Mutual Stabilization of V_L and V_H in Single-Chain Antibody Fragments, Investigated with Mutants Engineered for Stability†

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Received March 27, 1998; Revised Manuscript Received June 16, 1998

ABSTRACT: A set of six mutants of the levan binding single-chain Fv (scFv) fragment A48 (ABPC48), which have the identical light chain but differ gradually in the stability of the heavy chain, was generated. This was achieved by introducing one or both of the stabilizing mutations H−K66R and H−N52S into the V_H domain of the A48 wild-type protein, which is naturally missing the conserved disulfide bridge in V_H, and into the cysteine-restored variant A48cys scFv. The stabilizing effects of these two mutations in V_H, which had been selected in the context of a disulfide-free derivative of this scFv fragment [Proba, K., et al. (1998) *J. Mol. Biol. 275*, 245−253], were found to be additive and transferable to the cysteine-restored variant of the A48 scFv, thereby generating extremely stable V_H domains. The equilibrium denaturation of these scFv fragments was compared with the corresponding isolated V_L domain and two of the different isolated V_H domains. In the scFv fragment, the V_L domain was found to be stabilized by a more stable V_H domain, and, conversely, the V_H domain was stabilized by a more stable V_L domain. A folding intermediate with nativelike V_H and denatured V_L was found at equilibrium, if V_H was significantly more stable than V_L. In all other cases, a cooperative unfolding of the scFv was observed. We explain this observation with different contributions of intrinsic domain stability and extrinsic stabilization provided by the partner domain in the single-chain antibodies.

The folding and unfolding pathway of multidomain proteins has been extensively studied both in terms of their folding thermodynamics and kinetics (reviewed in *1*−*3*). However, there are still many unresolved problems. One particularly interesting question is to what extent the folding and/or stability of a domain or subunit is influenced by an interacting domain. Generally, domains are believed to be autonomous folding units which are mainly stabilized by interactions within themselves, yet it is clear that they are frequently involved in interactions with other domains.

Many multidomain proteins show complex folding behavior which cannot be described by two-state models. Intermediate states may either arise from the individual domains unfolding separately or from partially unfolded states, involving several domains. An experimental difficulty is that many multidomain proteins cannot be split into stable folded fragments or even subunits, because they critically depend on the presence of interdomain contacts to prevent aggregation. In the case of immunoglobulins, however, expression of single antibody domains has been possible and therefore allows an investigation of these questions. V_L[1] domains have been functionally expressed by themselves in several cases (*4*−*9*, among others). This might, however, be at least partially due to their potential to form homodimers (*10*). Isolated V_H domains often tend to aggregate (*11*). The solubility and stability of human V_H domains could in some cases be increased by "camelization" (*12*), i.e., introducing solubilizing residues found in camel antibodies, some of which do not have a light chain (*13*−*16*). However, at low temperature and concentration, some murine and human V_H domains can be prepared in soluble and native form without "camelization".

V_H and V_L domains can be covalently linked by a flexible linker to give the so-called single-chain Fv (scFv) fragments (*17*, *18*). The stability of scFv fragments and the mutual stabilization of the single domains within the scFv are important for the design of scFv fragments which are sufficiently stable for medical or technical applications. The detailed analysis of the influence of mutations in one domain on the domain itself and its partner domain will allow a more rational approach to engineering stable proteins, by focusing on the relevant parts of the heterodimeric molecule.

In the present study we have created a set of six mutants of the levan (poly-β-2,6-D-fructose) binding antibody scFv fragment A48 (*19*−*21*) with gradually increasing stability of the V_H domain, all in the context of an unchanged V_L domain. Two of the V_H domains, the most stable one and one of the less stable ones, and the V_L domain were also expressed on their own. The stabilities of the different proteins were then measured by denaturant-induced equilibrium unfolding. With this series of proteins, we investigated the nature of the equilibrium transitions of the variable scFv

† This study was funded by the Schweizerische Nationalfonds Grant 31-47302.96 and by a predoctoral fellowship to A.W. from the Fonds der Deutschen Chemischen Industrie.

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[1] Abbreviations: BBS, borate-buffered saline; EM, emission maximum; Fv, antibody variable fragment which consists of V_H and V_L; GdnHCl, guanidinium hydrochloride; PVDF, polyvinylidene fluoride; scFv, antibody single-chain Fv fragment; V_H, variable domain of the heavy chain; V_L, variable domain of the light chain.

S0006-2960(98)00712-0 CCC: $15.00
Published on Web 08/29/1998

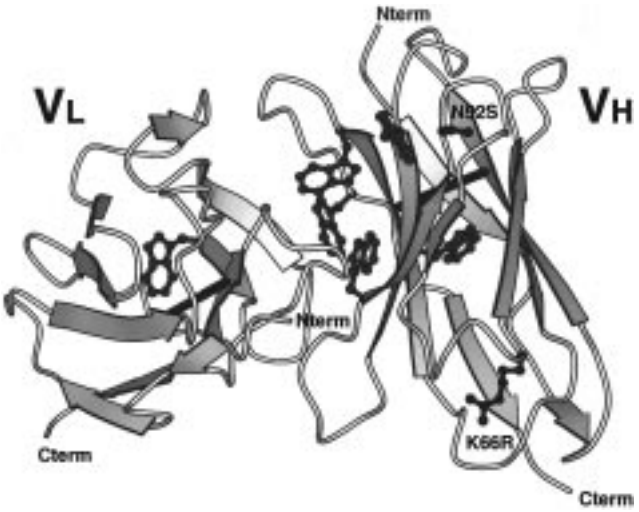

FIGURE 1: Homology model of the A48cys(H−K66R/N52S) Fv fragment. The two stabilizing mutations K66R and N52S in the heavy chain are labeled. The orientation of the model structure is chosen such that the five Trp residues in V_H and the single Trp residue in V_L, which dominate the fluorescence emission maximum in the denaturation transitions, are best visible. The conserved disulfide bridges in V_H and V_L are schematically represented as black bars.

fragments and showed under which conditions the two domains can stabilize each other. The nature of the equilibrium intermediates in two selected scFv fragments was probed by limited protease digestion experiments (*22*) in the presence of the denaturant concentration where equilibrium intermediate species were expected, based on the shape of the unfolding curves. The importance of mutual domain stabilization is shown to depend primarily on the relative intrinsic stability of the domains.

