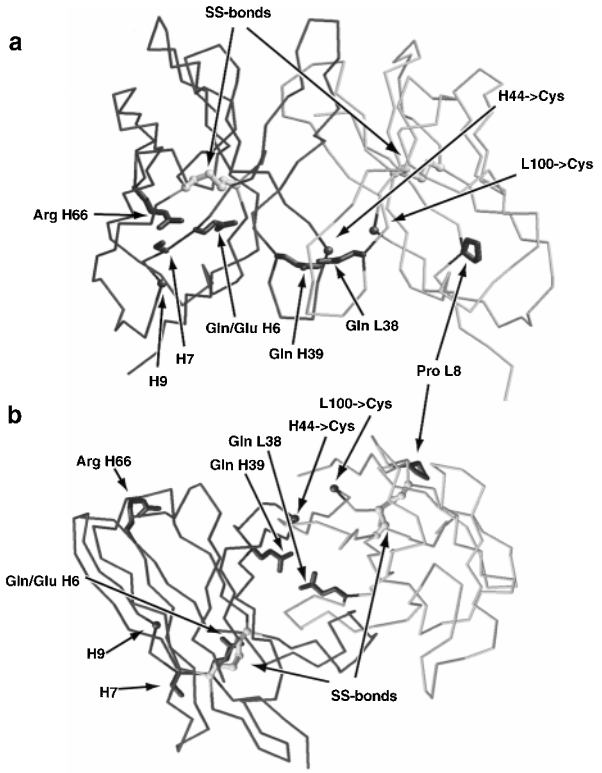


**Figure 6.** Location of mutations discussed in the text. The structure of the Fv fragment of the antibody hu4D5-8 is shown (PDB entry 1fvc). (a) side view; (b) top view. The $C^{\alpha}$ trace of the $V_H$ domain is shown in dark gray, that of the $V_L$ domain in light gray.

### *Increasing the intrinsic domain stability*

The intrinsic domain stability of both $V_H$ and $V_L$ domains can in principle be increased by introducing stabilizing point mutations, either in framework or in loop positions (Figure 6). The stabilizing effect of multiple point mutations introduced into one single domain has often been found to be additive, as a good first approximation, as long as the mutations do not interact with each other (e.g. observed by Frisch *et al.*, 1996; Proba *et al.*, 1997; Wörn & Plückthun, 1998a; Ohage & Steipe, 1999).

The easiest and most straightforward approach to introduce stabilizing mutations by rational engineering is to use sequence statistics (Steipe *et al.*, 1994). The underlying idea is that the immunoglobulin consensus sequence reflects the result of selective pressure put on stability and heterodimer interactions of all sequences present in the repertoire. The statistical occurrence of different residue types at a particular framework position may be expected to be related to its contribution to stability, and the consensus residue may thus be taken as being the best choice for stability (''canonical sequence approximation''). However, this is obviously a simplification because it ignores the interaction between those amino acid residues which should only be changed as groups: by paying attention to these structural subclasses, much higher stabilities could be reached (Willuda *et al.*, 1999).

By following the alignment to a general consensus sequence, stabilizing mutations in the McPC603 $V_\kappa$ domain correlated with the expectations from sequence information with about a 60% success rate (Steipe *et al.*, 1994). A similar approach led to the identification of stabilizing mutations in the $REI_{v\text{-}\kappa}$ domain (Frisch *et al.*, 1996) and in a $V_H$ domain (Wirtz & Steipe, 1999), while Willuda *et al.* (1999) used a subgroup-specific approach to $V_H$ engineering. Additionally, structural motifs such as idealized β-turns from non-antibody sequences can be included (Ohage *et al.*, 1997). The McPC603 $V_\kappa$ domain was thereby further stabilized to a free energy of folding of about −11 kcal/mol (*m* value of 3.7 kcal $mol^{-1}$ $M^{-1}$). Domains with stabilities in this range can be refolded under reducing conditions *in vitro*, as shown for the stabilized $V_\kappa$ domains of REI (Frisch *et al.*, 1996), that of McPC603 (Ohage & Steipe, 1999), the naturally stable $V_\kappa$ domain of 4D5 (M. Jäger & A.P., unpublished results) and the stabilized $V_H$ domain of an anti-levan antibody

(Wörn & Plückthun, 1998a). Even higher stabilities have been observed with subgroup-consensus $V_H$ domains (S. Ewert *et al.*, unpublished results). Very stable and disulfide-free antibody domains have also been obtained by evolutionary approaches (see below).

