J. Mol. Biol. (1997) **265**, 161–172

JMB

A Natural Antibody Missing a Cysteine in V_H: Consequences for Thermodynamic Stability and Folding

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While the disulfide bridge is highly conserved within the immunoglobulin fold, a few antibody variable domains lack one of the essential cysteine residues. In the levan binding antibody ABPC48 one of the essential cysteine residues (Cys H92) of the heavy chain variable domain is replaced by tyrosine. We expressed scFv fragments with the ABPC48 sequence and a mutant in which the V_H disulfide bond has been restored in *Escherichia coli*, purified both proteins by antigen affinity chromatography and characterized them by equilibrium denaturation. While the ABPC48 protein was found to be significantly less stable than an average scFv molecule, the restored disulfide increased its stability above that of other, unrelated scFv fragments, explaining why it tolerates the disulfide loss. Surprisingly, we observed that under some refolding conditions, the unpaired cysteine residue of functional scFv of ABPC48 is derivatized by glutathione. It is easily accessible to other reagents and thus appears to be solvent-exposed, in contrast to the deeply buried disulfide of ordinary variable domains. This implies a very unusual conformation of strand b containing the unpaired Cys H22, which might be stabilized by interactions with the tyrosine residue in position H92.

Keywords: disulfide bond; antibodies; scFv fragment; protein folding; stability

Introduction

Disulfide bonds are conserved across almost the whole immunoglobulin superfamily. In antibody variable domains (V_H and V_L), they connect the two β-sheets of the immunoglobulin domain, from strand b to strand f. The contributing cysteine residues L23 and L88 in V_L, and H22 and H92 in V_H (numbering according to Kabat *et al*., 1991) are almost perfectly conserved in all antibodies. Of 5605 human and mouse V_H sequences listed in the Kabat database (March 1996), 3355 contain sequence information for the two conserved cysteine positions H22 and H92. Of these, only 17 (0.5%) show a different residue in one of these positions, although in four cases a different alignment would bring a cysteine residue into the appropriate position. Similarly, of 3735 V_L sequences, 2985 contain sequence information covering both L23 and L88. Of these, 18 (0.6%) show a substitution of one of the cysteine residues by a different residue type, with two possible alignment errors.

Because of this extreme conservation of the disulfide bond it was probably already present at the very beginning of Ig-fold diversification and was maintained under selective pressure. Since every known human and mouse germ-line sequence of immunoglobulin variable domains contains both cysteine residues, and since the known disulfide-lacking domains are not particularly closely related, the observed cases are almost certainly due to random somatic mutations occurring in B-cell development.

Abbreviations used: BBS, borate-buffered saline; CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay; ESMS, electrospray mass spectrometry; Fv, variable fragment; GdnHCl, guanidinium hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; Ig, immunoglobulin; IPTG, isopropyl β-D-thiogalactopyranoside; IMAC, immobilized metal ion affinity chromatography; K_D, dissociation constant; MHA, Mes-Hepes-acetate buffer; PVDF, polyvinyldifluoride; SB, super broth; scFv, single-chain variable fragment; TBS, Tris-buffered saline; V_H, variable heavy chain; V_L, variable light chain.

0022–2836/97/020161–12 $25.00/0/mb960726

The almost perfect conservation of the disulfide bond also suggests that it contributes significantly to the stability of the Ig-fold. Indeed, mutagenesis experiments in the antibody McPC603, in which each of the cysteine residues of the variable domains has been mutated alone and in combinations, have shown that in no case were functional antibodies obtained, not in the case of a Fv, a scFv or a Fab fragment (Glockshuber *et al*., 1992).

From the study of a number of proteins in which a natural disulfide bond has been removed or a new disulfide bond has been introduced (Wetzel, 1987), it can be deduced that the disulfide bond generally stabilizes the protein, with the exception of some engineered examples. This stabilization has been suggested to be due to decreased entropy loss in folding (Flory, 1956) or, to enthalpy gain (Doig & Williams, 1991), but a review of the available data suggests that both models may apply but in different proteins (Betz, 1993). The predictability of the size of the stabilizing effect of a disulfide bond, if any, is still very poor, because of the complexity of the problem (Betz, 1993). Due to the large sequence variability of antibody variable domains, both through variations in the framework and changes in the CDRs, it is inevitable that the stability of antibody fragments covers a certain range. It may therefore not be surprising that some variable domains tolerate the removal of a disulfide bond and others do not.

While it is barely conceivable that the loss of a disulfide bond could be of any advantage, a few antibodies have apparently substituted one cysteine residue, even though for the majority of the sequences found lacking a cysteine residue in the Kabat database, the functionality of the protein has not been demonstrated. A monoclonal antibody isolated with a missing disulfide bond must at least tolerate the removal of the crosslink, since in the normal procedure of making hybridomas, it must survive two selection steps, one *in vivo*, where a continued antigen-stimulation of the B-cell is required for clonal expansion (Leanderson *et al*., 1992), the other *in vitro*, where hybridomas are screened for antigen binding.