The antibody A48, which was used as a model system in the scFv format, is naturally missing the conserved cysteine H-92 in the heavy chain, which is replaced by a tyrosine (*20*). Because of its natural occurrence, we call this protein lacking the V_H disulfide "wild-type". It has been shown before that the stability of the wild-type A48 can be significantly increased by mutating this Tyr H92 back to Cys and thereby reintroducing the disulfide bridge into V_H (*19*). The A48wt scFv fragment and the cysteine-restored A48 scFv fragment (termed A48cys) served as recipients for introducing the stabilizing mutations H−K66R and H−N52S (numbering according to Kabat et al. (*23*), where the "H" denotes the heavy chain). These mutations had been created by DNA shuffling (*24*) and selected by phage panning in the context of a completely disulfide-free derivative of the A48 scFv fragment, which is also missing the conserved disulfide bridge in the light chain (*25*). Although up to six mutations had been selected during phage panning per disulfide-free A48 scFv fragment, a detailed analysis of the selected mutations by equilibrium denaturation showed that mainly two mutations (K66R and N52S in the heavy chain) had a significant effect on the stability of V_H.[2] The position of these two mutations in the homology model of the A48cys(H−K66R/N52S) Fv fragment can be seen in Figure 1. These mutations were introduced alone or combined into the two A48 scFv fragments, with and without the V_H disulfide bond, to obtain a set of six scFv fragments with large differences in relative stabilities of V_H, always in the presence of the same V_L domain. This allowed analysis of the stabilizing contributions of one domain on the other. Note that in all A48 scFv fragments investigated here, the A48wt V_L domain used carries the conserved disulfide bond.

[2] Wörn, A., unpublished results.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. The two scFv mutants derived from the A48wt scFv fragment, the wild-type protein itself and the corresponding V_H domain with the stabilizing H−K66R mutation, which all lack the disulfide in V_H, were cloned into the vector pTFT74 (*26*, *27*) and expressed as cytoplasmic inclusion bodies in *E. coli* BL21DE3 [F^-, *ompT*$^-$, $r_B^- m_B^-$ (λimm21, *lac*I, *lac*UV5, *T7 pol*, *int*)] (*28*). In all scFv fragments and the isolated V_H lacking the disulfide bond, the unpaired CysH22 was replaced by an alanine, to prevent the formation of covalent oxidation products during equilibrium unfolding experiments. Purification of inclusion body protein, refolding, and antigen affinity chromatography were carried out as described previously (*19*). Since antigen affinity chromatography with the isolated V_H domain was not possible, the refolding mixture of the A48wt(H−K66R) V_H domain was concentrated by a factor of 10 by using an Amicon model 8200 ultrafiltration cell, dialyzed against 40 mM Tris, pH 8.5, to remove the arginine present from the refolding mix (*19*) and then eluted from a Sepharose Fast-Q anion-exchange column with a 0−500 mM NaCl gradient in the same buffer at pH 8.5. Pooled fractions were dialyzed against BBS (50 mM borate, 150 mM NaCl, pH 8.0).

The disulfide-restored A48cys scFv fragment and mutants derived from this protein were cloned into the secretion vector pLiscSEF (*29*) and were expressed in functional form in the periplasm of *E. coli* JM83 [λ^-, *ara*, Δ(*lac*, *proAB*), *rpsL*, *thi*, Φ80, *dlacZ*Δ*M*15] (*29*). They were purified to homogeneity by immobilized metal ion affinity chromatography (IMAC), using the C-terminal His-tag, followed by antigen affinity chromatography as described previously (*19*).

All scFv fragments were in a V_H-linker-V_L orientation, carrying an N-terminal FLAG (*30*) and a C-terminal Myc- and His-tag (*19*). A 20-mer nonrepetitive peptide linker was used in all cases to connect V_H and V_L (*31*). The V_L domain of the A48 scFv was expressed with the secretion vector pIG1 (*27*), the V_H domain of A48cys(H−K66R/N52S) with the secretion vector pIG3 (*27*). Both single domains were expressed by secretion to the periplasm of *E. coli* JM83 (*29*) and purified by using two columns directly coupled in-line (*32*). In this strategy, the eluate of an IMAC column, which exploits the C-terminal His-tag, was directly pumped onto a HQ anion-exchange column with a buffer of pH 8 (for V_H) or pH 8.5 (for V_L), respectively. Elution from the anion-exchange column was achieved with a 0−500 mM NaCl gradient at the respective pH. Pooled fractions were dialyzed against BBS.

Urea- and GdnHCl-Induced Unfolding/Refolding Equilibria. Solvent-induced denaturation was followed by the intrinsic fluorescence emission spectra of the proteins. Excitation was at 280 nm. Measurements were performed and analyzed as described before (*25*). Protein concentrations of 5 μg/mL were used in all cases, with the ex-

ception of the isolated V_L domain, which was measured at 10 μg/mL. All measurements were carried out in BBS at 20 °C, using a Shimadzu RF-5000 spectrofluorimeter. Denaturation curves were normalized to fraction unfolded protein, whenever the extent of the pre- and post-transition region was sufficient to calculate the corresponding baseline, which is required for normalization (*33*). As some of the urea denaturation curves of the cysteine-restored A48 scFv fragments lack a post-transition phase because of their high thermodynamic stability, these curves were not normalized.

