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The Nature of Antibody Heavy Chain Residue H6 Strongly Influences the Stability of a V_H Domain Lacking the Disulfide Bridge

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Monoclonal antibody mAb 03/01/01, directed against the musk odorant traseolide, carries a serine residue instead of the conserved Cys H92 in the heavy chain variable domain, and is thus lacking the highly conserved disulfide bridge. We investigated the energetic consequence of restoring the disulfide bond and the nature of residue H6 (Glu or Gln), which is poised to interact with Ser H92 in the recombinant scFv fragment obtained from this antibody. In the scFv fragment derived from this antibody, the stabilizing effect of Gln H6 over Glu was found to be as large as the effect of reintroducing the disulfide bond. We have analyzed the conformation and hydrogen bond pattern of Gln H6 and Glu H6 in antibodies carrying these residues and suggest mechanisms by which this residue could contribute to V_H domain stability. We also show that the unpaired cysteine H22 is buried, and conforms to the expected V_H structure. The antibody appears to have acquired two somatic mutations (Ser H52 and Arg H66), which had been previously characterized as having a positive effect on V_H stability. The overall domain stability is the decisive factor for generating functional, disulfide-free antibody domains, and several key residues play dominant roles.

Keywords: disulfide bond; scFv fragment; intrabodies; antibody engineering; protein folding

Introduction

Cysteine residues are amongst the most highly conserved residues in antibody variable domains. They form a buried intradomain disulfide bridge which connects the two β-sheets of the domain. The disulfide bond contributes significantly to the stability of the antibody domains. Only very few examples of antibodies naturally lacking a conserved disulfide bridge are known. Of 5605 human and mouse V_H sequences listed in the Kabat database (http://immuno.bme.nwu.edu, March, 1996), only 17 (0.5%) contain a different residue at position H22 or H92, although in four cases a different alignment would bring a Cys into the correct position. For V_L, only 18 (0.6%) out of 5605 sequences show a substitution of one of the Cys residues, including two possible alignment errors. Most of these sequences were determined at the DNA level after PCR amplification, and it has not been shown whether the published sequences, when expressed, will indeed yield a functional antibody fragment. Some of these sequences were suggested to represent pseudogenes or PCR errors (Cuisinier *et al*., 1993; Mitamura *et al*., 1996; Yang *et al*., 1996).

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Abbreviations used: BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; FR-I, framework I; GdnHCl, guanidinium hydrochloride; IB, inclusion body; IPTG, isopropyl-β-D-thiogalactopyranoside; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; PCR, polymerase chain reaction; PVDF, polyvinylidene fluoride; RT, room temperature; scFv, single-chain Fv fragment of an antibody; traseolide, 6-acetyl-1-isopropyl-2,3,3,5-tetramethyl-indane; V_H, heavy chain variable domain; V_L, light chain variable domain.

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One reason for the rarity of such mutations in natural antibodies is the limited stability of immunoglobulin variable domains. To be able to tolerate the loss of stability associated with the loss of a disulfide bond, a scFv fragment has to be exceptionally robust. In addition, the replacements which can be achieved by a single point mutation in the Cys codon (Gly, Ser, Arg, Phe, Tyr and Trp) do not represent optimal substitutions for a buried cysteine. So far, only one case of a Cys to Tyr substitution in the V_H domain of a functional antibody, ABPC48, has been characterized in detail (Rudikoff & Pumphrey, 1986; Proba *et al*., 1997, 1998).

Engineered removal of a disulfide bridge from the variable domains of the Fab, Fv and scFv fragments of the antibody McPC603 resulted in an unstable protein (Glockshuber *et al*., 1992). More recently, an Ala-Val pair was selected from a library as the optimal replacement for the two Cys residues forming the V_L disulfide bridge. Additional mutations selected in an evolutionary approach stabilized the scFv fragment ABPC48 sufficiently to tolerate the loss of the disulfide bonds in both domains (Proba *et al*., 1998). Both disulfide bonds of the c-*erb*2 (HER-2) binding scFv 4D5 could be replaced by Val-Ala pairs (Wörn & Plückthun, 1998a), as this antibody already contains several stabilizing mutations. Similarly, the disulfide bridge could be removed from the V_L domain REI after stabilizing the fragment by introduction of specific mutations (Frisch *et al*., 1996).

With such scFv molecules, which are not dependent on disulfide bonds for folding into a stable structure, functional expression in reducing environments would become possible (Proba *et al*., 1998). Their application as ''intrabodies'' (Jannot *et al*., 1996; Gargano & Cattaneo, 1997; Persic *et al*., 1997) may involve the titration of cellular factors to study their function, which may become an important technology for connecting function to sequences obtained in genome projects.

Here, we have studied a second example of a monoclonal antibody with a cysteine replacement in V_H. Sequencing of the antibody scFv fragment Tras-SE, derived from mAb 03/01/01, revealed an unusual deviation from the consensus sequence: cysteine H92 in the heavy chain is substituted by a serine residue, indicating that the conserved disulfide bridge of the heavy chain is missing. The sequence of Tras-SE was compared with the subgroup consensus to identify potential stabilizing mutations. Reversal of a PCR-induced mutation of residue H6 (Glu in the scFv, Gln in mAb 03/01/01) led to a significant stabilization of the scFv fragment, comparable to the stabilization achieved by the reconstitution of the disulfide bond. To understand this effect, the role of this residue in the immunoglobulin domain structure was investigated.

Results

Sequence of scFv Tras-SE

Hybridomas were generated against the commercial musk odorant 6-acetyl-1-isopropyl-2,3,3,5-tetramethyl-indane by immunizing mice with the succinyl derivative coupled to KLH (see Figure 2). The antibodies produced by different hybridoma cell lines were cloned as V_H-$(Gly_4Ser)_3$-V_L single-chain Fv (scFv) fragments. One of these fragments, scFv Tras-SE, derived from mAb 03/01/01, deviated from the consensus sequence for the mouse immunoglobulin subgroup V_H2B (Kabat *et al*., 1991) at two highly conserved positions: Cys H92 was replaced by Ser, thereby eliminating the intradomain disulfide bond, and Gln H6 was replaced by Glu (Figure 1). To distinguish whether these differences were already present in the monoclonal antibody or derived from cloning artefacts, the monoclonal antibody mAb 03/01/01 was analyzed at the protein level.

After enzymatic deprotection with pyroglutamate aminopeptidase (Podell & Abraham, 1978), the amino-terminal sequence of the heavy chain of mAb 03/01/01 was determined by Edman degradation. Three of the first six V_H residues are either Gln or Glu. In this stretch of sequence, Gln/Glu ratios of 3:1 (H3), 2:1 (H5) and 3:2 (H6) were found for the positions indicated. This is consistent with a Gln residue in position H6 of the monoclonal antibody. The minor amount of Glu found is probably due to hydrolysis of the side-chain amide. Glu H6 in the scFv fragment was thus most probably introduced by the degenerate V_H2B primer used to amplify the heavy chain of the Fv fragment (Figure 1). Treatment of denatured mAb 03/01/01 with Ellman's reagent revealed the presence of an unpaired Cys residue and thus confirmed that the Cys H92 Ser replacement had occurred prior to the cloning of the scFv fragment, suggesting the somatic hypermutation of the B-cell.

To assess the effect of the two mutations on stability and functionality of the anti-traseolide scFv, four different variants were constructed and compared: scFv Tras-SE (Ser H92, Glu H6), scFv Tras-CE (Cys H92, Glu H6), scFv Tras-SQ (Ser H92, Gln H6) and scFv Tras-CQ (Cys H92, Gln H6).