Disulfide-lacking sequences might be useful as a starting point for engineering of antibody frameworks with improved thermodynamic stability by reconstruction of the lost disulfide bond, and they allow insight into the general question of construction of more stable frameworks. Furthermore, disulfide-free antibodies would themselves be of great interest as intracellular reagents (Richardson & Marasco, 1995; Biocca & Cattaneo, 1995), and finally, they might even open new production avenues, by folding efficiently in the cytoplasm.

To investigate these questions, we decided to study the antibody ABPC48, which naturally carries the C92Y mutation in the heavy chain. This antibody was originally found as a myeloma protein (Lieberman *et al*., 1975) and was shown to bind its antigen levan, a homopolymer of D-fructose with predominantly β-(2 → 6) glycosidic linkages (Feingold & Gehatia, 1957). By Edman degradation, Rudikoff & Pumphrey (1986) demonstrated that the functional antibody contains a heavy chain with tyrosine replacing the normal cysteine residue. The partial cDNA sequence (Auffray *et al*., 1981) confirmed this mutation, and established the close relationship to other levan and inulin-binding antibodies, all containing the disulfide bond in question. Thus, the C92Y substitution must have arisen by somatic mutation in this member of the V_HX24 family (Bonilla *et al*., 1990).

We studied the antibody ABPC48 *in vitro* and in *Escherichia coli*, in different scFv formats. To understand the energetic consequences of the disulfide bond, we also restored it, by converting the tyrosine residue back to cysteine and measured the free energy of folding. By analyzing the behavior of ABPC48 in a variety of experiments, we are led to the surprising conclusion that the unpaired cysteine residue at position H22 in ABPC48 must be freely accessible to very large probes.

Results

Construction of scFv fragments

The synthetic V_L and V_H genes of ABPC48 were designed by backtranslation from the amino acid sequences, derived from cDNA. For V_L, the complete sequence was available (Legrain & Buttin, 1985), whereas the V_H sequence started at position H32 (Auffray *et al*., 1981). The sequence from residues H1 to H31 was assumed to be similar to the highly homologous levan-binding antibody U10. At position H31, most fructosan-binding antibodies contain Arg or Lys, while U10 contains Gly (Victor-Kobrin *et al*., 1990). We chose Gly, because U10 also binds levan, and indeed the recombinant proteins produced in this study were all able to bind levan.

For the investigation of the disulfide-free V_H of ABPC48, initially a scFv fragment in the V_L-$(Gly_4Ser)_3$-V_H format was constructed by complete gene synthesis. Although soluble protein was expressed in the *E. coli* periplasm, isolation of monomeric protein was not possible (data not shown). V_L and V_H were also put into the V_H-$(Gly_4Ser)_3$-V_L format, but still only insufficient amounts of monomeric protein could be obtained. Since dimerization or oligomerization of scFv fragments may be caused by too short a peptide linker (Desplancq *et al*., 1994), we changed the linker length in the V_H-linker-V_L construct to 30 residues, resulting in a V_H-$(Gly_4Ser)_6$-V_L construct, which yielded milligram amounts of monomeric scFv protein. This construct was used for scFv purification and characterization. To create the disulfide-restored variant of ABPC48, the ''back-mutation'' Y92C was introduced. We refer to the original, disulfide-lacking sequence as ABPC48 and to the disulfide-restored one as ABPC48-cys.

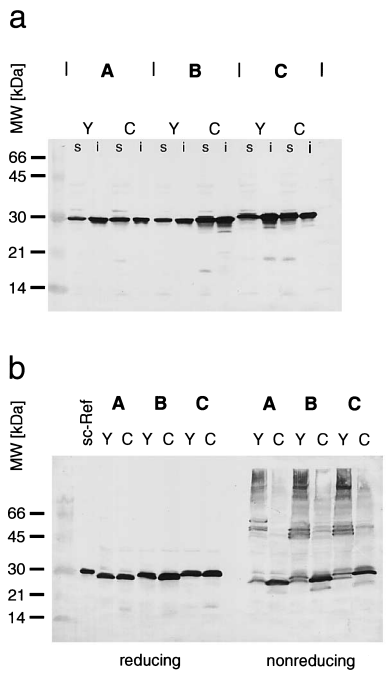

Figure 1. Western blots of samples from small-scale periplasmic expression cultures of the different scFv constructs. Y denotes ABPC48, which carries a Tyr at H92, and C denotes ABPC48-cys, which carries a Cys at H92. a, Samples of soluble (s) and insoluble (i) fractions after SDS-PAGE under reducing conditions; b, samples of soluble fractions after SDS-PAGE under reducing and non-reducing conditions. A, V_L-$(Gly_4Ser)_3$-V_H; B, V_H-$(Gly_4Ser)_3$-V_L; C, V_H-$(Gly_4Ser)_6$-V_L; scRef, purified ABPC48-cys scFv fragment (V_H-$(Gly_4Ser)_6$-V_L construct).