Limited Protease Digestion Studies. Limited protease digestion studies were performed with the A48cys(H−K66R/N52S) scFv fragment in the presence of 6 M urea, and with the A48wt scFv fragment in the presence of 4 M urea. Both scFv fragments were preincubated overnight at 10 °C and an additional 2 h at 20 °C prior to digestion in solutions containing the corresponding urea concentration in BBS, with the scFv fragment at 2 mg/mL. The thermolysin digestion was carried out at 20 °C in the presence of 5 mM $CaCl_2$ and 100 mM Tris-HCl pH 8.0 in a reaction volume of 50 μL. Both scFv fragments were digested at a final scFv concentration of 1 mg/mL, using a ratio of thermolysin to scFv of 1:200 (w/w) for the A48wt or 1:25 (w/w) for the A48cys(H−K66R/N52S). The reactions were stopped after defined time periods (10 s to 7 h) by adding EDTA to a final concentration of 50 mM, followed by addition of SDS−PAGE loading buffer and immediate heating of the mixture in a boiling waterbath for 5 min. Samples taken after different time points were analyzed by SDS−PAGE using an 18% polyacrylamide gel, which was stained by Coomassie Brilliant Blue. The limited digestion reaction of A48cys(H−K66R/N52S) in 6 M urea was also blotted onto a PVDF membrane by standard techniques for further analysis of the accumulating prominent digestion band by N-terminal sequencing and MALDI mass spectrometry.

RESULTS

Protein Expression and Purification. The scFv fragments derived from the A48wt scFv fragment, lacking the disulfide bond in V_H, yielded between 15 and 40 mg of scFv/L of *E. coli* BL21DE3 upon refolding and purification. Periplasmic expression of the disulfide-restored A48cys scFv fragment and its mutants allowed purification of about 1.2 mg of scFv/L of *E. coli* JM83. The isolated A48cys(H−K66R/N52S) V_H domain yielded 1.2−1.4 mg of purified protein/L upon secretion in *E. coli* JM83. The A48wt(H−K66R) V_H domain, which could not be expressed periplasmically, allowed purification of 1.4 mg of refolded protein/L of *E. coli* BL21DE3. The isolated V_H domains could not be concentrated above 30−40 μg/mL, because they were very prone to aggregation. The V_L domain of A48 eluted in the flow-through of the HQ column, with most contaminating proteins binding to the anion-exchange matrix at pH 8.5. The purification yield was 300−400 μg of V_L domain/L upon secretion in *E. coli* JM83.

Functionality of the isolated domains was demonstrated qualitatively by antigen binding ELISA. An equimolar mixture of either of the V_H domains with V_L, preincubated for 2 h on ice to allow F_V formation, gave a clear antigen binding ELISA signal, detected with an anti-His-tag antibody (*34*), which could be completely inhibited with soluble antigen. In contrast, the isolated domains employed at the same molar concentration did not produce any signal (data not shown).

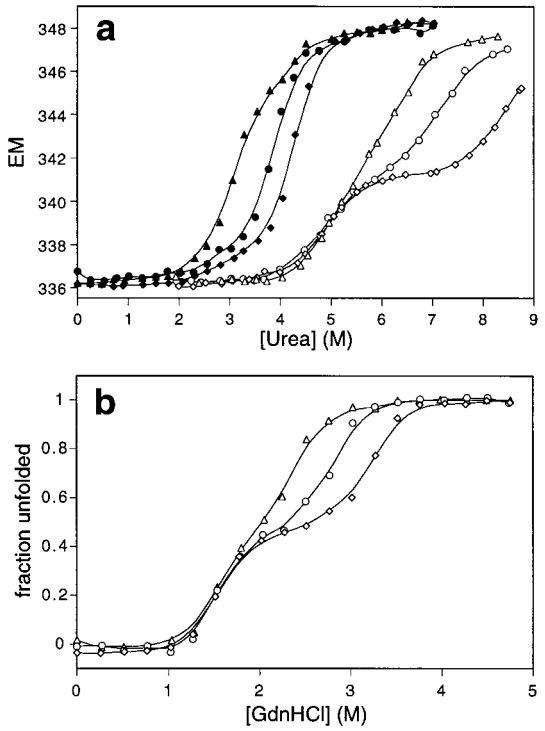

FIGURE 2: (a) Overlay of urea denaturation curves of scFv fragments A48wt (▲), A48(H−K66R) (●), A48(H−K66R/N52S) (♦), A48cys (△), A48cys(H−K66R) (○), and A48cys(H−K66R/N52S) (◊). Curves are not normalized to "fraction unfolded protein", due to the lack of a post-transition phase in some mutants in urea. (b) Overlay of normalized guanidinium hydrochloride denaturation curves of A48cys (△), A48cys(H−K66R) (○), and A48cys(H−K66R/N52S) (◊). All unfolding transitions (a and b) were measured by the change in emission maximum (EM) as a function of denaturant concentration at an excitation wavelength of 280 nm.

Unfolding Equilibria. Figure 2a shows two sets of urea-induced unfolding curves. The three curves on the left represent the wild-type A48 scFv fragment without, with one (H−K66R), or with two (H−K66R/N52S) stabilizing mutations in V_H. The remaining three curves on the right correspond to the same mutants in the context of the cysteine-restored A48cys scFv fragment. It can be seen that six scFv fragments with widely different urea denaturation behavior were obtained, which all contain the same disulfide-containing light chain. This shows that the stabilizing effect of the mutations H−K66R and H−N52S can be seen both in the wild-type fragment, which lacks the disulfide bond in V_H, as well as in the disulfide-restored form of the A48 scFv, which corresponds to a more "normal" antibody.

To analyze the effects of the mutations, it is customary to derive ΔG values from the shape and midpoints of the transition curve, using a six-parameter fit (*33*). Two reasons, however, make us present only a semiquantitative analysis at this point and report only the midpoints. First, the two-state nature of the curves is not clear for all experiments, and, second, some of the pre- and post-transition regions, notably the intermediate plateaus, are too short to reliably fit a straight line (Figures 2 and 3), which would result in a large uncertainty of the derived ΔG values. Of course, the