A very similar approach of using consensus sequences was also followed by Chowdhury *et al.* (1998) in order to produce an immunotoxin with increased stability at 37 °C. ''Unusual'' amino acid residues, defined as having less than 5 % frequency in the Kabat database, were identified by aligning the target sequence with sequences of known, well-expressing and stable scFv sequences, and then scanning all framework residues for deviations from the Kabat consensus. As a further restriction, ''unusual'' residues identified in this first step were only considered problematic if their hydrophobicity was incompatible with their solvent exposure (i.e. if buried residues were hydrophilic or exposed residues were hydrophobic, although this restriction misses some important globally stabilizing residues discussed below). These residues were then mutated to the consensus residue, if the Kabat consensus was identical to the consensus of known stable and well-expressing scFv fragments.

The human combinatorial antibody library (HuCAL) (Knappik *et al.*, 2000) was designed with the consensus sequence approach in mind. However, instead of averaging across all antibody sequences, separate consensus frameworks were built for the different subgroups (7 $V_H$, 4 $V_\kappa$ and 3 $V_\lambda$), and care was taken to not disrupt interacting residues in the averaging process. By using totally synthetic genes, these frameworks have also unique restriction sites and can receive any CDR. The great majority of these antibodies shows good expression properties. The 3D structures of the different HuCAL frameworks have all been modeled (A. Honegger, unpublished work) and the coordinates have been deposited (see Knappik *et al.*, 2000, for the list of PDB entries).

### *Generally useful stabilizing mutations*

In the studies cited above, a number of stabilizing point mutations have been identified, without being mentioned here individually. Many more mutations, partly in the framework, partly in the CDRs, have been described to be stabilizing or destabilizing in specific scFv fragments or isolated domains (Yasui *et al.*, 1994; Benhar & Pastan, 1995; Martineau & Betton, 1999; Raffen *et al.*, 1999; Jäger & Plückthun, 1999b). Below, only those residues will be listed, which are: (i) positioned in the framework (and thus, in principle, easily transferable to other scFv frameworks); and (ii) have been described to act stabilizing in more than one scFv fragment. This list is, however, expanding rapidly (Honegger *et al.*, unpublished results).

An Arg at position H66 (Figure 6) was found to be stabilizing in comparison with a Lys in the scFv fragment A48 (Proba *et al.*, 1998; Wörn & Plückthun, 1998a). Both residues are represented with approximately equal frequency in murine sequences, while the Arg clearly dominates in the human consensus at position H66. The Arg H66, which had been selected in the context of a cysteine-free variant of the scFv fragment A48 (Proba *et al.*, 1998), was also shown to stabilize the cysteine-restored A48 (Wörn & Plückthun, 1998a), while mutating the naturally occurring Arg H66 back to a Lys destabilized a GCN4-binding scFv fragment (Wörn *et al.*, 2000). This residue is part of a conserved charge cluster in $V_H$ domains and can make a double hydrogen bond to Asp residue H86.

A Pro in position 8 of kappa light chains (Figure 6), which is a highly conserved residue in the Kabat database, was shown to stabilize both a hemagglutinin-binding scFv (Spada *et al.*, 1998) and the levan-binding A48 scFv fragment (Wörn & Plückthun, 1999), but decreased the expression and refolding yield in the latter case. This residue has a *cis*-peptide bond whenever it is proline, and all human kappa germline sequences carry it. Nevertheless, a number of mouse κ-chains do not have a proline residue at this position and in all these cases, the peptide is *trans*.

The nature of the residue at position H6 (Figure 6) has been investigated several times. This residue is part of a complex hydrogen-bonding network in the core of $V_H$ (Langedijk *et al.*, 1998), and is either Glu or Gln. Framework I can have different conformational classes depending on the residue types in positions H6, H7 and H9 (Honegger *et al.*, unpublished results), and when these types are mixed, a destabilization results. Since H6 is usually randomized and part of the PCR primer, if an antibody sequence is amplified from a native mRNA pool, very frequently the ''wrong'' residue is introduced. Upon correcting this mistake, a stabilization is observed (Brégégère *et al.*, 1997; Kipriyanov *et al.*, 1997; de Haard *et al.*, 1998; Langedijk *et al.*, 1998; Willuda *et al.*, 1999).

In summary, an alignment to the consensus sequence, either to that of all antibodies or preferably to the relevant subgroup, is a convenient first step for identifying potentially destabilizing residues and removing them. However, a very important refinement is to pay attention to the structural classes of frameworks and to avoid mixing them (Willuda *et al.*, 1999). When following this approach, the initial identification of the stability-limiting property of the scFv as described by Wörn & Plückthun (1999) can be very critical. This avoids unnecessary mutagenesis in those scFv domains, which are not limiting the overall stability of the corresponding scFv fragment anyway (Figure 2).

### *Increasing the stability of the $V_H$-$V_L$ interface*

In the Fv fragment the contribution of contact domains to the interaction energy present in the natural Fab fragment is missing. As a consequence,

the relatively weak $V_H$-$V_L$ interaction energy of typical Fv fragments (see above) is directly reflected in their much lower and concentration-dependent stability compared to the corresponding scFv fragments (Jäger & Plückthun, 1999a), since the domains are unlinked in the Fv fragment. This relatively weak interaction energy, considering the substantial size of the $V_H/V_L$ interface of about 710 Å$^2$ (ranging from 583 to 874 Å$^2$) (Padlan, 1994), may be the result of a low selective pressure caused by the presence of the constant domains in the natural antibodies, and perhaps the need for maintaining a ''generic'' interface which allows essentially all $V_H$ to pair with all $V_L$. It follows that in most given scFv fragments, the interface leaves room for improvement.