Expression and protein purification

All three different scFv constructs, V_L-$(Gly_4Ser)_3$-V_H, V_H-$(Gly_4Ser)_3$-V_L and V_H-$(Gly_4Ser)_6$-V_L, yielded soluble ABPC48 scFv protein in periplasmic expression, and in all three cases the ratio of soluble to insoluble protein was significantly improved by the disulfide restoration (Figure 1a). However, only for the ABPC48-cys constructs, functional scFv fragments could be detected by ELISA in crude extracts of the expression cultures (data not shown). Analysis of expression samples by SDS-PAGE under non-reducing conditions revealed that the disulfide-lacking ABPC48 scFv was mainly dimerized or linked to other periplasmic proteins by disulfide bonds (Figure 1b). This may be the reason why preparation of ABPC48 protein from the *E. coli* periplasm was not possible, even though soluble protein was seen on reducing gels. Thus, a cytoplasmic expression system involving *in vitro* refolding had to be chosen (see below) to produce the protein.

In contrast, the disulfide-restored scFv protein could be expressed in functional form in the *E. coli* periplasm and purified to homogeneity from crude extracts by immobilized metal ion affinity chromatography, using the C-terminal His_5-tag, followed by antigen affinity chromatography. Typical yields are given in Table 1. The disulfide-lacking ABPC48 protein was expressed under control of the T7 promoter in the *E. coli* cytoplasm, resulting in inclusion body formation. After refolding, pure protein could be obtained by antigen affinity chromatography. The formation of disulfide bonds in the refolding mixture was induced either by addition of a mixture of reduced and oxidized glutathione, or simply by air oxidation, catalyzed by addition of $CuSO_4$, as described in Materials and Methods. For the disulfide-lacking ABPC48 protein, the purification yields depended strongly on the oxidizing conditions (Table 1). Under standard conditions (0.2 mM GSH, 1 mM GSSG), about 50 mg of antigen affinity-purified protein could be obtained from one liter of *E. coli* culture, but only 2.5 mg using air oxidation. The disulfide-restored ABPC48-cys protein was also prepared from inclusion body protein. Interestingly, under the same oxidation conditions found to yield high amounts of functional ABPC48 protein, only 1.2 mg of ABPC48-cys per liter of culture could be obtained (Table 1). Changing the conditions by using different GSH/GSSG ratios or air oxidation did not significantly improve the refolding yields. Obviously, the formation of the disulfide bond in the ABPC48-cys V_H is problematic.

Protein characterization

To avoid any artifacts caused by scFv dimers or oligomers in ELISA measurements and unfolding,

Table 1. Purification yields of ABPC48 and disulfide-restored ABPC48-cys scFv fragments from expression and refolding conditions

Protein	Disulfide[a]	Expression[b]	Oxidation procedure[c]	Yield (mg/l)[d]
ABPC48	–	Cyto	0.2 mM GSH, 1 mM GSSG	50
ABPC48	–	Cyto	Air	2.5
ABPC48-cys	+	Peri	None, native	1.0
ABPC48-cys	+	Cyto	0.2 mM GSH, 1 mM GSSG	1.2
ABPC48-cys	+	Cyto	1.9 mM GSH, 0.1 mM GSSG	1.5
ABPC48-cys	+	Cyto	Air	1.5

[a] Presence of disulfide Cys22-Cys92 in V_H.
[b] Expression system refers to T7 system for cytoplasmic inclusion bodies or periplasmic secretion using a *lac* promoter.
[c] See Materials and Methods for details.
[d] The yields were normalized to mg protein per liter of bacterial culture.

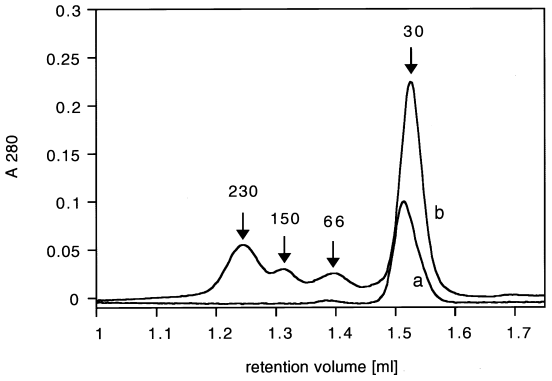

Figure 2. Gel permeation chromatography of purified ABPC48-cys scFv fragment on a Superose 12 column. a, Sample of scFv fragment; b, molecular mass standard.

purified scFv proteins were routinely checked by gel-permeation chromatography. In all cases the purified proteins were found to be monomeric. As an example, the chromatography of the periplasmically expressed disulfide-restored scFv fragment after purification by IMAC and antigen affinity chromatography is shown (Figure 2).