Production, purification and characterization of the antibody fragments

All antibody variants were produced as cytoplasmic inclusion bodies in *Escherichia coli,* refolded and purified by affinity chromatography on an antigen column, using elution either with an excess of free antigen or with acid (pH 2.7), followed by immediate neutralization. Removal of the hydrophobic antigen from affinity-purified, antigen eluted scFv by dialysis did not yield satisfactory results. The material obtained in this way showed a significantly reduced antigen binding capacity, either due to incomplete removal of the very

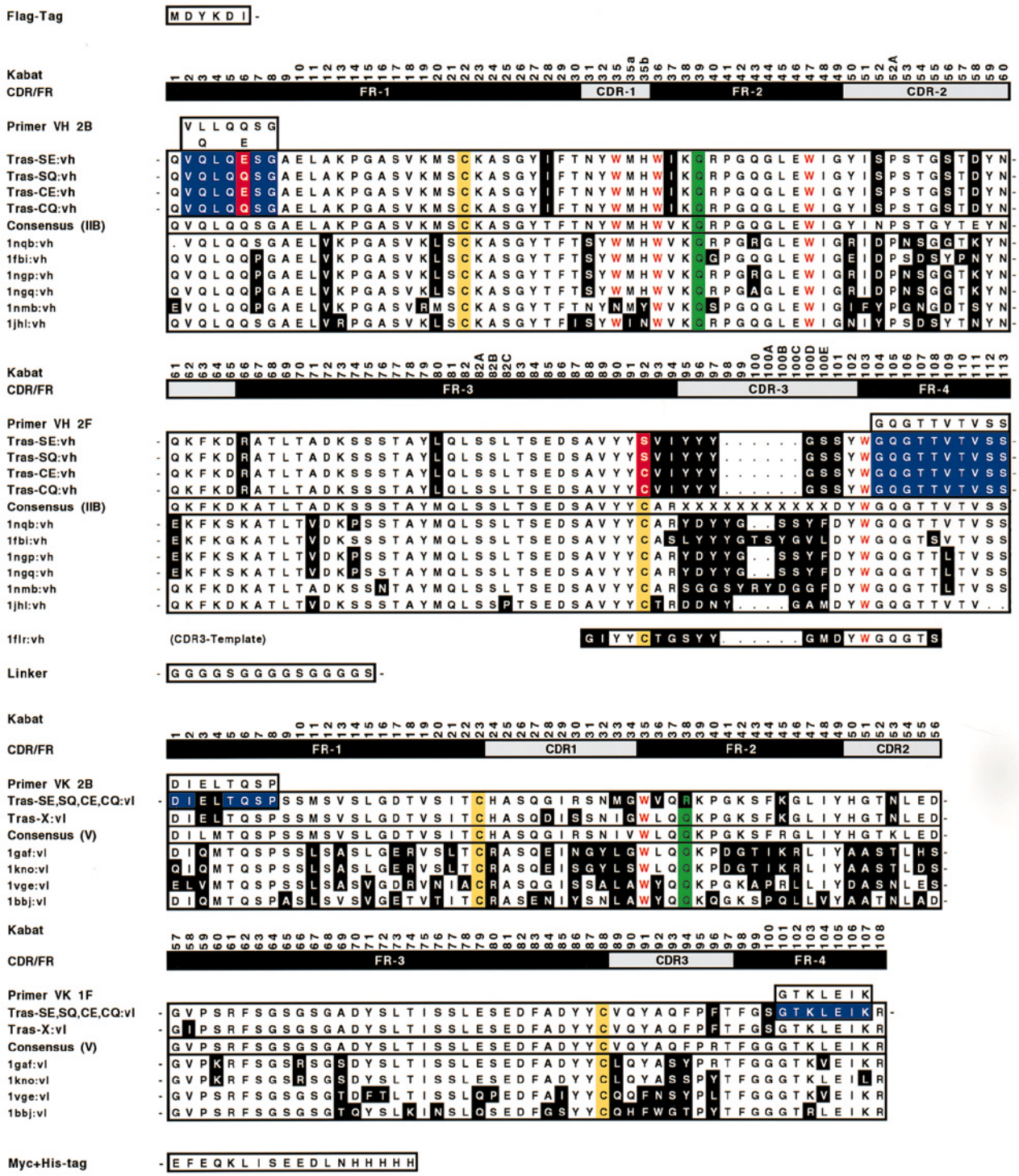

Figure 1. Amino acid sequences of the heavy and light chains of scFv Tras-SE. Sequence regions encoded by the primer are highlighted by blue background, positions H6 and H92 by red background, Cys residues by yellow background, the Gln L38/Gln H39 pair by green background and Trp residues in red lettering. Framework (FR), complementarity determining regions (CDR), tags and peptide linker are indicated. The scFv proteins thus consist of FLAG-V_H-linker-V_L-Myc-His-tag, as indicated. The V_H and V_L sequence positions are numbered according to the system of Kabat *et al*. (1991). The different Tras sequences are compared with the consensus of the most closely related sequences in the Kabat database (http://immuno.bme.nwu.edu/) and the sequences of the most closely related domains in the Brookhaven protein structure database (http://www.pdb.bnl.gov/). Sequence positions deviating from the consensus are indicated by white letters on a black background. The CDR-HIII region was modelled from a different template (PDB entry 1FLR; Whitlow *et al*., 1995), which is given for only this part of the sequence.

hydrophobic antigen or due to denaturation during the extensive dialysis required to remove the antigen. While Tras-SQ, Tras-CE and Tras-CQ could be purified in ligand-free form by acid elution from the affinity column, Tras-SE was not sufficiently stable to be purified by this method. From 1 l of *E. coli* culture about 25 mg of affinity-purified antigen-eluted Tras-SQ, Tras-CE or Tras-CQ could be obtained, while only about 2 mg of Tras-SE was obtained under the same conditions.

Gel filtration in the same buffer as used for affinity chromatography (20 mM Hepes, 100 mM NaCl, pH 7.6) showed that all variants were monomeric under the conditions used (data not shown). Mass spectrometric analysis showed the proteins to have the correct molecular mass. The Ellman assay under denaturing conditions proved the presence of the free sulfhydryl group in mAb 03/01/01 and the scFv Tras-SQ mutant, but not in the two disulfide-restored variants containing the Ser H92 Cys mutation, indicating that disulfide formation had occurred in the refolded protein. The scFv Tras-SE protein could not be analyzed by the Ellman assay, since high concentrations of protein are needed to have a reliable spectrophotometric measurement. K_D values were determined by fluorescence titration with increasing concentrations of antigen to a constant amount of active scFv protein. The values for Tras-SE, Tras-CQ and Tras-CE agreed within the precision of the measurement ($3.0\ (\pm 1.5) \times 10^{-8}$ M, data not shown).

Urea denaturation curves

To determine the stability of the scFv Tras variants, the changes in fluorescence emission during urea denaturation were followed. Binding of the ligand traseolide to the scFvs led to significant quenching of the fluorescence intensity: at saturating concentrations, the intensity was reduced to about 20% of the initial value. The wavelength of the emission maximum was not significantly affected by ligand binding (Figure 3A and B). Similarly, a large amount of traseolide antigen