The presence of the linker already constitutes stabilization compared to the Fv fragment, but we need to discuss the consequences of different linkers. In principle, both the length and the sequence of the linker can have an effect on stability (Robinson & Sauer, 1998) and this effect is usually expressed as the effective molarity, i.e. the molarity an Fv fragment would have to have in order to result in the same properties. In antibodies, the situation is complicated by the existence of a ''domain-swapping'' phenomenon (Arndt *et al.*, 1998) (Figure 3), where dimeric (Essig *et al.*, 1993; Holliger *et al.*, 1993; Whitlow *et al.*, 1993, 1994; Desplancq *et al.*, 1994), trimeric and even tetrameric assemblies (Hudson & Kortt, 1999) can form with short linkers. Therefore, only a certain range of linker lengths are directly comparable for stability measurements. Linkers with different sequences and slightly different length have been compared (Hennecke *et al.*, 1998), all giving rise exclusively to monomers, and without any detectable effect on stability. However, Huston and co-workers (personal communication) have observed that increasing linker length from 15 to 20 and 25 residues (i.e. in the range where monomeric scFv fragment was present) slightly increased scFv stability in GdnHCl equilibrium unfolding, perhaps by increasing the fraction of monomers in that model system. Therefore, including linkers of 25 residues as a matter of routine may be a general way of slightly increasing the stability of some scFv fragments by favoring monomer formation, which is the thermodynamically stable form of scFv fragments at typically used concentrations (Arndt *et al.*, 1998). Optimization of the linker sequence, on the other hand, is unlikely to be a strategy of great promise to stabilize antibody scFv fragments.

The most widely used strategy to stabilize the $V_H$-$V_L$ interface is to engineer an interface disulfide bond into the contact surface between the two domains. While this strategy has initially been used alternatively to the peptide linker (Glockshuber *et al.*, 1990; Brinkmann *et al.*, 1993), it is now also routinely employed in addition to the linker (Young *et al.*, 1995; Rajagopal *et al.*, 1997; Wörn & Plückthun, 1999). In the original proof-of-principle experiment, the best positions for a disulfide bond had been identified from geometric calculations, based on the crystal structure of the given antibody (Glockshuber *et al.*, 1990). Subsequently, similar modeling was used to identify suitable conserved framework residues that can be mutated to cysteine residues, and several possibilities were found (Reiter *et al.*, 1995). The combination H44-L100 (Figure 6) has been most frequently used in scFvs with satisfactory results (e.g. Brinkmann *et al.*, 1993; Reiter *et al.*, 1994a,b; Rajagopal *et al.*, 1997; Wörn & Plückthun, 1999), and in a particular Fv fragment, this combination gave the highest yield after refolding of the combinations tested (Reiter *et al.*, 1995). However, due to slight variations in domain orientation, a property still extraordinarily difficult to model, different pairs of cysteine residues might be preferable in different antibodies (e.g. see Rodrigues *et al.*, 1995; Dooley *et al.*, 1998). Rodrigues *et al.* (1995) imposed a further restriction on the selection of suitable interface residues by searching a position for the disulfide bond, which is completely buried, in order to minimize the risk of cleavage in the serum. Residues H101 and L46 were found to fit these requirements best, although this may not be transferable to other scFvs, since residue H101 is already part of the CDRs, and mutating this residue to a Cys may impede antigen-binding in other scFv fragments.

Such engineered interface disulfide bonds were shown to increase the stability of scFv fragments against thermal aggregation (Young *et al.*, 1995; Rajagopal *et al.*, 1997), denaturants (Wörn & Plückthun, 1999), non-polar and polar solvents, surfactants and proteases (Dooley *et al.*, 1998) and were also more potent during *in vivo* applications in tumor imaging and therapy (reviewed by Reiter *et al.*, 1996).

However, the interface disulfide bond as a means to stabilize the $V_H$-$V_L$ interface in scFvs has some drawbacks. The disulfide-bridged variants usually have to be produced by refolding from inclusion body protein, since the functional yield upon periplasmic secretion is severely decreased by the presence of the interface disulfide (Young *et al.*, 1995; Wörn & Plückthun, 1999). Furthermore, it cannot be used to stabilize cytoplasmically expressed intrabodies, because disulfide bond formation requires an oxidizing environment. Thus, additional strategies to stabilize the $V_H$-$V_L$ interface in scFvs would be desirable.