The functionality of ABPC48 and ABPC48-cys protein was compared by antigen-binding ELISA with immobilized levan and inhibition titration, using solubilized levan. An exact K_D determination is not possible with this homo-oligomeric antigen. As shown in Figure 3, both proteins exhibited almost identical inhibition curves, suggesting similar antigen affinity.

The presence of free sulfhydryl groups was tested using Ellman's reagent under denaturing conditions. As expected, no sulfhydryl could be detected in the disulfide-restored protein ABPC48-cys, but surprisingly, no signal was found for the ABPC48 protein either, which had been refolded in the presence of glutathione. To check the proteins for their identity and possible modifications, they were further characterized by electrospray mass spectrometry (ESMS).

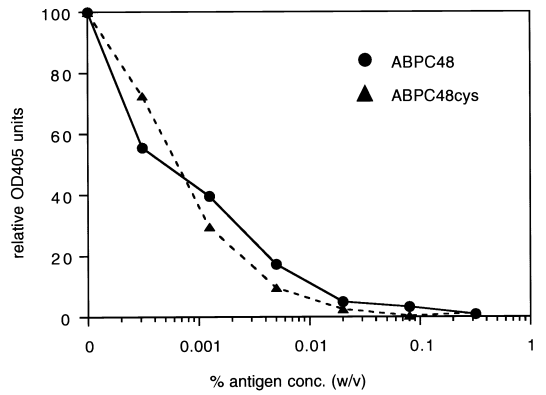

Figure 3. Relative ELISA signals of ABPC48 and ABPC48-cys scFv fragments, as a function of competing soluble antigen (hydrolyzed levan) concentration. The plate was coated with levan.

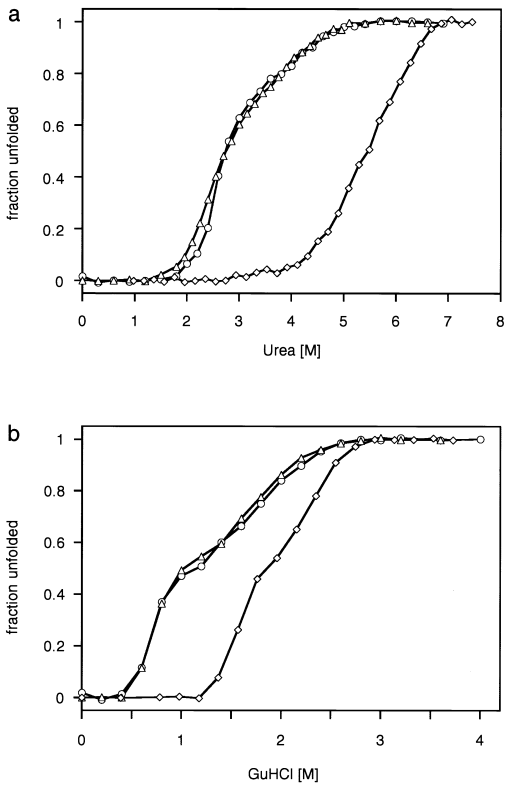

Figure 4. Equilibrium denaturation curves of different scFv fragments. (○) Refolded ABPC48, non-derivatized; (△) refolded ABPC48, GSH-derivatized; (◇) periplasmically expressed ABPC48-cys. a, Urea denaturation; b, GdnHCl denaturation.

The ABPC48-cys protein exhibited the expected mass, whereas the ABPC48 mass was found to be 305 mass units too high. Thus, the ABPC48 protein becomes derivatized by glutathione during the refolding process, but can nevertheless be purified by antigen affinity chromatography, has normal ELISA behavior and must therefore be functional. Further ESMS data revealed that ABPC48 protein, refolded in the absence of glutathione, is easily derivatized by a serine protease inhibitor (4-(2-amino-ethyl)-benzene sulfonyl fluoride), which was routinely added to the purified protein solutions. ABPC48 protein with the expected mass could be obtained by reducing the glutathione-derivatized protein under mild conditions with DTT and repurification using the same antigen affinity chromatography. Thus, Cys22, which is normally deeply buried as part of the disulfide bond to Cys92, is accessible in this protein, although the scFv fragment maintains antigen binding.

The stability of ABPC48 and disulfide-restored ABPC48-cys proteins was compared by urea and guanidinium equilibrium denaturation experiments. The relative behavior of the proteins was independent from the denaturant used (Figure 4). Interestingly, ABPC48 derivatized by glutathione and non-derivatized ABPC48 protein exhibit identical denaturation curves, suggesting that the structure of this protein is not disturbed by the

additional three amino acid residues of glutathione, covalently attached to Cys22. This is not compatible with ''burying'' glutathione in the domain core and demands that it is solvent-exposed and, by implication, so is the sulfur atom of Cys22.