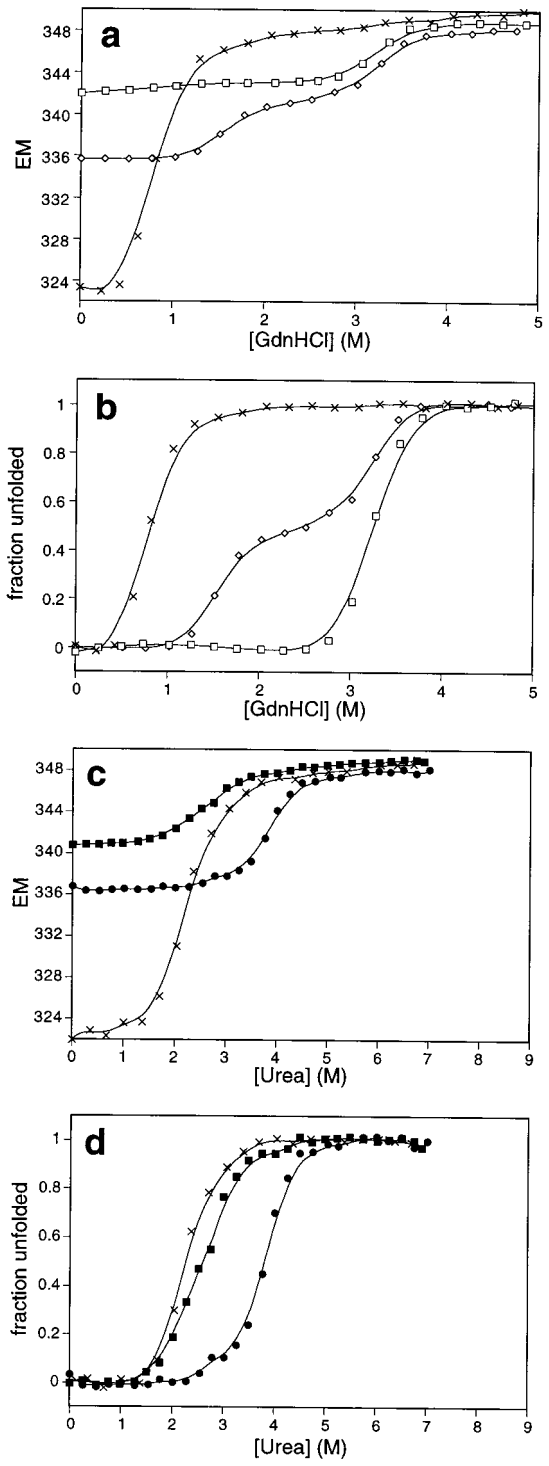

FIGURE 3: Overlay of denaturation curves of the scFv fragments A48cys(H−K66R/N52S) and A48wt(H−K66R) with the constituent individual domains. (a) Overlay of guanidinium hydrochloride denaturation curves of A48cys(H−K66R/N52S) (◊), and the constituent isolated V_H (□) and V_L (×) domains. The emission maximum is shown directly and the data are not normalized. (b) The same data as in a, but after normalization. (c) Overlay of urea denaturation curves of A48(H−K66R) scFv (●) and the constituent isolated V_H (■) and V_L (×) domains. The emission maximum is shown directly and the data are not normalized. (d) The same data as in c, but after normalization. All unfolding transitions (a−d) were measured by the change in emission maximum (EM) as a function of denaturant concentration at an excitation wavelength of 280 nm.

midpoint of a denaturation transition is not an exact representation of the stability of a protein, because the slope of

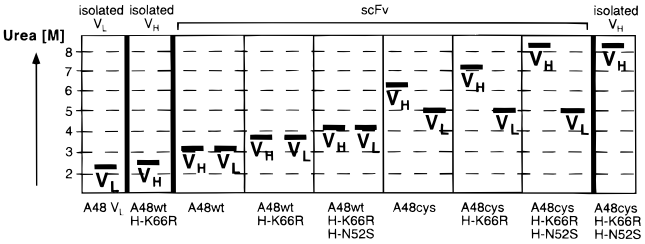

FIGURE 4: Schematic representation of denaturation midpoints of the light-chain variable domain (V_L) and the heavy-chain variable domain (V_H) within the scFv fragment A48 and its derived mutants. Note that V_L is chemically identical in all cases, whereas the nature of V_H is indicated. A48wt carries H22-Ala and H92-Tyr, and A48cys carries two cysteines at these positions and thus the restored disulfide bridge in V_H. H−K66R and H−N52S are stabilizing mutations of the heavy chain that were introduced alone and in combination into both the A48 wild-type and the A48cys scFv fragments. In addition the denaturation midpoints of the isolated V_L domain as well as of the isolated A48wt(H−K66R) and the A48cys(H−K66R/N52S) V_H domains are shown. Because of uncertainties in deriving ΔG values from the transition region, only denaturation midpoints (see text) are given and the comparison of the protein stabilities can only be semiquantitative. It can be seen that V_L is stabilized by the V_H-V_L interface until a limit is reached, where V_L cannot be further stabilized, and it denatures in a separate reaction with a midpoint at 5 M urea in all A48cys derived mutants.

the transition (*m* value) also affects the calculation of ΔG values and there might be compensating effects between midpoints and slopes. Yet, as there is no dramatic difference between the slopes of the different transitions (see Figure 3), the midpoints of the transitions clearly allow a semiquantitative estimate of the relative stabilities and the derived conclusions are independent of the exact numbers.

A schematic representation of the denaturation midpoints of the V_H and the V_L domain in these mutants, as well as of the isolated V_L and the investigated isolated V_H domains, is given in Figure 4. In the case of the three A48wt-derived proteins, lacking the disulfide bridge in V_H, the midpoint of the transition region was at 3.1 M urea for the A48wt, at 3.8 M urea for the A48wt(H−K66R), and at 4.2 M urea for the A48wt(H−K66R/N52S) scFv fragment, and only a single transition was apparent. In contrast to the wild-type derived scFv fragments, the cysteine-restored mutants showed a superimposable first part of the transition region, all starting unfolding at 4 M urea. Above 5.5 M urea, the urea denaturation curves of the three A48cys derived scFv fragments began to differ significantly (Figure 2). The second part of A48cys(H−K66R) was shifted by about 0.8 M, and the corresponding part of A48cys(H−K66R/N52S) by about 2 M to higher urea concentrations, compared with A48cys, resulting in a clear two-step transition for the most stable mutants. Even at 8.5 M urea concentration the post-transition plateau was not reached for the A48cys(H−K66R) and the A48cys(H−K66R/N52S), indicating very stable proteins indeed. Therefore, the denaturation experiments with the cysteine-restored A48 mutants were repeated with the stronger denaturant GdnHCl. Figure 2b shows that the same qualitative results were obtained. The transition region in GdnHCl-induced unfolding started at 1 M GdnHCl, and the step in the transition curve occurred at 2 M GdnHCl. The unfolding curves of all scFv fragments were found to be essentially reversible (data not shown), indicating that equilibrium properties are observed.