Tan *et al.* (1998) have investigated the possibility to stabilize the interface of the S5 scFv fragment by replacing the highly conserved Gln pair between framework positions H39 and L38 (Figure 6), which form two hydrogen bonds in a head-to-tail alignment across the $V_H$-$V_L$ interface, with different amino acid combinations. This study showed that the Gln pair is not essential for the stability of the interface. In an attempt to bury more surface area in the interface the conserved Gln residues were replaced with Met residues in different permutations, and Met-Ala pairs were constructed in

both orientations. Moreover, Lys-Glu pairs were introduced in both orientations, to form a salt bridge across the interface. While the authors report $\Delta G$ values with a maximum apparent increase in $\Delta G$ of 2.1 kcal/mol for the L-Q38 K/H-Q39E double mutant, compared with the wild-type, the *m* values are conspicuously low (see above) and cover a wide range, while the midpoint of the transition changes much less, which would be unexpected if a true two-state transition is measured for all fragments. It also remains to be tested if these mutations are transferable to other scFv fragments.

Further interface engineering in scFv fragments has been performed by introducing ''knob-into-hole'' mutations, but mainly in order to drive hetero-dimerization of bispecific diabodies, rather than to increase the stability of monomeric scFv (Zhu *et al*., 1997). Another approach was explored by Brinkmann *et al*. (1997) by engineering a scFv fragment, in which the natural connectivity between the two domains is permuted. The resulting permutated Fv (pFv) was, however, not more stable than the corresponding scFv, even though it might be useful in special situations, if the classical peptide linker interferes with antigen-binding.

The individual optimization of a particular $V_H$-$V_L$ interface may be conceivable by an evolutionary approach. However, currently no generally applicable mutations have been identified which are equally effective as interface disulfide bonds for stabilizing the $V_H$-$V_L$ interface in scFv fragments, apart from ''correcting'' obvious point mutations.

## Evolutionary approaches for stability engineering of scFvs

If evolutionary approaches are applied to increase scFv stabilities, the direct and unequivocal coupling of the function (binding) to the stability of the fragment is the crucial step. As all selection systems work by physically coupling the genotype to the binding phenotype (Figure 7), it is essential to ensure that binding is limited exclusively to the folded state, and no enrichment of partially unfolded molecules occurs. However, all selection systems will select for a ''compromise'' between increases in affinity and increases in the number of active molecules. The latter may be increased by a true improvement of the stability or of the folding efficiency, resulting in reduced aggregation. The challenge is therefore to direct the selection pressure mostly to the stability component (Figure 5).

The principal ways of achieving this have been reviewed by Forrer *et al*. (1999) for phage display, but the analogies to other display systems are readily apparent (Figure 8). In this section, we will briefly review these strategies and discuss the key results regarding antibodies, using phage display, ribosome display and intracellular expression.

### *Selection for scFv stability by phage display*

During phage panning (Winter *et al*., 1994) scFv fragments displayed as fusion proteins to the gene3

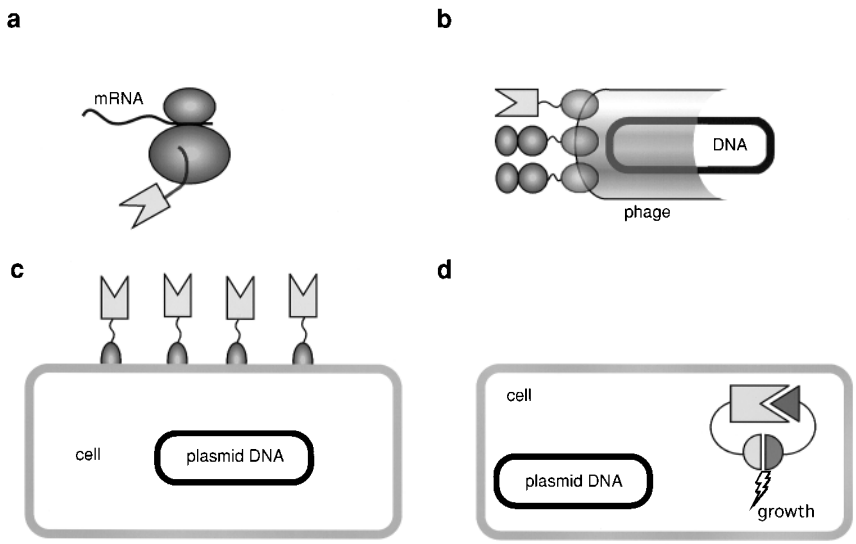


**Figure 7.** The linking of phenotype and genotype in selection systems for antibodies. (a) In ribosome display, mRNA and protein product are linked by the ribosome; (b) in phage display, the antibody is fused to the minor coat protein g3p of filamentous phage, while the DNA is on the inside of the phage; (c) in bacterial cell surface display, the antibody is displayed on the outer surface of the cell, while the genetic information is encoded on a plasmid inside the cell. These extracellular or *in vitro* methods can be tailored for stability selection (Figure 8). Additionally, antibodies can be screened and selected intracellularly (d) (Wörn *et al*., 2000; Visintin *et al*., 1999), and selection for growth puts high demands on the stability of the antibody. Intracellular screening is achieved by fusing the antigen and antibody to two protein halves which, when brought together by the antibody-antigen interaction, allow cellular growth.