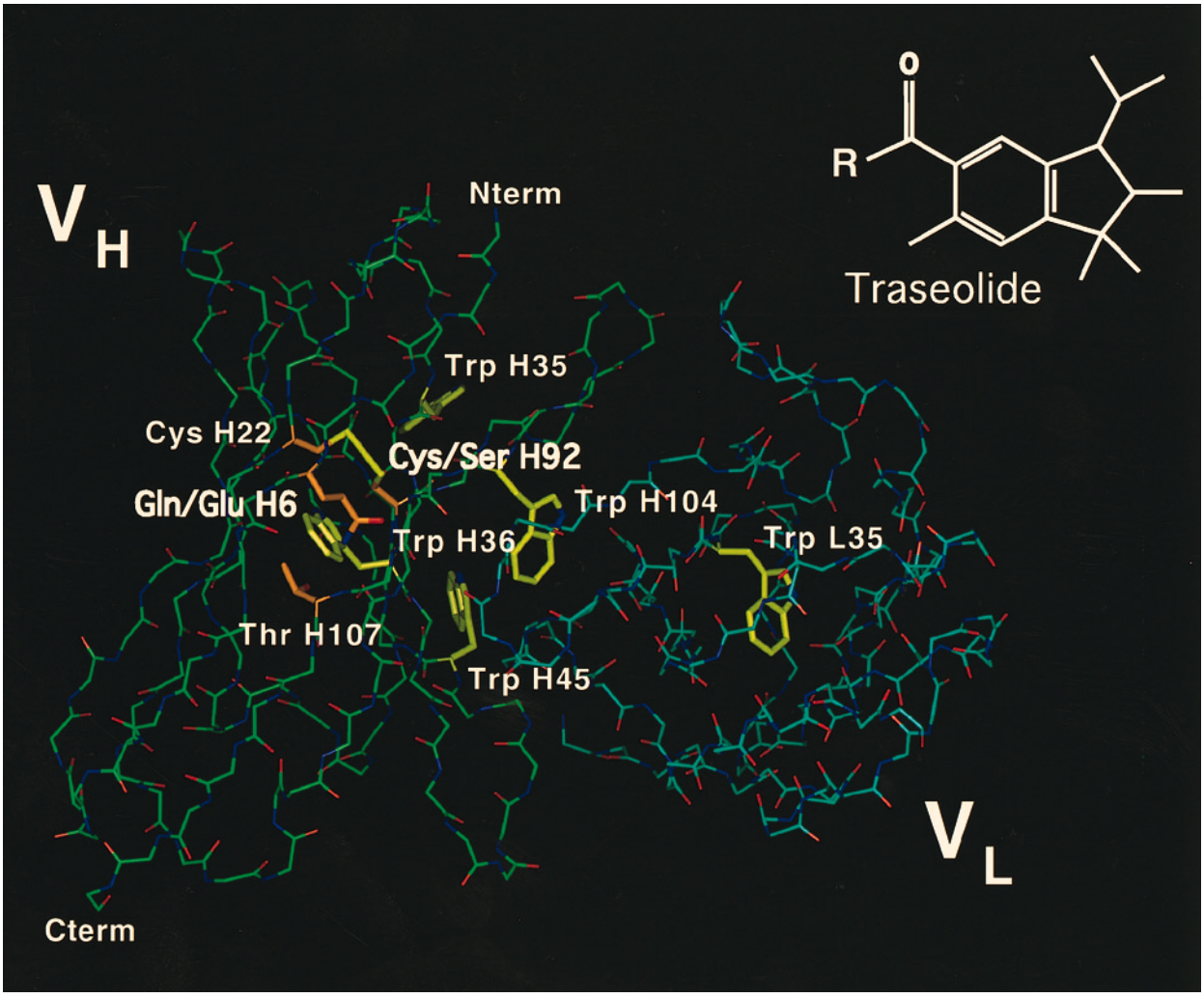

Figure 2. Model of the Tras-CQ scFv with the mutated sequence positions H6 (Glu/Gln) and H92 (Ser/Cys) indicated and the positions of the five Trp residues highlighted. Top right: structure of the antigen traseolide (6-acetyl-1-isopropyl-2,3,3,5-tetramethyl-indane), where R=CH_3. For immobilization, a succinyl derivative was used (R=HOOC-CH_2-CH_2).

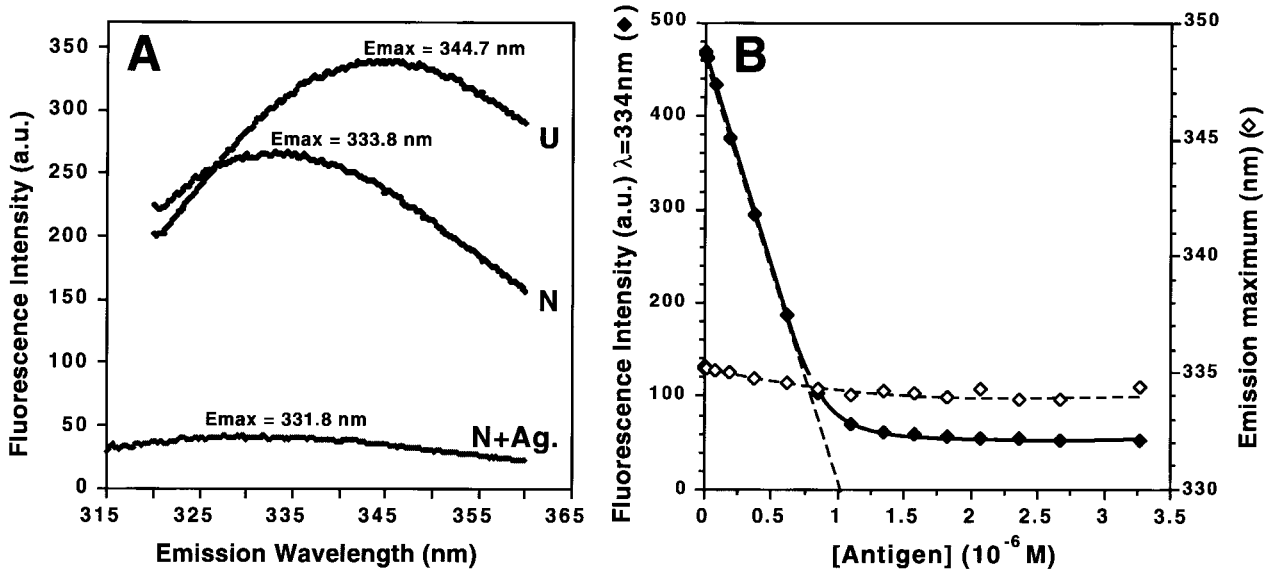

Figure 3. Fluorescence titration of scFv Tras-CQ. A, Emission spectra of fully antigen-quenched native Tras-CQ (N + Ag), unliganded native Tras-CQ (N) and fully unfolded Tras-CQ (U). B, Active-site titration of Tras-CQ: (◇) wavelength of the emission maximum as a function of antigen concentration; (◆) fluorescence intensity at 334 nm as a function of antigen concentration. This experiment was carried out at a protein concentration of 1.0 μM, much higher than the K_D value (3.0(±1.5) × 10^{-8} M, data not shown), and demonstrates specific binding of traseolide with a stoichiometry of 1:1 and thus fully active scFv (Brewer *et al.*, 1974).

added to a solution of free Trp and Tyr in buffer, mixed in concentrations representing the scFv fragment, did not change the emission maximum (data not shown). Measurable quenching of the fluorescence intensity was only observed at traseolide concentrations much higher than those leading to maximal quenching of the scFv fluorescence signal.

Complete urea denaturation of the scFv fragment gave a 40% increase in maximal fluorescence intensity over the non-liganded, native scFv, concomitant with a red shift of the emission maximum by about 15 nm (Figure 3A). Therefore, following the wavelength of the emission maximum as a function of urea concentration will give an indication of the relative stabilities of the different scFv variants (Figure 4A, C and E), while the curves of fluorescence intensity against urea concentration will be dominated by the dequenching effect of ligand dissociation (Figure 4B, D and F).

The fluorescence measurements were found to be highly reproducible, but not fully reversible (data not shown). In addition, the initial emission maximum (in the absence of urea) of the Glu H6-containing variants Tras-SE (335 nm) and Tras-CE (334 nm) was higher than that of Tras-SQ (332 nm) and Tras-CQ (331 nm) (Figure 4A, C and E). This may be due to the presence of non-native, soluble species in the Glu H6-containing proteins. Furthermore, Tras-CQ showed an increase of the initial emission maximum after prolonged storage. However, the Glu/Gln substitution may also have a direct influence on the initial emission maximum, since the buried side-chain of H6 makes direct van der Waals contact with the conserved core Trp in position H36 (Figure 2). To avoid any bias due to normalization, the raw data are shown, in which the fluorescence maximum or emission intensity at 345 nm is plotted as a function of urea concentration (Figure 4).

The urea denaturation curves clearly do not follow the simple sigmoid shape predicted by a two-state model, indicating a more complex process of unfolding and precluding the calculation of ΔG values for the different scFvs.

The shift in emission maximum

The urea denaturation curve of the originally obtained scFv Tras-SE clearly showed two distinct transitions separated by a stable intermediate state. Reintroduction of the conserved Cys H92 residue shifted the lower transition to higher urea concentration, indicating a higher thermodynamic stability (Figure 4A). The denaturation curve of Tras-CE did not show a distinct intermediate, but neither did it show the steepness of a fully cooperative unfolding transition. Mutating Glu H6 to Gln had a stabilizing effect at least comparable to or even slightly larger than that of restoring the disulfide bridge (Figure 4C). Combining the two mutations in scFv Tras-CQ brought little additional stabilization, as indicated by the transition midpoints, but the different initial emission maxima of the two curves are apparent (Figure 4E and F). The effects of the two mutations are not even additive, let alone synergistic. Although the complexity of the denaturation curves precludes any quantitative

interpretation of scFv stability, scFv Tras-CQ does not show exceptionally high stability (For comparison: using the same protocol, we determined the urea denaturation midpoints of the scFvs of antibody McPC603 at approximately at 1.7 M, wild-type ABPC48 at 2.8 M, 4-4-20 at 4.1 M, disulfide-restored ABPC48 at 5.5 M and 4D5 at 6.4 M, data not shown).