For the scFv constructs used in this investigation, the equilibrium denaturation is clearly not consistent with a two-state model. The plateau at intermediate denaturant concentration is more pronounced in the guanidinium-induced denaturation than in that induced by urea. As is clearly visible in Figure 4 for both denaturants, the stability of the disulfide-restored ABPC48-cys protein is significantly increased over that of the disulfide-lacking ABPC48. An exact quantification of ΔG_u values for the whole scFv fragments is not possible, because of the lack of two-state behavior of the equilibrium denaturation and the poorly defined intermediate plateaus. By comparison with the denaturation midpoints of other scFv fragments, we may guess the ΔG_u values to be about 3 to 3.5 kcal/mol for the ABPC48 scFv and about 6 to 6.5 kcal/mol for the ABPC48-cys construct. This difference is in the range that can be expected by addition of a disulfide bond as a stabilizing structural element (Wetzel, 1987; Betz, 1993). Considering measured ΔG_u values of about 5 kcal/mol for several scFv fragments (Pantoliano *et al*., 1991; Knappik *et al*., 1993), the stability of the disulfide-restored mutant scFv fragment of ABPC48 is above average, explaining why it may tolerate the loss of a disulfide bond.

Model structures

Using homology modeling, different models of the ABPC48 V_H domain were built (Figure 5). All amino acid substitutions leading to the disulfide-restored V_H domain of ABPC48-cys could be accommodated without problems, leading to a model whose stereochemical quality (as judged by the program PROCHECK; Laskowski *et al*., 1993) was comparable with that of the template structure. The substitution of Cys92 by tyrosine in a ''classical'' model, which conserves the main-chain conformation of the disulfide-bonded template structure and buries Cys22, could be accommodated through minor rearrangements of the core packing. However, such a model is clearly not able to accommodate the extension of the unpaired cysteine residue by glutathione or 4-(2-aminoethyl)-benzene sulfonyl fluoride, nor is it compatible with the high level of reactivity of the free sulfhydryl group.

In the ''alternative'' model, the side-chain OH group of Tyr92 might insert into the H-bond between HN of Cys22 and the main-chain carbonyl group of Leu78, thus stabilizing a local distortion of strand b (Figure 5C), with a potential additional H-bond from the HN of Leu78 to the tyrosyl OH group and from the tyrosyl OH group to the CO of Leu78. The H-bond between HN of Leu78 and CO of Cys22 would be weakened or broken, but may be replaced by water or by a H-bond from Lys75 to the CO of Cys22. This might allow the main-chain to be sufficiently rotated for the cysteine side-chain to be facing outwards, especially since the small side-chains of Ala23 and Ala24 would be less likely to produce steric conflict than the larger side-chains found in many other antibodies in these positions.

In this model, the torsion angles of Cys22 are placed into the ''generously allowed'' region (as defined in PROCHECK), while the torsion angles (Table 2) of neighboring residues are all in the allowed or most favored regions. Such a model could explain the observed accessibility of the unpaired cysteine residue with minimal disturbance of the immunoglobulin structure, which must remain intact since both ABPC48 and ABPC48-cys are able to bind the antigen (Figure 3). While in a ''classical'' model, the volume of both the tyrosine side-chain and the remaining cysteine side-chain would have to be accommodated in the core of the domain, necessitating some rearrangement of other core residues, in the alternative model, the tyrosine side-chain fills the space normally occupied by the disulfide bridge, hardly requiring any additional volume. Since Cys22 lies in the outer sheet of the scFv fragment and faces outwards, even large molecules can be coupled to this residue without disturbing the overall structure or the V_H/V_L dimer interface, or interfering with antigen recognition.

Discussion

Disulfide bonds in antibodies

The disulfide bond between the b-strand and the f-strand in the immunoglobulin domain is remarkably well conserved, and has been maintained in all known germline genes of all antibodies. Only very few antibody sequences have been reported in which one of the cysteine residues is mutated, most likely by somatic mutation. One of the reasons for this conservation appears to be the relatively low stability of the domain, which then requires additional stabilization by the disulfide. It follows that more stable domains may tolerate the removal of the disulfide and, conversely, that those that do tolerate removal may be intrinsically more stable than average.

Of the sequences reported in the Kabat database, only 17 out of 3355 human and murine V_H sequences are missing one of the two cysteine residues forming the disulfide bond, and 18 out of 2985 V_L sequences. To our knowledge, ABPC48 is the first antibody with a naturally missing disulfide that has been investigated for folding and stability (Rudikoff & Pumphrey, 1986). We must propose that the unpaired cysteine residue becomes solvent-exposed in order to explain that this residue can be modified without loss of function. By this conformational change, new hydrogen bonds are formed according to the model, thus probably compensating for the loss of free energy from the missing disulfide.