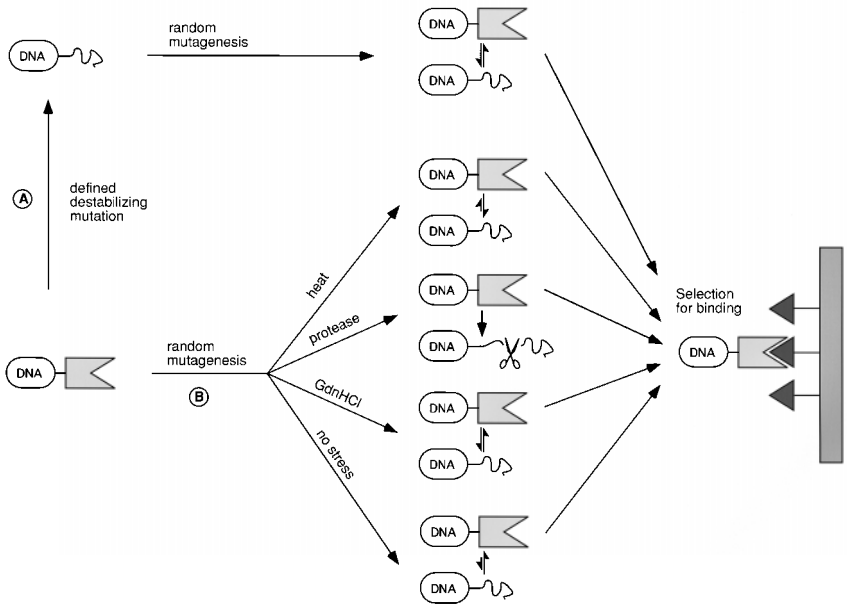


**Figure 8.** Selection for stability with display technologies. Pathway A: a defined destabilizing mutation (or set of mutations) is introduced into the starting scFv (e.g. removal of disulfide bonds). The resulting fragment is too unstable to fold, and it is denatured, even without incubation under adverse conditions. Randomization of this destabilized variant results in a pool of scFv fragments, some of which are stable enough to bind to immobilized antigen and thus get selected. In the last step the defined destabilizing mutations are removed again and the resulting fragments should, in general, have increased stability, compared with the starting scFv. Pathway B: the starting scFv fragment is directly subjected to random mutagenesis, generating fragments with decreased, unchanged or increased stability. This pool is subjected to an incubation under adverse conditions (heat, protease, GdnHCl), favoring denaturation of less stable variants, and then selected on immobilized antigen. Only antibody fragments surpassing a certain threshold stability will be selected. Note that heat incubation fulfills these requirements best, since GdnHCl denaturation is partially reversible (as indicated by the reversible arrow) and protease treatment of displayed scFvs is problematic for different reasons, as discussed in the text. If the pool is not subjected to any stress incubation (''no stress''), the enrichment factor for fragments with increased stability will be much smaller, but some enrichment for stability is still possible due to a weak correlation of production yield and stability.

protein of filamentous M13 phage are bound to immobilized antigen, usually in successive rounds of binding and amplification (by reinfection of bacteria). Different strategies have been used, in the attempt to bias the selection towards increasing stability (Figure 8). The initial deliberate reduction of protein stability by site-directed mutagenesis followed by classical phage panning is a very attractive option, since it imposes a strong selection pressure for stabilizing mutations (Braisted & Wells, 1996; Ruan *et al.*, 1998). In the case of antibodies, the initial destabilization is easily achieved by removal of the conserved intradomain disulfide bonds (Figure 8, pathway A). This has been done in the case of the scFv fragment A48 (Proba *et al.*, 1998), where some globally stabilizing mutations could be selected after partial randomization of the cysteine-free sequence and subsequent phage panning. The selected mutations also stabilized the same scFv fragment after the disulfide bonds had been reintroduced (Wörn & Plückthun, 1998a). The energetic consequences have been summarized in Figure 9.

Jung *et al.* (1999) tested different stress incubation conditions during phage panning in order to stabilize the already very stable 4D5Flu scFv fragment even further. The phage particles displaying randomized 4D5Flu protein were incubated either at high temperature (up to 60 °C) or increasing GdnHCl concentration (up to 3.5 M) before the actual panning steps, and the stress was increased stepwise in successive panning rounds. Temperature-stress guided selection turned out to yield the most stable scFv mutant, which was about 4 kcal/mol more stable than the original 4D5Flu, besides having an approximately 20-fold better binding constant. Temperature-stress worked better than the chemical denaturation, presumably because it results in irreversible denaturation of scFv protein on the phage particle. GdnHCl incubation, on the other hand, denatures the protein in an, at least partially, reversible manner. The GdnHCl-incubated phage particles displaying scFv protein had to be diluted 1:10 into native buffer during the binding steps, as high GdnHCl concentrations interfered with antigen-binding. Thus, binding may have occurred with molecules which had refolded on the phage, rather than stable molecules which were never unfolded in the first place.