To compare these results with the corresponding isolated domains, GdnHCl curves were measured for the isolated A48cys(H−K66R/N52S) V_H domain and the isolated V_L domain. They are overlayed on the curve of the A48cys-(H−K66R/N52S) scFv fragment in Figure 3b. While this V_H domain had the same midpoint of denaturation as the second part of the denaturation curve in the corresponding scFv (3.3 M GdnHCl), the isolated V_L domain unfolded significantly earlier than the first transition in the scFv fragment, with a midpoint of denaturation below 1 M GdnHCl. In the V_L denaturation experiments, different protein concentrations did not affect the slope of the denaturation curve, suggesting that dimerization did not play a significant role in this V_L domain at the concentrations under investigation (data not shown). The GdnHCl transitions of the isolated domains and the A48cys(H−K66R/N52S) scFv fragment before normalization are shown in Figure 3a for better demonstration of the unfolding intermediate formed in this scFv fragment.

An overlay of the urea denaturation curves, normalized to fraction unfolded protein, of the A48wt(H−K66R) scFv fragment with the corresponding V_H and V_L domains is shown in Figure 3d. The isolated V_L domain and the isolated A48wt(H−K66R) V_H domain showed similar transitions in urea denaturation, with a midpoint of denaturation at 2.6 M urea for V_H and 2.3 M urea for the V_L. The A48wt(H−K66R) scFv fragment had a midpoint of denaturation at 3.8 M urea, which is higher than either domain alone. The corresponding data before normalization are shown for comparison in Figure 3c.

Since the position of the fluorescence emission maxima (EM) in the native and denatured proteins are informative about the nature of these states, we want to report these values. The EM of all completely unfolded proteins was between 348 and 350 nm (consistent with the EM at 350 nm of pure Trp in aqueous solution (*35*)). The native scFv fragments had their EM at 336 nm, consistent with the more or less buried nature of their five V_H Trp residues and the one V_L Trp residue. The EM of the native V_H domain carrying the disulfide bridge was at 342 nm, and the disulfide-free V_H domain derived from the wild-type A48 scFv fragment had a maximum at 341 nm. The higher EM of the isolated V_H domains compared with the corresponding scFv fragments is probably due to the three Trp residues, which are exposed in the isolated V_H domains, but which are part of the interface in the native scFv. The slightly higher EM in the native V_H domain carrying the disulfide bridge compared with the V_H domain lacking the disulfide can be explained by quenching of the fluorescence emission of the conserved Trp H36 by the disulfide bond. The V_L domain in its native conformation showed an EM of 323 nm. The signal is mainly caused by the single Trp L35 residue, which is strongly quenched in the native state by the neighboring disulfide bond (*36*). Figure 1 indicates the position of the Trp residues in V_H and V_L in the homology model of the A48cys(H−K66R/N52S) Fv fragment.

The fluorescence transitions in the scFv fragments and in the isolated domains were also followed by measuring fluorescence intensity at 350 nm (data not shown). The fluorescence intensity of the isolated V_L domain increased, that of both isolated V_H domains under investigation decreased upon unfolding, and transitions were found at essentially the same

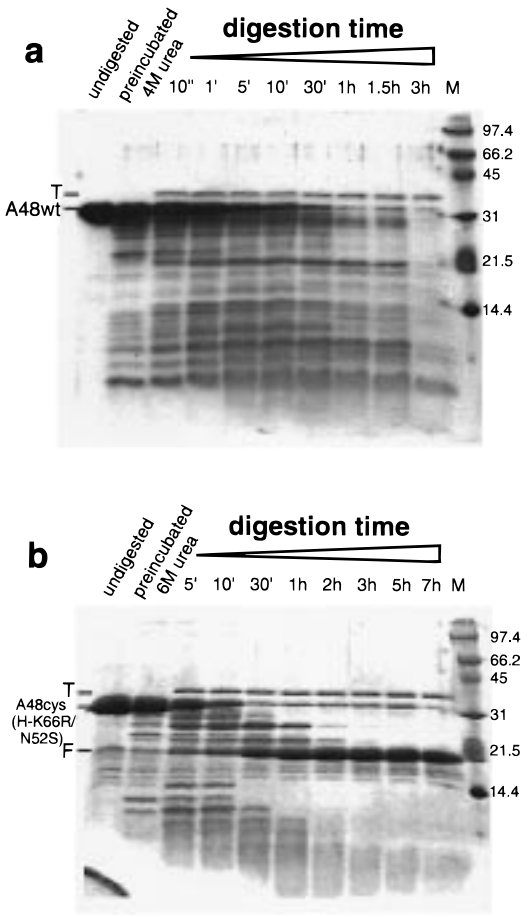

FIGURE 5: (a) Limited thermolysin digestion of the A48wt scFv fragment in the presence of 4 M urea with a 1:200 (w/w) ratio of thermolysin (**T**) to scFv. No digestion band accumulates, suggesting that a structured core is not present in this scFv fragment under these conditions. (b) Limited thermolysin digestion of A48cys(H−K66R/N52S) scFv fragment in the presence of 6 M urea with a 1:25 (w/w) ratio of thermolysin (**T**) to scFv. Even after extensive digestion a core fragment (**F**) accumulates, comprising the entire V_H domain and some additional amino acids (see text).

denaturant concentration as in the emission maximum data. However, the intensity data scattered much more than the fluorescence maxima data. We believe that this effect is partially caused by the fact that small errors in protein concentration, e.g., caused by protein binding to the wall of the cuvette at intermediate denaturant concentrations, influence fluorescence intensity, while not affecting the emission maximum. As small differences in the midpoint of transitions are being discussed, we thus decided to use emission maximum data only, which were found to be very reproducible.