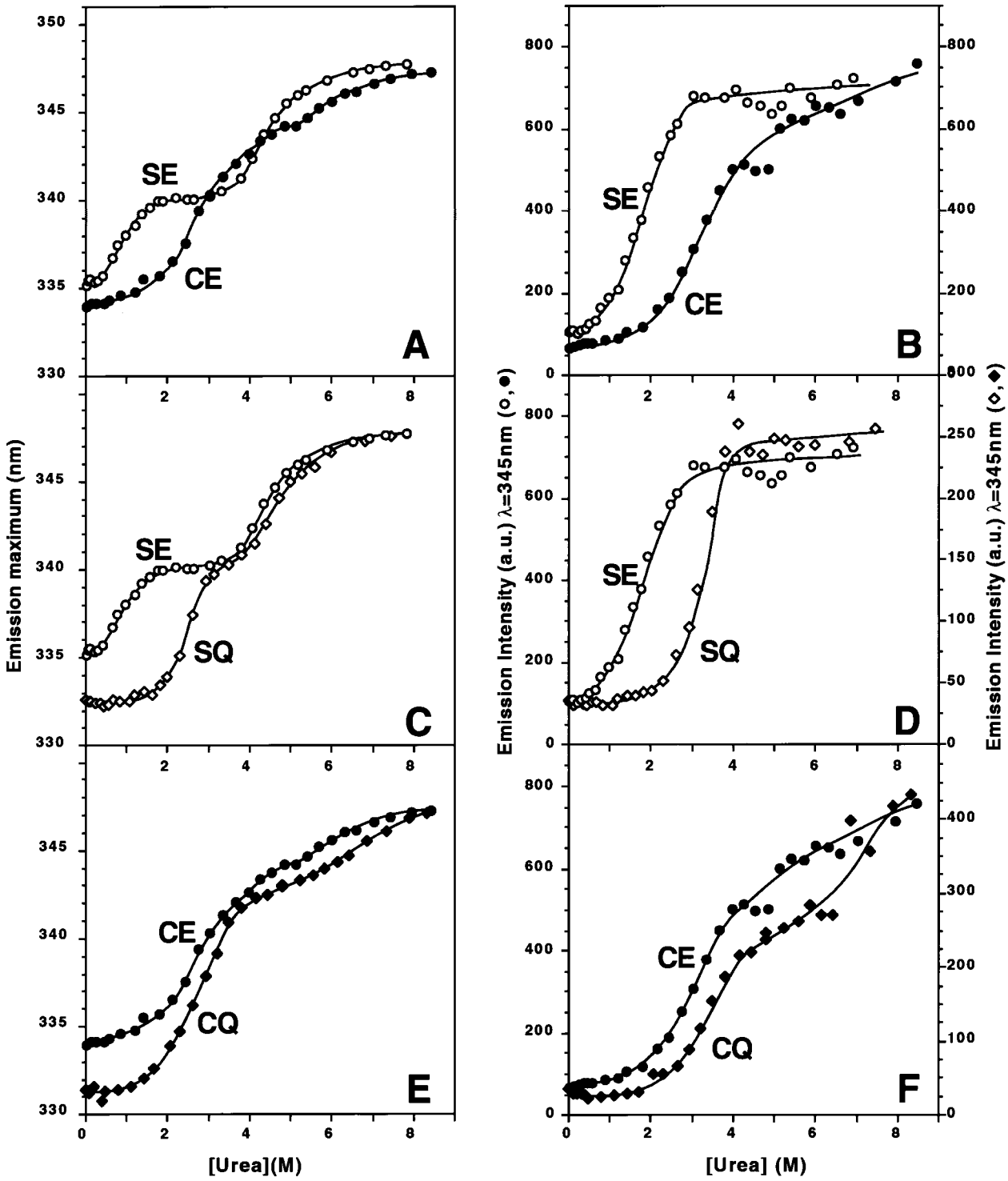

Figure 4. Urea denaturation curves of scFv Tras fragments. Shift in fluorescence emission maximum (A, C, E) and changes in fluorescence intensity measured at 345 nm (B, D, F) upon urea denaturation: (○) scFv Tras-SE; (●) scFv Tras-CE; (◇) scFv Tras-SQ; (◆) scFv Tras-CQ. The figures depict: A, B, the influence of introducing the disulfide bridge in scFv Tras-SE by mutating Ser H92 to Cys; C, D, the influence of bringing FR-I back to consensus by mutating Glu H6 Gln in the presence of Ser H92; and E, F, the influence of bringing FR-I back to consensus in the disulfide restored variant scFv Tras-CE. a.u., arbitrary units.

Change in emission intensity

In the presence of antigen, the change in emission intensity in urea denaturation experiments was dominated by the dequenching effect of antigen dissociation, which led to a fivefold increase in emission intensity going from the liganded scFv to the unliganded scFv, compared to a 40% increase in intensity going from the unliganded scFv to the denatured scFv (Figure 3A). Comparing the urea denaturation curves of the different constructs followed by the emission maximum and by emission intensity shows that ligand dissociation is coupled to the first unfolding transition and the intermediate seen in the unfolding of Tras-SE and Tras-SQ in Figure 4C is no longer able to bind the ligand.

To check whether the presence of the antigen would influence the stability of the scFv fragments, urea denaturation experiments were repeated in the absence of the antigen traseolide. No significant shift in the denaturation curves was found (Figure 5).

Discussion

The monoclonal antibody mAb 03/01/01 was found to be functional, although Cys H92, which contributes to the V_H disulfide bond, was replaced by a Ser residue. Despite an additional deviation from the consensus introduced by the PCR primer used to clone the antibody variable domains (Gln H6 Glu), the Tras-SE scFv derived from this antibody could be produced in functional form by refolding from cytoplasmic inclusion bodies and antigen affinity purification. Either reconstruction of the disulfide bond in the fragment Tras-CE or restoration of Gln H6 in fragment Tras-SQ led to a comparable increase in stability and increased the refolding yield by more than tenfold. Combining the two mutations in the fragment Tras-CQ brought little further improvement in either stability or yield. The scFv Tras variants in which one or both residues were changed to the consensus bound to the affinity column and could be specifically eluted in good yields. The antigen affinities of the variants agreed within a factor of 2, indicating that neither the Ser H92 nor the Glu H6 mutation is required for maintaining the binding characteristics. Thus, there is no evidence that in either antibody the loss of the disulfide was positively selected during somatic mutation by being linked to improved antigen binding. Instead, it seems to be an accident which is tolerated because of the sufficient stability of the V_H domain.

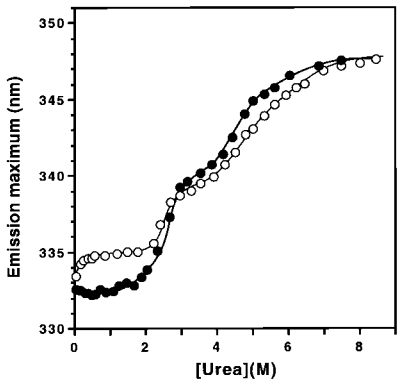

Figure 5. Influence of antigen on stability of scFv. Urea denaturation curves of scFv Tras-SQ in the presence (○) and in the absence (●) of antigen.

Urea denaturation curves

Five tryptophan residues contribute to the fluorescence spectrum of the Tras scFv fragments: V_H and V_L each contain a fully buried, highly conserved Trp in the domain core (H36 and L35), two conserved Trp residues contribute to the hydrophobic interface between the two domains (H45, H104) and there is a fully solvent-exposed Trp (H35) in CDR I of the heavy chain (Figure 2). The fluorescence of the native scFv is strongly quenched by the antigen: a saturating concentration of traseolide (1 μM) reduced the fluorescence intensity to 20% of the signal obtained in the absence of ligand, without changing the wavelength of the emission maximum (Figure 3B). In contrast, denaturation of the scFv fragment increased the wavelength of the emission maximum by about 15 nm with a concomitant increase in fluorescence intensity by about 40% over that of the unliganded native form (Figure 3A).

The interpretation of the fluorescence emission spectra is complicated by the fact that in the two fragments containing Glu H6 (Tras-SE and Tras-CE) the emission maximum of the native species is about 3 nm higher than that for the two fragments containing Gln (Tras-SQ and Tras-CQ). This might be due to the interaction of the H6 residue with the core Trp H36, which is located in close proximity (Figure 2). However, the scFv preparations showed lower antigen binding capacity than expected from the protein concentration, and thus a contamination of the preparations with a non-native, but soluble, form of scFv with unknown fluorescence properties cannot be excluded.

The presence of a stable folding intermediate

The urea denaturation curves of the scFv fragments investigated do not show the steep sigmoid transition curves expected from a two-state model of a fully cooperative folding/unfolding process involving both domains. This is particularly true for scFv Tras-SE, which clearly shows two transitions separated by a stable intermediate at 2 to 3 M urea. For Tras-SQ, the transition midpoints lie more closely together, and for the disulfide-restored fragments Tras-CE and Tras-CQ, this intermediate is no longer detectable, although the behavior at concentrations of urea above 4 M is difficult to interpret (Figure 4A, C and E).