The temperature-stress guided selection should be a general way of selecting for high stability by phage display, although affinity and stability selection could not be completely separated, even under these conditions. Moreover, an upper stability limit is set by the thermal stability of the filamentous M13 phage itself, which lies at about 60 °C (Jung *et al.*, 1999). After incubation at even higher temperature the reinfection rate dropped dramatically, probably because of irreversible heat denaturation of phage coat proteins, which are essential for bacterial infection. Thus, the stability of scFv fragments that are still completely native at 60 °C cannot be improved by the described procedure, unless thermostable phages are devel-

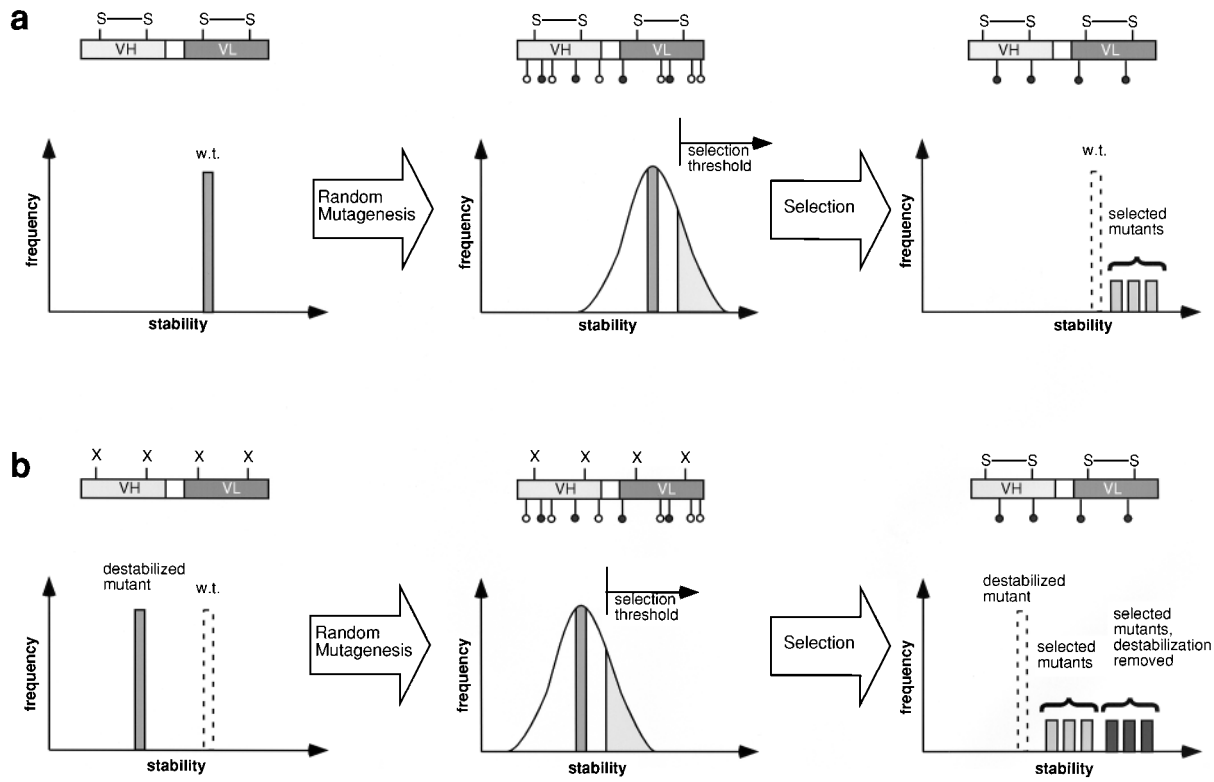


**Figure 9.** Energetic consequences of mutant selection. (a) Direct selection of mutants of an already stable w.t. scFv. After random mutagenesis, many mutations are obtained, favorable ones (black dots) and unfavorable ones (white dots). A distribution of energies is obtained, and the selection threshold must be chosen such that only molecules more stable than w.t. are retained. The selected mutants will thus be of higher stability than the w.t. (b) The w.t. is deliberately destabilized at first, indicated by the mutations ''X'', which might e.g. be mutations of the cysteine residues forming the disulfide bonds. The energy of the w.t. (broken lines) is thus reduced (gray bar). After random mutagenesis, again a series of mutants is obtained with a distribution of stabilities. The selection threshold can be very low this time, it only has to be high enough to impede the selection of the unstable mutant used as the starting material. The selected mutants (white bars) can then be stabilized further by removing the destabilizing mutations deliberately introduced in the beginning (e.g. by reintroducing the disulfide bonds). Thereby, the initially lost energy is regained, converting the light gray bars to the dark gray ones.

oped for this purpose, as proposed by Jung *et al.* (1999).