Limited Protease Digestion Studies. The digestion of A48wt scFv with thermolysin in the presence of 4 M urea resulted in a digestion pattern without accumulation of a prominent digestion band (Figure 5a). Although some digestion bands appeared stronger than others, no band accumulated proportional to the disappearance of undigested scFv protein. During digestion of A48cys(H−K66R/N52S) in 6 M urea, on the other hand, even after extensive digestion one band clearly accumulated (Figure 5b), whereas all other digestion bands became very weak. N-terminal sequencing and MALDI-MS analysis characterized that band as a fragment comprising the entire V_H domain, plus the linker and the first 10 amino acids of V_L. This indicates that at

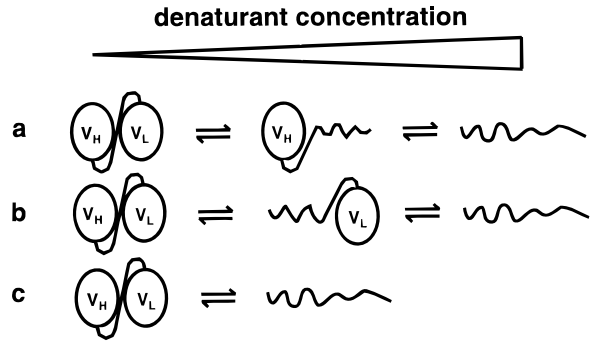

FIGURE 6: Schematic representation of denaturant-induced unfolding equilibria of scFv fragments. (a) If V_H is more stable than V_L, an intermediate equilibrium state is reached, where V_L is denatured and V_H is still native. At higher denaturant concentration V_H also denatures. This situation is observed in the case of the A48cys(H−K66R/N52S) scFv fragment. (b) Hypothetical scheme, not observed for any A48 scFv fragment in this study. If V_L is much more stable than V_H, an intermediate equilibrium state with a denatured V_H and a structured V_L could theoretically be expected, before V_L denatures as well. However, this intermediate state does not seem to form at equilibrium in the case of A48 scFv fragments with a V_H domain of low stability, and model c is found instead. (c) V_L denatures together with V_H in a cooperative reaction. This situation is found if V_L is more stable than V_H or of similar stability, because V_L critically depends on the V_H−V_L interface for stability. This coupled equilibrium is observed in the A48wt derived mutants.

the plateau region in Figure 2a the V_H domain is resistant against proteolysis, whereas the V_L domain is not. This is not an intrinsic property of these domains, since the wt scFv fragment does not give such an accumulation of a band, even at lower urea concentration. Instead, the accumulation of a band seems to correlate with the presence of a plateau in the equilibrium denaturation experiments, and we suggest that it identifies which domain is still stable in the plateau region. Some degradation had taken place in both scFv fragments during the overnight incubation in urea, prior to the addition of thermolysin. This might be due to a minor contamination with *E. coli* proteases which can act on the partially unfolded scFv fragments (Figure 5a,b, second lane).

DISCUSSION

Two types of denaturation curves of the six scFv fragments, which differ in the heavy chain, but not in the light chain, were found. Either one single transition was observed for the less stable fragments or a more or less pronounced intermediate plateau was seen, for the most stable fragments. In the latter group (Figure 2, open symbols), the first transition is identical for the three mutants and we interpret this as unfolding of the light chain. At the plateaus in the denaturation curves (Figure 2), the heavy chain still appears to be folded (schematically represented in Figure 6a). The shift in EM upon unfolding of V_L is proposed to be mainly caused by the three interface Trp residues of V_H becoming solvent exposed. Whether, in the process of unfolding, it is the interface, which first disrupts at 4 M urea, followed by the immediate denaturation of V_L or whether V_L denatures "on" the interface cannot be distinguished from our equilibrium experiments and must be the subject of further kinetic investigations.

Additional evidence for the presence of a defined equilibrium folding intermediate during unfolding of A48cys(H−K66R/N52S) comprising native V_H and denatured V_L comes from the fact that the unfolding curve of the isolated A48cys(H−K66R/N52S) V_H domain is superimposable with the second part of the corresponding scFv denaturation curve (Figure 3a). Moreover, limited thermolysin digestion of A48cys(H−K66R/N52S) in the presence of 6 M urea (the beginning of the plateau region) results in the accumulation of a core fragment comprising the entire V_H domain and some additional amino acids from the linker and the light chain (Figure 5b). This clearly suggests that the V_H domain is structured under these conditions. The fact that the accumulating core fragment also includes the nonrepetitive peptide linker (used to connect V_H and V_L (*31*)) and the 10 N-terminal V_L amino acids is probably due to the preferred cleavage sites of thermolysin. Despite its generally broad sequence specificity, thermolysin is known to have a tendency to cleave preferentially before Leu residues (*22*). Indeed, the cleavage site, identified by MALDI-MS in the A48cys(H−K66R/N52S), is at the first Leu residue after the C-terminus of V_H.

Because of the presence of this intermediate and the fact that the A48cys(H−K66R/N52S) V_H domain can be expressed by itself and is very stable, we conclude that this stable V_H does not depend on the V_H−V_L interface for stability. However, the reverse is not true. The isolated V_L domain starts unfolding at very low denaturant concentrations (Figure 3) with denaturation midpoints at 0.8 M GdnHCl and 2.2 M urea. Its stability in isolation is therefore significantly lower compared to its stability in the context of this scFv fragment, i.e., in the presence of V_H. When it is part of a stable V_H−V_L interface, the same V_L domain starts unfolding only at 4 M urea (Figure 2a).

In the A48 wild-type scFv and mutants derived from this protein, which all lack the V_H disulfide, V_H is limiting for the stability of the scFv fragments, and the transition region of the unfolding curves of the various scFv fragments starts around 2−3 M urea, depending on which stabilizing mutations have been introduced into V_H. One might argue that there is a stable V_L in these scFv fragments, which starts unfolding only at 4 M urea, just as in the cysteine-restored scFv fragments. Such a stable V_L would be hardly detectable in the spectrum, since it has only one Trp residue, while V_H has five. Therefore, the further shift in emission maximum caused by a putative late denaturing V_L domain would be very small, compared with the shift in emission maximum caused by the denaturation of V_H and concomitant interface destruction, and one would not expect an obvious plateau to be visible in the transition region of the denaturation curve or an obvious transition at 4 M urea. However, since V_L expressed on its own is very unstable, it is very likely that V_L—lacking its stabilizing interface because of early V_H denaturation—starts unfolding as soon as the unstable V_H is denatured. In addition, a "stable core", consisting of a structured V_L domain and a denatured V_H domain, cannot be enriched in limited digestion experiments of A48 wt with thermolysin in the presence of 4 M urea (Figure 5a).