One factor which may contribute to the poor cooperativity of unfolding of the Tras scFv frag-

ments is a somatic mutation found in position L38. The Gln residue usually found in this position forms a double hydrogen bond across the dimer interface to the homologous Gln H39. At least for V_L dimers, the disruption of this pair of Gln residues has been shown to have deleterious effects on production (Chan *et al*., 1996). In the Tras scFvs, Gln L38 is replaced by Arg. In the natural antibody, the loss of V_H/V_L interaction energy would be compensated for by the interaction of the constant domains. In the context of a scFv, the substitution may lead to the destabilization of the V_H/V_L interface and thus to the uncoupling of the unfolding transitions of V_H and V_L.

Since both mutations Glu H6 Gln and Ser H92 Cys predominantly shift the first transition, it can be reasonably assumed that the first transition seen for Tras-SE affects the heavy chain structure. However, complete unfolding of V_H would lead to an exposure of three out of the four buried Trp residues of the scFv. We cannot assume that the four buried Trp residues contribute equally to the overall fluorescence spectrum, since they are quenched to a different degree in the native, liganded fragment. Still, if the intermediate state was one in which the V_H domain is fully denatured and, as a consequence, the dimer interface is disrupted, we would expect the fluorescence spectrum of the intermediate to be closer to that of the denatured state than to that of the native state, particularly since the fluorescence of the core Trp in the native V_L domain is highly quenched and contributes little to the spectrum of the native scFv (Tsunenaga *et al*., 1987; Cowgill, 1967).

As Figure 4C shows, the intermediate state of Tras-SE and Tras-SQ has a fluorescence emission maximum which is red-shifted only half-way between the native and denatured state. We must therefore assume that in the intermediate not all of the V_H Trp residues are fully exposed. Restoration of Gln H6 shifts the first transition, while the denaturation curve at higher urea concentrations is unchanged (Figure 4C). In contrast, the disulfide-restored fragments show a more complex behavior at high urea concentration (Figure 4E), indicating the superposition of more than one transition with mid-points too close together for the individual transitions to be discernible. The shape of the curves is too complex to determine whether two or more transitions contribute to it. It is possible that in these fragments the V_H domain is sufficiently stabilized that a different factor, e.g. the stability of the domain interface, becomes limiting for the overall stability of the scFv fragment. Different relative domain stabilities have been shown to change the order of domain unfolding and dissociation upon increasing denaturant concentration (Wörn & Plückthun, 1998b). The curves of fluorescence intensity *versus* urea concentration (Figure 4C and D) indicate that for Tras-SE and Tras-SQ ligand dissociation is linked to the first unfolding transition. Therefore, in the intermediate state the ligand binding site is sufficiently perturbed to abolish ligand binding.

Solutions for the replacement of the disulfide bridge

In immunoglobulin domains as well as in other proteins, the best pair of substitutions for the two Cys residues has been found to be a combination of Ala and Val (Frisch *et al*., 1996; Liu *et al*., 1997; Proba *et al*., 1998; Wörn & Plückthun, 1998a). In natural antibodies we can only expect to find substitutions which require a single point mutation, with an unpaired Cys residue remaining in the fragment. Thus the substitutions that we find in nature may not represent the best possible solution in terms of stability, but only a viable compromise. In place of the mutated Cys, we may find Ser, Gly, Phe, Tyr, Arg or Trp. At first sight, none of these substitutions looks promising: the greater bulk of the aromatic residues and of the Arg side-chain would be difficult to accommodate in the tightly packed core of the domain, Gly would introduce a destabilizing cavity, and to bring the hydrophilic Ser side-chain into the core of the protein would be energetically unfavorable because of desolvation. Unless the hydrogen bonding requirements can be satisfied in the native structure, the substitution would be highly destabilizing, although it represents an isosteric substitution as nearly as possible.

Previous work on the antibody ABPC48 (Rudikoff & Pumphrey, 1986; Proba *et al*., 1997) showed that the removal of the second disulfide bond was only possible in the presence of additional stabilizing mutations, selected in an evolutionary approach (Proba *et al*., 1998). The same two mutations (Ser H52 and Arg H66) which were found to have the strongest stabilizing effect are also present in Tras-SE (Figure 1), probably representing somatic mutations acquired by the B-cell to give sufficient stability to the original antibody mAb 03/01/01, since they differ from the sequence consensus of subgroup 2B (Figure 1).

The two monoclonal antibodies naturally lacking the V_H disulfide bond, whose scFv fragments have been investigated in our lab, have found different solutions to the problem the Cys substitutions present.

In the scFv fragment of the anti-levan antibody ABPC48 (Proba *et al*., 1997), the unusual reactivity of the remaining, unpaired Cys and the ability of the scFv fragment to refold and bind antigen, even when this Cys residue is quantitatively modified with a bulky substituent such as glutathione, forced us to propose a highly unusual ''cysteine outward'' structure. The substitution of Cys H92 by Tyr has been accommodated by a conformational change in the opposing strand, flipping Cys H22 to a solvent-exposed conformation to make room for the greater bulk of the Tyr side-chain as well as providing hydrogen bonding partners for the side-chain OH group (Proba *et al*., 1997).

In mAb 03/01/01 (this study), Cys H92 has been replaced by a serine residue. There is no evidence of the V_H domain structure of scFv Tras-SE significantly deviating from the canonical structure of V_H domains. By electrospray mass spectrometry we found the molecular mass expected from the sequence for all scFv Tras variants after refolding in the presence of glutathione, and under denaturing conditions, the free SH group could be detected using Ellman's reagent. The Ser residue replacing Cys H92 probably satisfies its hydrogen bonding potential by interacting with the conserved hydrogen bond network found in the core of immunoglobulin variable domains.

The structural role of Glu/Gln H6 in disulfide-bridge-containing variable domains

The importance of FR-I for folding and stability of antibody fragments has been recognized before (Benhar & Pastan, 1995; DeHaard & Kazemier, 1995). McCartney *et al*. (1995) found that changing FR-I sequences obtained by PCR amplification to the consensus for the corresponding subtype resulted in a dramatic improvement of scFv refolding yields. One of the residues these authors changed was H6, which can be either Gln or Glu. Recently, Kipriyanov *et al*. (1997) found that substitution of Glu encoded by a PCR primer at position H6 in FR-I by Gln led to a more than a 30-fold increase in the production of an otherwise very poorly soluble scFv, bringing it back to average levels, with very little effect on the affinity. However, none of the authors provided an explanation of how residue H6 might affect the folding stability or efficiency of the scFv fragment.

The fully buried Glu or Gln residue in position 6 is a highly conserved feature in immunoglobulin variable domains, distinguishing them from other types of immunoglobulin domains. The variable domains show characteristic structural adaptations to accommodate the hydrogen bonding needs of this polar side-chain in the core of the domain (A. Honegger *et al*., unpublished results). In lambda and kappa V_L domains, Gln L6 is as highly conserved as the Cys residues contributing to the disulfide bond, and the conformation of the kink in the first strand as well as the preference for *cis*-Pro in position L8 of kappa chains are adaptations needed to accommodate the hydrogen bonding network in the core of the domain (Spada *et al*., 1998).

We found that there is a strong correlation between the type of residue found in position H6 and the conformation of the FR-I region of the V_H domain (Figure 6, and A. Honegger *et al*., unpublished results). V_H domains can be divided into structural subclasses based on whether the residue in position H6 is a Glu or a Gln. Although the burial of an unpaired charge in the core of a protein is usually considered to be very destabilizing (Dao-pin *et al*., 1991; Langsetmo *et al*., 1991; Tissot *et al*., 1996), 45% of the mouse and 52% of the human V_H sequences in the Kabat database (http://immuno.bme.nwu.edu, July 1997) have Glu in position H6, while 54% of all mouse and 45% of the human sequences contain Gln. The two different side-chains assume different conformations to satisfy their different hydrogen bonding potential.

The side-chain oxygen of Gln and one of the two oxygen atoms of Glu take up the same position, accepting hydrogen bonds from main-chain NH groups of the opposite sheet (Cys H92, Gln H105 and/or Gly H106). The second oxygen of Glu and the NH_2 of Gln point in different directions. The second oxygen atom of Glu accepts a hydrogen bond from its own main-chain NH and from Gly H106 (Figure 6A), while the side-chain NH_2 of Gln donates hydrogen bonds to the main-chain carbonyl oxygen of Tyr H90 and to the side-chain OH of Thr H107 (Figure 6C). This in turn imposes constraints on the main-chain conformation of positions H6 to H10. While Gln H6 is more permissive, Glu H6 seems to require a Pro or Gly in position H9. In the Tras fragments, the H9 position holds an Ala residue, the residue type most frequently found in conjunction with Gln H6. This conformational preference may explain the beneficial effects of a Glu to Gln substitution reported in some normal, disulfide-containing scFvs, where Glu appears in a Gln-type sequence context (e.g. see Kipriyanov *et al*., 1997): Glu (H6), Ala(H9)), but not in others, where Glu occurs in its native sequence context.