Yet another approach of selecting for protein stability by phage display is the selection for resistance against proteases as a property of native, correctly folded proteins (Kristensen & Winter, 1998; Sieber *et al.*, 1998; Finucane *et al.*, 1999). However, these strategies may not be ideal for scFv fragments, due to proteolytic susceptibility of the scFv linker and the long, unstructured CDR regions at least in some antibodies. Moreover, selection for protease resistance would somehow have to be alternated with selection for binding, in order to keep selection pressure on scFv functionality.

### *Selection for scFv stability by ribosome display*

Ribosome display (Hanes & Plückthun, 1997) is an *in vitro* method for the selection of scFv fragments (Figure 7). *In vitro* translated protein is bound to immobilized antigen under conditions that leave the scFv and its corresponding mRNA attached to the ribosome. After washing away unbound mRNA-ribosome-scFv complexes, complexes remaining bound to immobilized antigen are dissociated, and the eluted mRNA is amplified by RT-PCR. By using *Taq* polymerase during the amplification steps, random mutations are introduced into the pool, making this method ideal for increasing the variability of the pool in parallel to the selection for binding. As this procedure is carried out entirely *in vitro*, it is very conveniently combined with PCR-based mutagenesis methods, such as error-prone PCR (Fromant *et al.*, 1995) or DNA shuffling (Stemmer, 1994). Ribosome display has been successfully used to select for novel high-affinity binders against a given antigen from a synthetic library (Hanes *et al.*, 2000), as well as from an immune library (Hanes *et al.*, 1998).

In an attempt to select for increasing stability of a hag-peptide-binding scFv by ribosome display, the *in vitro* translation steps between successive binding and amplification rounds were performed at increasing DTT concentration, going from 0.5 to a maximum of 10 mM (Jermutus *et al.*, 2001). Normally, transcription and translation are carried out

separately during ribosome display, and translation is performed under oxidizing conditions. Reducing conditions prevent disulfide bond formation, such that the *in vitro* translated proteins are kept in a reduced state. As discussed above (section on disulfide bonds), the disulfide bond is very critical for scFv stability, and only intrinsically very stable fragments should be able to fold into the correct native structure in the presence of high concentrations of DTT. Indeed, some of the mutants selected by this procedure, but not the original starting scFv, were shown to be able to refold completely under reducing conditions *in vitro*. In addition, GdnHCl-denaturation curves indicate the selection of molecules which have increased stability with and without disulfide bonds (Jermutus *et al*., 20001).

These results show that selection for stability is possible with ribosome display by simply using the destabilizing strategy of reducing the disulfide bonds during *in vitro* protein folding and antigen-binding.

### *Selection for scFv stability by functional cytoplasmic expression*

Selection for functional expression of scFv fragments directed to the reducing cytoplasm should, on average, yield more stable fragments than selection under oxidizing conditions, for the reasons described above.

Selection for high functional expression in the bacterial cytoplasm has been performed for a randomized pool derived from scFv13, which binds and activates an inactive mutant of β-galactosidase (Martineau *et al*., 1998). By coexpressing the randomized library of scFv13 with the mutant β-galactosidase in *lac*$^-$ bacteria and plating on limiting lactose, scFv mutants with significantly improved soluble cytoplasmic expression could be selected after four successive rounds of mutation and selection. The best mutant, scFv13R4, contained seven mutations and was purified in soluble form from the cytoplasm, and thus in the absence of disulfide bonds which would confer the stability necessary for medical applications, but with a reported yield of >170 mg from 1 l of culture in an optimized shake flask culture. ScFv13R4 could also be renatured efficiently under reducing conditions *in vitro*. Although further *in vitro* analysis showed that scFv13R4 is clearly more stable than scFv13 under reducing conditions (Martineau & Betton, 1999), conflicting results between thermal stabilities (Martineau *et al*., 1998) and stabilities in urea unfolding (Martineau & Betton, 1999) were found for their relative stability under oxidizing conditions.

The limitation of the approach by Martineau *et al*. (1998) is that the selection procedure is scFv-specific and not transferable to fragments binding different antigens. To overcome this limitation a yeast two-hybrid *in vivo* assay has been used for the cytoplasmic selection of scFv fragments, which allows, in principle, selection of scFvs against any cytoplasmically expressed antigen (Visintin *et al*., 1999). The *in vitro* stability of the selected scFv fragments has, however, not been investigated in this study. Nevertheless, stable fragments are likely to be selected in this setup, especially at higher temperature, since some fragments were shown to be active at 20 °C, but not at 30 °C (Visintin *et al*., 1999) and since performance and *in vitro* stability of cytoplasmically expressed intrabodies seem to correlate (Wörn *et al*., 2000).