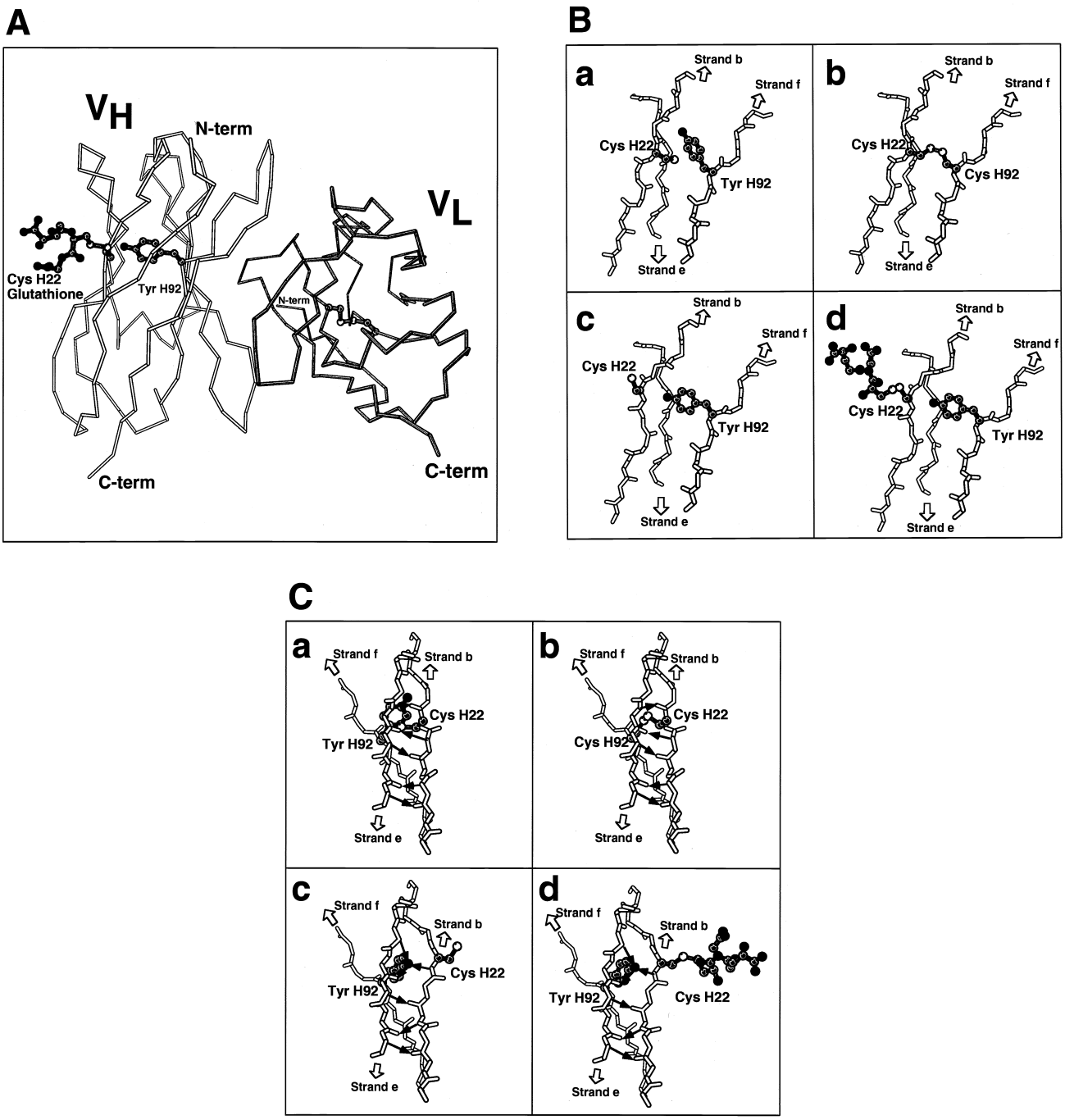

Figure 5. A, Overview of the alternative model of ABPC48 with cysteine H22 derivatized by glutathione. B, Detail: a, classical model of ABPC48; b, disulfide-restored mutant ABPC48-cys; c, alternative model; d, alternative model with cysteine H22 derivatized by glutathione. C, Hydrogen-bonding pattern: a, classical model of ABPC48; b, disulfide-restored mutant ABPC48-cys; c, alternative model; d, alternative model with cysteine H22 derivatized by glutathione.