Of 58 non-redundant V_H structures from the Brookhaven database (http://www.pdb.bnl.gov), 30 contain glutamine in position H6. Of these, 16 conform to the hydrogen bonding pattern described above. Nine show the same side-chain conformation, but with the positions of the side-chain oxygen and nitrogen swapped. Since nitrogen and oxygen atoms cannot be distinguished by their electron density at the resolution of these structures and such a conformation would allow fewer hydrogen bonds, this swap may represent a misinterpretation of the electron density. Five structures show Gln H6 in a Glu-like conformation, but their sequences all correspond to subgroups which normally contain Glu in this position. Frequently, crystals have been grown from hybridoma-derived Fab fragments, while the sequence has been determined with PCR primers, which may change the nature of residue H6. Of 28 structures containing glutamate in H6, 20 structures agree to the consensus pattern, the remaining eight show Glu in Gln-like conformation, three of them in a Gln-type sequence context (Figure 6).

As this analysis shows, both Gln H6 and Glu H6 side-chains, although fully buried, can participate in a hydrogen bonding network which satisfies their hydrogen-bonding requirements.

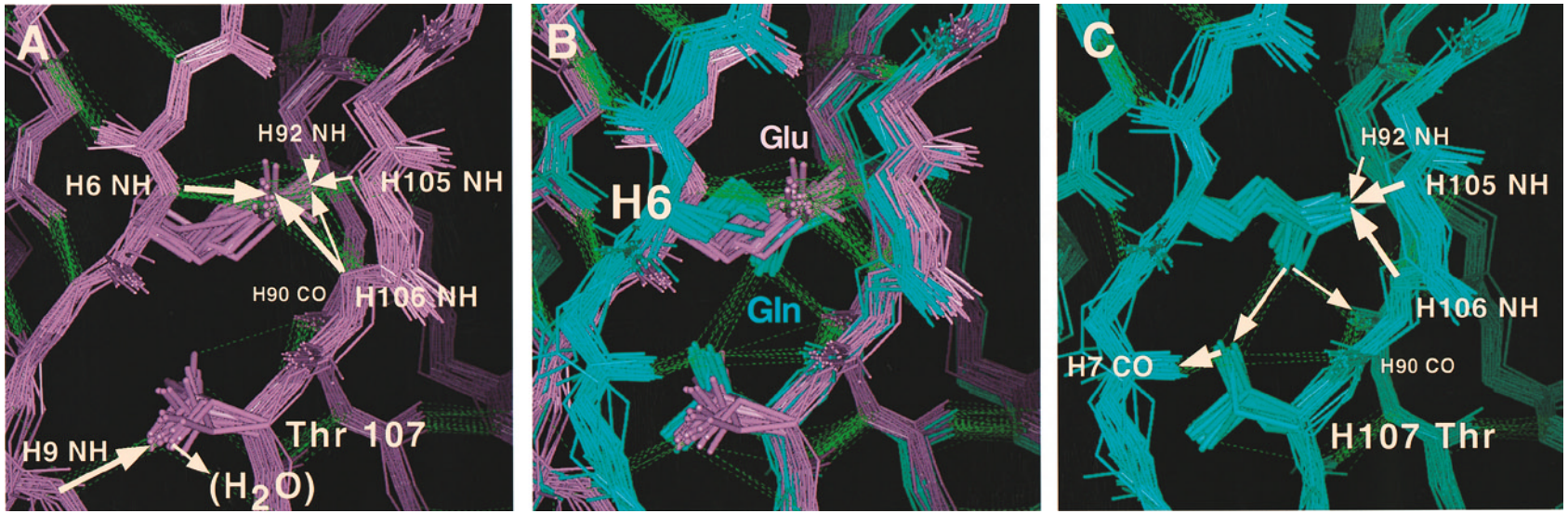

Figure 6. Conformation of H6 in experimental V_H structures. Thirty-eight non-redundant V_H structures from the PDB database (http://www.pdb.bnl.gov) were superimposed by a least squares fit of the C^{α} positions of residues H3 to H7, H20 to H24, H35a to H40, H44 to H50, H67 to H71, H78 to H82, H88 to H94 and H102 to H108. Pink, structures containing Glu H6 in a Glu-type sequence context (A, B); cyan, structures containing Gln in a Gln-type context (B, C). Consensus hydrogen bonds are indicated by white arrows pointing from the donor to the acceptor.

Hydrogen bonding pattern in the Tras scFv fragments

All possible conformations were considered in order to rationalize the strong effect that the Glu to Gln substitution has on the stability of the disulfide-free Tras scFv fragment. Figure 7 shows the possible hydrogen bonding pattern for the different mutations. In Figure 7A the consensus hydrogen bonding pattern for V_H domains containing Gln H6 is emphasized. Although the side-chain oxygen can only accept two hydrogen bonds with good geometry at the same time, three potential donors are within reach: the main-chain NH groups of Cys H92, Gln H105 and Gly H106. The side-chain NH_2 donates hydrogen bonds to the main-chain C=O of Tyr H90 and the side-chain OH of the highly conserved Thr 107, which in turn donates a hydrogen bond to the main-chain C=O of residue H7.

Replacing Gln by Glu without conformational adaptation (Figure 7B) replaces the hydrogen bond donor NH_2 by the hydrogen bond acceptor O^-. Thr 107 can therefore only form one hydrogen bond, either to H6 or to the main-chain C=O of residue H7. However, this is not the conformation