Another alternative is to apply some of the established cytoplasmic selection methods (Pelletier *et al*., 1999), which reconstitute the activity of a split protein upon cognate recognition between the binding partners (reviewed by Mendelsohn & Brent, 1999), to the selection of scFv fragments (Koch, Mössner & A.P., unpublished results). This should provide a general method to obtain a subset of stable molecules from a library, and a general selection method, if the library consists mostly of stable members.

## Stability and bacterial expression behavior

The stability of scFv fragments is often believed to be directly correlated with expression yield. This is, however, only partially true.

In cytoplasmic bacterial expression, where the protein is made in reduced form, indeed a good correlation between stability and functional yield of soluble scFv protein or isolated immunoglobulin domains was observed in a number of cases (Frisch *et al*., 1994; Martineau *et al*., 1998; Proba *et al*., 1998; Ohage & Steipe, 1999). Most likely, stability is usually the limiting factor for folding of an scFv or domain in the cytoplasm, since the intradomain disulfide bonds cannot form and only stable, but not unstable fragments, can fold efficiently.

The situation appears to be more complex in the bacterial periplasm. Although many stable scFv fragments were also reported to express well (e.g. by Wörn & Plückthun, 1998b, 1999; Jung & Plückthun, 1997; Jung *et al*., 1999), some other very stable scFvs show only poor periplasmic expression yield, such as e.g. the A48$^{++}$(H2L1) scFv fragment described by Wörn & Plückthun (1999). Periplasmic expression of scFv proteins is limited by the overall *in vivo* folding efficiency. As periplasmically expressed proteins do form the intradomain disulfides, they are more stable than when expressed in the reducing cytoplasm. In many cases stability may thus no longer be limiting for the *in vivo* folding efficiency. Knappik & Plückthun (1995) identified mutations in loops of scFvs that affect aggregation and *in vivo* folding yield, but not the overall protein stability. The absence of a linkage between thermodynamic stability and expression behavior has also been observed in P22 tailspike protein (Schuler & Seckler, 1998). Moreover, mutations disrupting hydrophobic patches at the antibody variable/con-

stant domain interface of the 4-4-20 scFv were shown to increase soluble periplasmic expression without influencing thermodynamic stability (Nieba *et al.*, 1997). For some mutations in this region of scFv fragments, high stability and high solubility may even mutually exclude each other. It follows that compromises have to be made, since hydrophobic residues in the V-C interface region can be important for stability, but, at the same time, deleterious for soluble expression yield (Nieba *et al.*, unpublished results; Jung & Plückthun, 1999).

Overall, the multiple factors influencing functional periplasmic expression of scFv fragments are only partially understood and clearly require more careful investigations.

### Conclusions and future directions

Essentially all applications of scFv fragments may benefit from an increase in the stability of the fragment, be it the cytoplasmic expression as intrabody, the numerous extracellular applications in human patients or in preclinical experiments in mice, or the biotechnological applications of scFvs in adverse environments.

The increasing number of reports describing rational and evolutionary methods for stability engineering of scFv fragments offer a wide variety of possibilities. It cannot be easily judged whether rational or evolutionary methods will dominate in the future. Rational approaches will become more sound with increasing biochemical understanding of protein stability and an increasing statistical basis of scFv structures, properties and functions. On the other hand, evolutionary methods could still offer completely new and possibly better solutions to the stability problem in individual cases and may, once established, be easier and faster to perform. The best results will most likely be achievable by combining both rational and evolutionary strategies.

Despite the great powers of evolutionary approaches to solve quickly a given problem, the repeated discovery of the same mutations would obviously not be very economic. Therefore, it will be vital to pool this knowledge and combine it with an analysis of existing frameworks. Such analysis of single domains and framework types, particularly the characterization of the natural frameworks, exemplified by the different subgroups, can uncover clusters of residues which can only be exchanged as groups, and will be important in understanding the molecular logic of why different antibody subgroups have evolved to particular sequences (Ewert, Honegger & A.P., unpublished results). Moreover, the identification of the guiding structural principles will allow a design of future libraries which capture as much structural diversity as possible, yet without compromising on stability. Making large libraries is necessary but not sufficient: the fraction of sequence space that can be searched with even the most advanced methods is tiny compared to the possibilities, and engineering stability into them is thus a vital component for securing functionality of the library.

It thus appears that we will need to combine advances from three fronts: (i) an increased biophysical understanding of stability, folding and its determining factors; (ii) more efficient and especially far more accurate prediction methods of the relevant properties; and (iii) further improved methods of evolutionary fine tuning. Antibodies are reagents of great general utility and the enormous unexplored biosphere within us and around us renders this effort worthwhile.

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