Besides the cysteine H92 to tyrosine mutation, the other amino acid substitutions (Arg, Gly, Phe, Ser and Trp) that can be expected by a single base substitution of cysteine codons have all been observed in this position. In the light chain position L23, the Cys to Tyr substitution is by far the most frequently observed. However, in most cases the functionality of the proteins has not been demonstrated. While we do not know whether this inversion of the Cys22 conformation is particular to ABPC48 or occurs in several other antibodies as well, the present analysis of ABPC48 shows that the disulfide bond can be functionally replaced in a more imaginative way than by merely filling the hole left by the missing cysteine residue. Thus, even if an energetically favorable isosteric substitution or set of substitutions of the cysteine residue may not be possible, other solutions may exist to restore

Table 2. Torsion angles of the main-chain atoms of the β-strands b, e and f, shown in Figure 5B

Main-chain positions	Phi (deg.) Y	C	2fbj	Psi (deg.) Y	C	2fbj	Omega (deg.) Y	C	2fbj
A. *Strand b*									
H20	–108	–81	–74	114	119	151	–168	–172	174
H21	–170	–114	–123	145	101	147	151	176	179
H22[a]	–145	–89	–130	–92	118	103	–175	179	180
H23	–166	–88	–84	156	132	126	152	–172	179
H24	–77	–115	–105	76	122	140	–168	–173	172
B. *Strand e*									
H76	63	58	64	23	56	27	–175	–169	–176
H77	–106	–136	–109	131	133	141	164	179	159
H78	–91	–109	–100	126	123	143	–173	–168	–178
H79	–104	–111	–128	140	112	158	167	168	177
H80	–135	–95	–140	109	102	103	171	172	173
C. *Strand f*									
H90	–105	–98	–105	132	109	116	–167	–177	178
H91	–124	–99	–100	138	129	132	159	166	154
H92[a]	–74	–91	–86	98	116	148	–180	–175	177
H93	–102	–106	–140	99	121	137	–162	–165	174
H94	–124	–114	–86	156	169	135	176	172	–175

Y, Alternative model of ABPC48, which carries a tyrosine residue at H92, and Cys22 exposed to solvent. C, Model of ABPC48-cys, which carries a cysteine residue at H92. 2fbj, Template structure for model building.
[a] Indicates the cysteine positions.

some of the energy loss caused by the missing disulfide, as found in the natural antibody ABPC48.

Since the disulfide bond appears to contribute significantly to stability, folding of antibodies under reducing conditions does not normally lead to functional protein. There is, however, great interest in the expression of antibodies inside the cell (''intrabodies'': Richardson & Marasco, 1995; Biocca & Cattaneo, 1995) to act as a protein analog of antisense RNA. In the cytoplasm, the cell takes special measures to keep a reducing milieu, at the expense of NADPH (Holmgren, 1985). Nevertheless, some biological activity of whole antibodies or fragments is often seen, despite this apparent contradiction. Biocca *et al*. (1995) recently showed that antibodies in the cytoplasm indeed form no disulfides, and the residual activity seen is most likely a crossreactivity of partially folded molecules with the antigen. Thus, it would be desirable to properly design molecules that fold to the native structure under reducing conditions, and have sufficient stability. The analysis of these naturally occurring antibodies may be the first step along the way towards this goal.

The unpaired cysteine problem

While in the laboratory both cysteine residues can be easily removed, the simultaneous mutation of both sites would be an extraordinarily rare event in somatic mutations of antibodies in an immune response. Thus, all natural antibodies that have lost a disulfide bond in the variable domains carry an unpaired cysteine residue. This may have a very different consequence in higher cells *versus E. coli*.

It was not possible to detect any functional scFv protein of the original ABPC48 sequence in the *E. coli* periplasm, but functional whole antibody could be isolated from ascites (Vrana *et al*., 1976). This is not merely a function of the scFv format, since no functional ABPC48 Fab fragment could be obtained from the bacterial periplasm (data not shown). We observed several dominant bands of scFv protein crosslinked to other proteins *via* disulfide bonds on SDS-PAGE under non-reducing conditions. The main disulfide-forming machinery in the *E. coli* periplasm consists of the proteins DsbA, which is present in an oxidized state and is continuously reoxidized by the membrane-bound DsbB (Bardwell *et al*., 1993), and the presumed disulfide isomerases DsbC and perhaps DsbD (DipZ), but no glutathione is present. Since DsbA is such a strong oxidant, it will crosslink to the cysteine residues to form a mixed disulfide. In the normal course of events the second cysteine residue to be engaged in the disulfide bond will displace DsbA, which becomes reduced in the process. If the ''normal'' partner of the activated cysteine residue is missing, another cysteine residue from this or another protein may displace DsbA, thereby forming a wrong disulfide and a misfolded, aggregation-prone product. An incorrect disulfide bond would be resolved by the isomerase DsbC and perhaps other factors. In the present case, however, there is no partner for Cys22.

In the endoplasmic reticulum, the formation of disulfide bonds is carried out by protein disulfide isomerase (PDI) in the presence of glutathione (Freedman, 1995). In cysteine-lacking mutants of lysozyme expressed in yeast, it was found that some mutants carried a covalently bound glutathione residue (Taniyama *et al*., 1990). If our *in vitro* refolding results for the ABPC48 sequence are taken into account, it seems possible that this derivatization also happens to the whole antibody, expressed in the eukaryotic system. There

is no information available about the state of the single cysteine residue in V_H of ABPC48 isolated from ascites, however, and therefore this remains speculative.