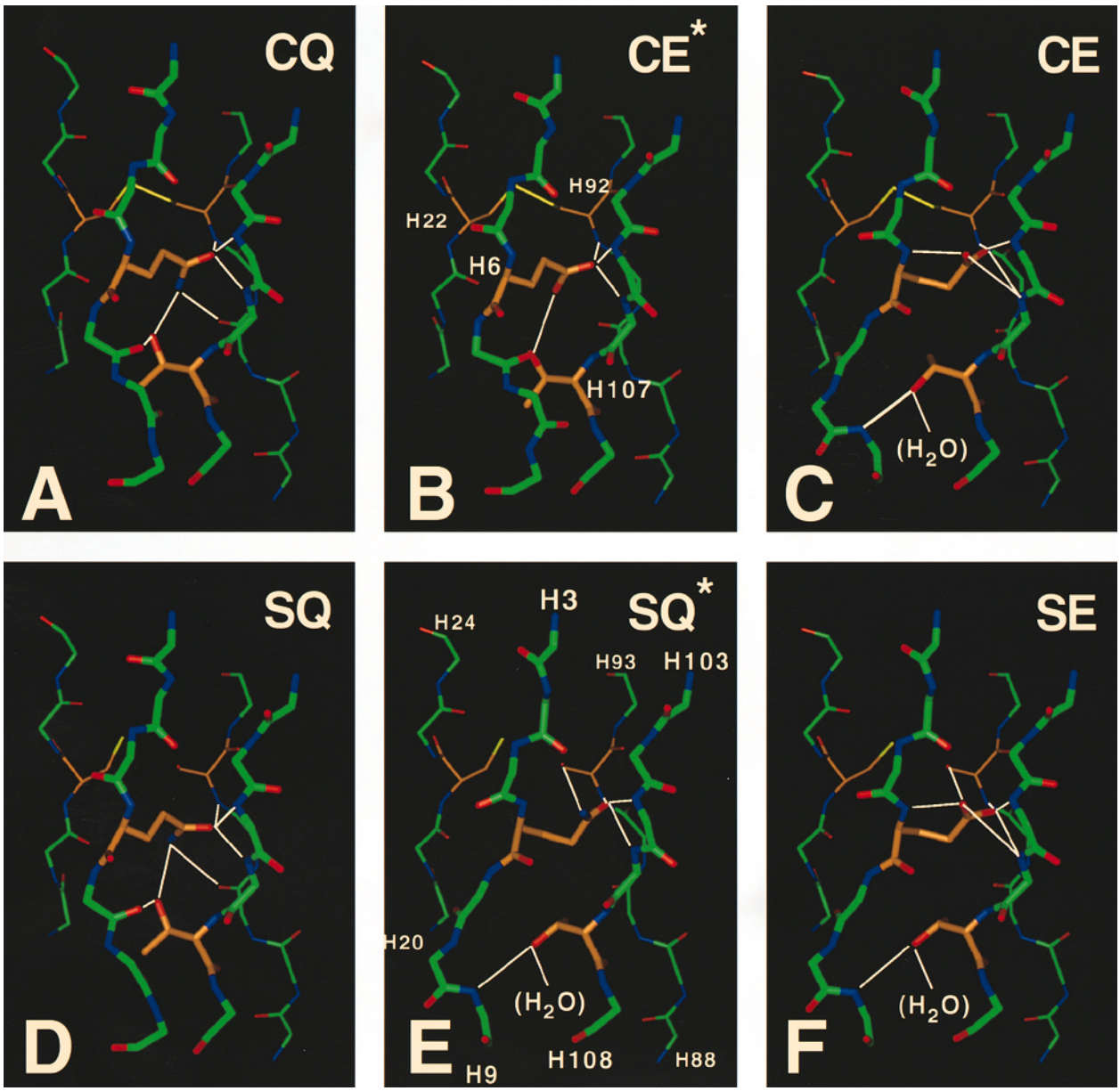

Figure 7. Hydrogen-bonding network in the core of V_H. A, B, C, V_H domains with intact disulfide bond between H22 and H92. A, Consensus pattern for H6 Gln in a Gln-type sequence context. B, Gln H6 replaced by Glu without additional structural adaptation. C, Consensus pattern for H6 Glu in a Glu-type sequence context. D, E, F, Potential interactions of Ser H92 with Glu/Gln H6. D, Gln side-chain of backbone in Gln consensus conformation. E, Gln side-chain in Glu-like conformation. F, Glu side-chain and backbone in Glu consensus conformation. For an explanation see the text.

found in the majority of structures containing Glu H6. In Glu-containing structures, H6 is rotated to accept a hydrogen bond from the H6 main-chain NH and from the main-chain NH of Gly H106 (Figure 7C). Depending on the local sequence the main-chain conformation of residues H7 to H9 changes in such a way that a hydrogen bond from the main-chain NH of H9 to the rotated side-chain of Thr H107 can be formed.

Interactions between Glu/Gln H6 and Ser H92

Substitution of a buried disulfide bridge by polar residues like Ser would be expected to cause a decrease in stability relative to alanine mutants (Liu *et al*., 1997). However, the V_H disulfide bridge and Glu/Gln H6 hydrogen bonding to the opposing sheet run in parallel directions, connecting neighboring strands of the two β-sheets. It is likely that upon substitution of Cys H92 by Ser the hydrogen bonding network will be rearranged to incorporate the side-chain OH of the Ser residue. This interaction would be optimal if the Gln side-chain assumed a conformation similar to the one usually seen for the Glu side-chain (Figure 7E). If the strong stabilizing effect of the Glu to Gln substitution is mainly due to a better integration of the Ser residue into this hydrogen bonding network, the lack of synergy or even additivity of the stabilizing effects of the Ser H92 Cys and Glu H6 Gln substitutions clearly follows: if the disulfide bond is restored, there is no longer a Ser OH group present for the Glu or Gln side-chain to interact with.

We cannot exclude, however, the possibility that Gln H6 takes on the ''standard'' conformation also in the Ser H92 variant, if the overall energy, even leaving Ser H92 unsatisfied, is more favorable. The stabilizing effect of the Glu to Gln substitution would then be due to unfavorable interactions between the Glu-imposed main-chain conformation and the Gln-typical sequence context, particularly the presence of Ala H9. In this case, the failure of disulfide restoration to further improve the stability of the scFv could be explained if factors other than the intrinsic stability of the V_H domain become limiting for the stability of the scFv fragment, e.g. the dissociation of the interface, which in Tras has been weakened by the Gln L38 to Arg substitution. The different factors influencing equilibrium unfolding of scFv fragments have recently been investigated (Wörn & Plückthun, 1998a).

Conclusions

An antibody fragment lacking the conserved disulfide bridge in the heavy chain has been characterized. In contrast to ABPC48, where the Cys H92 Tyr mutation has been accommodated by a change in main-chain conformation leading to a fully exposed Cys H22 (Proba *et al*., 1997), in scFv Tras the hydrophilic serine residue replacing Cys H92 is probably integrated in a conserved hydrogen bonding network spanning the core of immunoglobulin variable domains. The FR-I residue H6 plays a crucial role in stabilizing the scFv by interacting with the buried serine residue. Replacement of Glu H6 by Gln stabilizes the fragment to a level comparable to the reconstitution of the disulfide bond, but the same mutation has hardly any effect on the corresponding disulfide-restored fragment. This suggests that the stabilizing effect of the Glu H6 to Gln mutation is indeed due to a direct interaction between Gln H6 and Ser H92, which would be abolished upon disulfide restoration. Alternatively, upon increased stabilization of the V_H domain the interface energy may limit the overall stability of the scFv fragment, obscuring the additive effects of the two mutations on the intrinsic stability of V_H and altering the order of equilibrium unfolding transitions. Since the two interpretations hinge on the side-chain orientation of a single residue, they can only be distinguished by crystallography, which is currently being attempted.

Materials and Methods

Materials and strains

For all cloning experiments the *E. coli* K12 strain JM83 (λ^-, *ara*, Δ(*lac*, *pro*AB), *rpsL*, *thi*, ϕ80, *dlac*ZΔM15) (Yanisch-Perron *et al*., 1985) was used. The scFv proteins were expressed as cytoplasmic inclusion bodies in the *E. coli* strain BL21(DE3) (F^-, *ompT*$^-$, $r_B^-r_B^-$, (λimm21, *lac*I, *lac*UV5, T7pol, *int*$^-$)) with the bacteriophage T7-based system (Studier & Moffatt, 1986).

Immunization, fusion and subcloning

The commercial musk odorant traseolide (Quest International, The Netherlands) was used as antigen (see Figure 2). For immunization, the succinyl derivative of the odor molecule was coupled *via* the carboxyl group to keyhole limpet hemocyanin (KLH, Pierce), and for screening assays it was conjugated to bovine serum albumin (BSA, Sigma). Conjugations were performed using the hydroxysuccinimide method (Anderson *et al*., 1964). Immunization and subsequent preparations of hybridomas were performed by MCA Development (Groningen, the Netherlands) and production of monoclonal antibodies was carried out at Unilever Research Laboratories (Vlaardingen, The Netherlands). Screening for antigen specificity was performed by means of standard ELISA with BSA-traseolide coated on the microtiter plate. On the basis of antibody isotype and binding characteristics, stable cell lines were chosen for further characterization.

mRNA was isolated from the monoclonal hybridoma cell line mAb 03/01/01 by an acid guanidinium thiocyanate extraction (Chomczynski & Sacchi, 1987). Using reverse transcriptase, the first strand cDNA was synthesized using primers hybridizing to the constant domains (C_γF: 5′-CCCGAATTCTGGATAGACAGATGGGGGTGTCGTTTTGGC and C_κF: 5′-CCGAATTCGATGGATACAGTTGGTGCAGCATCAGCCCG) and partially sequenced from the C terminus to determine the subclass. Subsequently, cDNA was amplified by means of PCR with subclass-specific primers and subcloned in a suitable vector to complete sequencing (V_H2B: 5′-AGGTSMARCTGCAGSAGTAWGG; V_H2F: 5′-TGAGGA-

GACGGTGACCGTGGTCCCTTGGCCCC; V_K2B: 5′-GACATTGAGCTCACCCAGTCTCCA; V_K1F: 5′-GTTAGATCTCCAGCTTGGTCCC). Primer V_H2B is degenerate by using nucleotide mixtures in several positions (M = A or C, R = A or G, S = C or G, W = A or T).

Construction of plasmids

The V_H-$(Gly_4Ser)_3$-V_L construct was obtained by cloning V_H and V_L in the vector pHJ300 (Knappik & Plückthun, 1994). For cytoplasmic expression, the gene was digested at the unique restriction sites *Eco*RV and *Eco*RI at the 5′ and 3′ ends of the antibody genes and subcloned in pTFT74 (Freund *et al*., 1993; Ge *et al*., 1995) which places expression under control of the T7 promoter.

The mutant antibody fragments Tras-CE, Tras-CQ and Tras-SQ were constructed by site-directed mutagenesis using PCR with primers containing the desired mutation. After sequencing the mutated part, the gene was digested and ligated in the original plasmid. The different scFv constructs used in this study are summarized in Figure 1.