We therefore suggest a cooperative unfolding of both domains, V_H and V_L, whenever V_H is less stable than V_L or about equal in stability (Figures 6c and 3c,d). An equilibrium intermediate state with a denatured V_H domain and a native V_L domain, as depicted in Figure 6b, does not seem to be formed in the case of any of the A48 scFv fragments investigated. This does not rule out, of course, that more stable

V_L domains in conjunction with poorly stable V_H domains may give rise to such behavior. The V_L domain of A48 does, however, stabilize a V_H domain of lower or equal stability, as shown for the A48wt(H−K66R) V_H domain, which denatures at lower urea concentrations by itself than in the corresponding scFv fragment (Figure 3d). However, this stabilizing effect is smaller than the stabilizing effect a very stable V_H has on V_L (Figure 4). It will be interesting to see whether the asymmetry of the two antibody domains in their dependence on the V_H-V_L interface for stability within the scFv fragment is a general phenomenon which is also valid for other scFv fragments.

The stabilization of one domain by another, observed in the mutants of the A48 scFv fragment, has so far only been conclusively demonstrated in the case of the wild-O Fv fragment, derived from the hen egg-white lysozyme binding monoclonal antibody D1.3, and its separated V_H and V_L domains (*37*). In this case, the isolated domains denatured at a temperature about 20 °C lower than when they were associated with each other in the Fv fragment. However, an obvious plateau in the denaturation transition of scFv fragments, as present in the A48cys(H−K66R/N52S) scFv fragment, has not been reported until now, to our knowledge. This suggests that in previous studies unfolding schemes as depicted in Figure 6b or 6c (however, not as in Figure 6a) were followed (for example, *29*, *38*, and *39*).

Additional evidence for the mutual stabilization of the domains within a scFv fragment comes from deuterium exchange experiments[3] with the scFv McPC603. Protons in the isolated V_L domain of the McPC603 exchange rapidly (indicating poor stability), but these proton exchange rates in the very same V_L are significantly slowed solely by the presence of the V_H domain, for which stable NH protons with protection factors around 10^5 can be measured (*40*, *41*). The rapid exchange of the V_L protons, indicating a low protection factor, is consistent with the low ΔG value of unfolding, observed by fluorescence for that V_L domain (*9*).

The idea of the interdomain stabilization in multidomain proteins has been explored in several other model proteins. Stabilizing effects of the domain−domain interface region on one of the domains have been demonstrated in many cases. In γB-Crystallin, for example, the stability of the C-terminal domain at pH 2 is significantly increased compared with the isolated domain by the presence of the N-terminal domain (*42*). The A domain of diphtheria toxin was shown to be stabilized by interaction with the B domain (*43*). In the case of aspartate transcarbamoylase, the midpoint of thermal denaturation of the regulatory subunit is increased by 16 °C, from 51 to 67 °C, solely by the presence of the catalytic domain (*44*).

A thermodynamic model developed by Brandts et al. (*45*) explains the total stability of multidomain proteins as the sum of increments of the separate domains and the contributions of an interface free-energy term ΔG_{AB}. This term ΔG_{AB} is assumed to go to zero as soon as one of the domains involved in pairwise interaction unfolds. The interface term ΔG_{AB} is responsible for the observation that, regarding denaturation within the scFv fragment, a reasonably stable V_L domain stabilizes a V_H domain of lower or equal stability [e.g., in A48wt(H−K66R)] and a stable V_H stabilizes a less stable V_L [e.g., in A48cys(H−K66R/N52S)]. One particularly interesting aspect in our system is that the same A48 V_L domain acts either as the more stable, *stabilizing* partner, or as the less stable, *stabilized* partner within the scFv fragment, depending on the stability of the pairing V_H domain. Moreover, this analysis suggests that the most stable A48cys(H−K66R/N52S) scFv fragment can only profit from stabilizing V_L, whereas the less stable wt scFv fragments, lacking the V_H disulfide, will mostly profit from engineering of V_H, since the contribution of a stable V_H to the interface energy ΔG_{AB} is more pronounced.

A further aspect of our study is that the strategy we have followed to find the V_H-stabilizing mutations K66R and N52S (*25*) might point out a general way of obtaining globally stabilizing mutations in scFv fragments. Random mutations had been introduced into the disulfide-free variant of a scFv fragment, followed by a selection method (e.g., phage panning) to regain functionality. The disulfide bridges have then been reintroduced into the selected scFv fragments and very stable fragments were obtained, with an additive stabilizing effect of the disulfide bond and the selected mutations. By removing both disulfide bonds, the scFv fragment is severely destabilized and, in the starting antibody, essentially nonfunctional. Selection in the absence of the conserved disulfide bridge will therefore put a high evolutive pressure on the positive selection of stabilizing mutations. These can then be transplanted to the disulfide-containing framework. This detour provides a more powerful selection of stabilizing mutations than if it was carried out in the disulfide-containing molecule directly, since the function is strictly dependent on these mutations in the disulfide-free background, while it is only somewhat improved in the disulfide-containing background. However, the final selection of mutations is based on a sum of parameters, depending on the selection method employed, including binding constant, stability, toxicity, or expression rate. Therefore, only a fraction of the selected mutations will indeed increase the stability of the scFv fragments, making a detailed analysis of the contribution of single mutations necessary.

In summary, the analysis of the stability-limiting domains, the mutual stabilization of domains in a scFv fragment, and the selection strategy for stabilizing mutations are all important building blocks in engineering scFv fragments for stability. These concepts will be particularly important in the design of optimized scFv libraries.

[3] Freund, C., et al., unpublished.

ACKNOWLEDGMENT

MALDI-MS spectra were kindly recorded by Martin Münchbach, ETH Zürich. We thank Dr. Annemarie Honegger for preparing the homology model of the A48cys(H-K66R/N52S) Fv fragment presented in Figure 1.

REFERENCES

1. Wetlaufer, D. B. (1981) *Adv. Protein Chem. 34*, 61−92.
2. Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231−297.
3. Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117−237.
4. Anthony, J., Near, R., Wong, S. L., Iida, E., Ernst, E., Wettekind, M., Haber, E., and Ng, S. C. (1992) *Mol. Immunol. 29*, 1237−1247.

5. Stevens, P. W., Raffen, R., Hanson, D. K., Deng, Y. L., Berrios-Hammond, M., Westholm, F. A., Murphy, C., Eulitz, M., Wetzel, R., Solomon, A., Schiffer, M., and Stevens, F. J. (1995) *Protein Sci. 4*, 421−432.
6. Schormann, N., Murrell, J. R., Liepnieks, J. J., and Benson, M. D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9490−9494.
7. Frisch, C., Kolmar, H., and Fritz, H. J. (1994) *Biol. Chem. Hoppe-Seyler 375*, 353−356.
8. Hurle, M. R., Helms, L. R., Li, L., Chan, W., and Wetzel, R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5446−5450.
9. Steipe, B., Schiller, B., Plückthun, A., and Steinbacher, S. (1994) *J. Mol. Biol. 240*, 188−192.
10. Stevens, F. J., Solomon, A., and Schiffer, M. (1991) *Biochemistry 30*, 6803−6805.
11. Ward, E. S., Güssow, D., Griffiths, A. D., Jones, P. T., and Winter, G. (1989) *Nature 341*, 544−546.
12. Davies, J., and Riechmann, L. (1994) *FEBS Lett. 339*, 285−290.
13. Hamers-Castermann, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E. B., Bendahman, N., and Hamers, R. (1993) *Nature 363*, 446−448.
14. Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J. A. R. G., and Hamers, R. (1994) *Protein Eng. 7*, 1129−1135.
15. Desmyter, A., Transue, T. R., Ghahroudi, M. A., Thi, M. H., Poortmans, F., Hamers, R., Muyldermans, S., and Wyns, L. (1996) *Nature Struct. Biol. 3*, 803−811.
16. Spinelli, S., Frenken, L., Bourgeois, D., de Ron, L., Bos, W., Verrips, T., Anguille, C., Cambillau, C., and Tegoni, M. (1996) *Nature Struct. Biol. 3*, 752−757.
17. Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S. M., Lee, T., Pope, S. H., Riordan, G. S., and Whitlow, M. (1988) *Science 242*, 423−426.
18. Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., and Oppermann, H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5879−5883.
19. Proba, K., Honegger, A., and Plückthun, A. (1997) *J. Mol. Biol. 265*, 161−172.
20. Rudikoff, S., and Pumphrey, J. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7875−7878.
21. Auffray, C., Sikorav, J. L., Ollo, R., and Rougeon, F. (1981) *Ann. Immunol. (Inst. Pasteur) 132D*, 77−88.
22. Heinrikson, R. L. (1977) *Methods Enzymol. 47*, 175−189.
23. Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991) *NIH Publication No. 91-3242*, National Institutes of Health, Bethesda, MD.
24. Stemmer, W. P. C. (1994) *Nature 370*, 389−391.
25. Proba, K., Wörn, A., Honegger, A., and Plückthun, A. (1998) *J. Mol. Biol. 275*, 245−253.
26. Freund, C., Ross, A., Guth, B., Plückthun, A., and Holak, T. A. (1993) *FEBS Lett. 320*, 97−100.
27. Ge, L., Knappik, A., Pack, P., Freund, C., and Plückthun, A. (1995) in *Antibody Engineering* (Borrebaeck, C. A. K., Ed.) 2nd ed., pp 229−266, Oxford University Press, New York.
28. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−130.
29. Knappik, A., Krebber, C., and Plückthun, A. (1993) *Biotechnology 11*, 77−83.
30. Knappik, A., and Plückthun, A. (1994) *Biotechniques 17*, 754−761.
31. Tang, Y., Jiang, N., Parakh, C., and Hilvert, D. (1996) *J. Biol. Chem. 271*, 15682−15686.
32. Plückthun, A., Krebber, A., Horn, U., Knüpfer, U., Wenderoth, R., Nieba, L., Proba, K., and Riesenberg, D. (1997) in *Antibody Engineering, A Practical Approach* (Mc Cafferty, J., Hoogenboom, H. R., and Chiswell, D. J., Eds.) pp 203−252, Oxford University Press, New York.
33. Pace, C. N. (1990) *Trends Biotechnol. 8*, 93−98.
34. Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Plückthun, A. (1997) *Biotechniques 22*, 140−149.
35. Schmid, F. V. (1997) in *Protein Structure, A Practical Approach* (Creighton, T. E., Ed.) 2nd ed., pp 261−297, Oxford University Press, New York.
36. Cowgill, R. W. (1967) *Biochim. Biophys. Acta 140*, 37−41.
37. Yasui, H., Ito, W., and Kurosawa, Y. (1994) *FEBS Lett. 353*, 143−146.
38. Pantoliano, M. W., Bird, R. E., Johnson, S., Asel, E. D., Dodd, S. W., Wood, J. F., and Hardman, K. D. (1991) *Biochemistry 30*, 10117−10125.
39. Nieba, L., Honegger, A., Krebber, C., and Plückthun, A. (1997) *Protein Eng. 10*, 435−444.
40. Freund, C., Gehring, P., Holak, T. A., and Plückthun, A. (1997) *FEBS Lett. 407*, 42−46.
41. Freund, C., Honegger, A., Hunziker, P., Holak, T. A., and Plückthun, A. (1996) *Biochemistry 35*, 8457−8464.
42. Mayr, E. M., Jaenicke, R., and Glockshuber, R. (1997) *J. Mol. Biol. 269*, 260−269.
43. Ramsay, G., and Freire, E. (1990) *Biochemistry 29*, 8677−8683.
44. Edge, V., Allewell, N. M., and Sturtevant, J. M. (1988) *Biochemistry 27*, 8081−8087.
45. Brandts, J. F., Hu, C. Q., and Lin, L. N. (1989) *Biochemistry 28*, 8588−8596.

BI980712Q