Cytoplasmic scFv expression

One ml of LB medium (100 μg/ml ampicillin) was inoculated with bacteria from a glycerol stock (stored at −80 °C) and incubated for four hours at 37°C. Twenty-five ml of LB medium (100 μg/ml ampicillin) was inoculated with the 1 ml culture and incubated overnight at 37°C. One liter of LB medium (100 μg/ml ampicillin) was inoculated with the overnight preculture and incubated at 37°C (5-liter flask, 180 rpm). Expression was induced at an $A_{660} \approx 2$ by addition of IPTG to a final concentration of 0.5 mM. Incubation was continued for about four hours. After centrifugation (8000 *g*, five minutes, 4°C), cells were resuspended in 10 mM Tris-HCl buffer (pH 8.0) containing 2 mM $MgCl_2$ and disrupted by sonification. Inclusion body (IB) protein was isolated following a standard protocol (Buchner & Rudolph, 1991). The size of the IB pellets obtained for the different scFv fragments varied; the antibody fragment scFv Tras-SE yielded a brownish pellet, while bigger, clean and white pellets were obtained for the three other fragments. This correlates with protein yield after purification: Tras-SE yields were only 10% of the other scFv fragments.

Purification of inclusion body protein and refolding were carried out as described by Proba *et al*. (1997) except that the formation of disulfide bonds was allowed by the presence of 0.5 mM of both reduced and oxidized glutathione in the refolding buffer. Varying the oxidation conditions by changing the ratio of oxidized to reduced glutathione (1:5, 1:1 and 5:1) did not substantially change the refolding yield. After incubation of one to four days at 4°C the refolding mixture was applied to an antigen affinity column.

Antigen affinity chromatography

The antigen traseolide was activated at the carboxylate group by reacting it with equimolar amounts of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, Fluka) and *N*-hydroxysuccinimide (NHS, Fluka). The activated ligand was coupled to EAH-Sepharose 4B (Pharmacia) in 60% ethanol for 16 hours at RT. To avoid non-specific hydrophobic interactions on the column only 1% of the available groups on the gel were coupled to the ligand, and the remainder was quenched with 1 M acetic acid.

The protein was adsorbed in a batch procedure to circumvent inefficient large-scale concentration steps. For this purpose, 5 ml of activated and equilibrated gel material was placed in 2 l of refolding buffer and incubated overnight on a cell production roller system at 4°C. The gel material containing the active scFv was washed on a glass filter with 30 gel volumes of buffer (20 mM Hepes, 150 mM NaCl, pH 7.6) and poured into a column. The elution was performed in two different ways: (1) specific elution with antigen, with washing buffer containing 60 μM traseolide; or (2) non-specific elution with acidic buffer (100 mM glycine, pH 2.7) and immediate neutralization with 1/4 volume of 1 M Tris (pH 8.0). For the specific elution, the buffer containing the antigen was incubated for at least five hours at 4°C in the column before starting the elution. In both methods the purified protein is eluted as a single peak. Purity of the protein was controlled by Coomassie-stained SDS-PAGE. If necessary the purified scFv was applied to a gel filtration column (PD10, Pharmacia) to remove excess of antigen or was concentrated by centrifugal filtration with an Ultrafree concentrator (Millipore). Yields were calculated from absorption at 280 nm, using calculated extinction coefficients (Gill & von Hippel, 1989).

Gel filtration chromatography

Samples of purified scFv proteins were analyzed on a Sephadex 75 column, equilibrated with 20 mM Hepes, 150 mM NaCl (pH 7.6), on a SMART-system (Pharmacia). The sample volumes were usually 25 μl (containing about 2 to 5 μg protein) and the flow rate was 60 μl/min. As M_r standards, lysozyme (14 kDa), carbonic anhydrase (31 kDa), bovine serum albumin (66 kDa) and catalase (230 kDa) were used.

Ellman's assay

Two hundred μl of protein solution (minimal 100 μg) was mixed with 1 ml of assay buffer (0.3 M Tris-HCl (pH 8.0), 1 mM EDTA, 6 M GdnHCl) and 10 μl of dithio-bis-nitrobenzoic acid stock solution (DTNB, 10 mM in 0.3 M Tris-HCl, pH 8.0). After 15 minutes of incubation at RT, the A_{405} value was measured (Riddles *et al*., 1983). As a negative control, the protein was measured in the absence of denaturant. The mAb 03/01/01 values were compared with those for a mAb which does contain the disulfide bridge in V_H.

Electrospray mass spectrometry

Data were collected on a Sciex API III+ triple quadrupole mass spectrometer (Perkin Elmer, Thornhill, Canada). The ion spray voltage was 5000 V, the dwell time 0.7 ms and the scan step size 0.2 amu. The samples (10 pmol/μl scFv protein in 10 mM ammonium acetate, pH 7.0) were injected into the ion source at a flow rate of 7 μl/min. Molecular masses (Da) for the scFv fragments were: Tras-SE: 28.518 (det.), 28.514 (calc.); Tras-SQ: 28.513 (det.), 28.513 (calc.); Tras-CE: 28.533 (det.), 28.530 (calc.); Tras-CQ: 28.530 (det.), 28.529 (calc.).

Amino acid sequence analysis

The amino terminus of 100 pmol of mAb 03/01/01 was deblocked with pyroglutamate aminopeptidase (Podell & Abraham, 1978) according to the supplier's protocol (Boehringer Mannheim, Germany). The heavy and light chains of the deblocked protein were separated on SDS-PAGE, electroblotted on a PVDF membrane (Problott™) and stained with Coomassie Brilliant Blue. The heavy chain fragment was cut out and sequencing was carried out by automated Edman degradation.

Thermodynamic stability

Sample preparation

A stock solution of 9 M urea was prepared in 20 mM Hepes, 150 mM NaCl (pH 7.6). ScFv protein/denaturant mixtures (1.7 ml) contained a final protein concentration of 0.2 μM (10 μg) and increasing urea concentrations varying from 0 to 8 M. The urea concentration of the separate solutions were measured after the experiment by refractive indices (Pace & Scholtz, 1997). After overnight incubation at 4°C and an additional two hours at 17°C prior to the measurement, the fluorescence emission of the samples was recorded.

Fluorescence spectroscopy

Emission spectra of the samples were recorded as described by Proba *et al*. (1997). With increasing denaturant concentrations, the maxima of the emission wavelength of the recorded spectra shifted from about 332 to 348 nm. The maxima of the averaged spectra were determined at each urea concentration by fitting to a Gaussian function. The change in emission intensity was measured at a constant emission wavelength, the maximum of the denatured state.

Fluorescence titration

A 2 ml volume of 20 mM Hepes, 150 mM NaCl (pH 7.6), containing 0.1 to 0.5 μM active scFv Tras was placed in a cuvette with an integrated stirrer at 17°C. Aliquots of concentrated traseolide stock (0.6 μM) were added and after 12 minutes of equilibration a spectrum was recorded from 315 to 360 nm (excitation wavelength 280 nm). The emission intensity at 335 nm was used for determining the degree of complexation as a function of traseolide concentration. The K_D value was determined by a direct fit as described (Jung & Plückthun, 1997). To determine the influence of the presence of the antigen on the wavelength of the emission maximum, the emission maximum at every antigen concentration was also determined by fitting the spectra to a Gaussian function (see above).

Molecular modelling

The structures of the Tras scFv fragments were predicted by homology modelling using the Homology and Discover modules of the program InsightII version 95 (Biosym/MSI, San Diego, CA). The V_L domain model was based on the X-ray structure of Fab Tras-X (94% identity, 97% similarity; Figure 1), another monoclonal antibody directed against the same antigen with highly homologous V_L but a very different V_H (S. Spinelli & C. Cambillau, LCCMB-CNRS, Marseille, France, unpublished results).

The V_H model was predominantly based on the X-ray structure of the Fv fragment of the murine antibody D11.15 (PDB entry 1JHL; Chitarra *et al*., 1993; 75% identity, 80% similarity; Figure 1), which belongs to the same mouse immunoglobulin subgroup (Kabat *et al*., 1991). As template for a Glu-containing FR-I, the structure of anti-fluorescein antibody 4-4-20 (PDB entry 1FLR; Whitlow *et al*., 1995) was used. The CDR III conformation was based on the CDR III conformation in the V_H domain of anti-fluorescein antibody 4-4-20 (PDB entry 1FLR).

To align different V_H domains for the comparison of their conformation, a least-squares fit of the C^{α}-positions of residues H3-H7, H20-H24, H35a-H40, H44-H50, H67-H71, H78-H82, H88-H94 and H102-H108 was performed.

Statistical analysis of substitution frequencies and sequence correlations

Kabat database dump files were downloaded (ftp://ttwu.bme.nwu.edu/pub/database/) and human and mouse variable domain amino acid sequences extracted and converted to the one-letter code. Sequences less than 90% complete or containing multiple undetermined residues in the regions of interest were eliminated. Where necessary, the alignment was corrected to shift the gaps to the closest positions where they could be accommodated in the three-dimensional structure. From this alignment, position-dependent amino acid substitution frequencies were determined.

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