Folding and structure of ABPC48

While the model-building of ABPC48-cys with the restored disulfide led to a satisfactory model by all criteria (see Results), accommodating the Cys to Tyr exchange without significant alteration of the main-chain conformation required some adjustments in the core packing. More importantly, however, several lines of experimental evidence demand a structural change that exposes the sulfur atom of Cys22. First, the scFv protein is a monomer and can be antigen affinity purified even if Cys22 is derivatized by glutathione. Second, the glutathione moiety can be removed by mild reduction, apparently not changing the properties of the protein. Third, in equilibrium unfolding studies, no difference between the presence and absence of glutathione in the molecule is visible, which precludes its disturbing or even stabilizing the hydrophobic core in any way.

We thus propose that in the ABPC48 structure, Cys22 is exposed as detailed in our model (Figure 5). During folding, a mixed disulfide with glutathione is formed that survives into the native state since it is not attacked by Cys92, which is absent. Interestingly, if Cys92 is present, the folding yield drops dramatically under otherwise identical conditions (Table 1), even though the final product is much more stable (Figure 4). In this ''canonical'' form with both cysteine residues present, the glutathione-derivatized Cys22 still might be present in the alternative conformation, but we have not isolated any functional ABPC48-cys protein with one, or even two glutathione moieties. Rather, all affinity-purified ABPC48-cys scFv had the molecular mass expected from the sequence. This strengthens the hypothesis that Tyr92 has an active role in stabilizing the alternative conformation of ABPC48, and ABPC48-cys has the canonical structure of all V_H domains.

It might be argued that for the non-derivatized ABPC48 protein we cannot formally rule out a dynamic equilibrium between the alternative and the canonical conformations, which would involve the opening and closing of the hydrogen bonds between strands b and e, to allow the cysteine side-chain to flip in and out. However, the denaturation curves of the glutathione-derivatized and the non-derivatized protein are virtually identical. If the non-derivatized protein existed in two forms in dynamic equilibrium, they would have to be exactly of the same energy, so that the derivatization of the cysteine residue makes no difference. Furthermore, the energy barrier between the two distinct conformations would be expected to be high enough to observe distinct populations of derivatized and non-derivatized protein after purification and mass spectrometry, which was not the case. Therefore, we have no evidence for a dynamic equilibrium.

Conclusions

We believe that by the further analysis of this and similar systems, a deeper understanding of immunoglobulin folding can be achieved, leading to the design of disulfide-free and/or more stable frameworks, expanding the range of applications and recombinant production avenues. Further analysis of naturally occurring disulfide mutants may uncover the critical prerequisites to eliminate the most crucial component of the immunoglobulin fold.

Materials and Methods

Molecular biology and expression

Gene synthesis

In a variation of the method of Prodromou & Pearl (1992), the scFv gene in the orientation V_L-$(Gly_4Ser)_3$-V_H was synthesized by recursive PCR. The gene was assembled from a mixture of 20 oligonucleotides, encoding the complete sequences of both strands. The oligonucleotides varied in length from 59 to 81 nt and were designed to produce overlaps of about 16 nt (Figure 6b). For amplification, two additional short primers (20 and 19 nt) were added in 20-fold molar excess. To perform the PCR assembly reaction, the oligonucleotides (2 pmol each), end primers (40 pmol each), dNTPs (0.2 mM), 10× Vent-buffer and 2.5 units of Vent DNA polymerase (NEB) were mixed in a volume of 50 µl. PCR was carried out over 25 cycles (one minute at 94°C, two minutes at 58°C, one minute at 72°C).

scFv constructs and expression vectors

The different scFv constructs tested in our investigation are summarized in Figure 7. The V_L-$(Gly_4Ser)_3$-V_H construct was initially obtained by total gene synthesis (see above) and inserted into vector pIG6 (Ge *et al*., 1995) allowing expression of the gene under control of the *lac* promoter. Export of the protein to the *E. coli* periplasm is initiated by the *ompA* signal sequence. The FLAG,

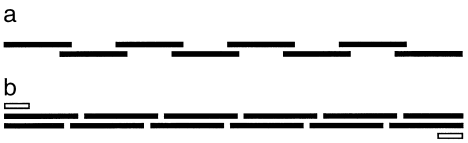

Figure 6. Schematic representation of oligonucleotide assembly in PCR-mediated gene synthesis. The oligonucleotides making up the gene are given in black and the PCR primers in white. a, Assembly following the method of Prodromou & Pearl (1992). Because of the presence of a certain percentage of single-base deletion molecules in the oligonucleotides even after gel purification, a large fraction of seqenced clones contain a single-base deletion somewhere in the gene. b, Oligonucleotide assembly as it was used in this work. Note that the whole gene is encoded by a double strand. In our hands it appears that this method is more robust against sequence errors.

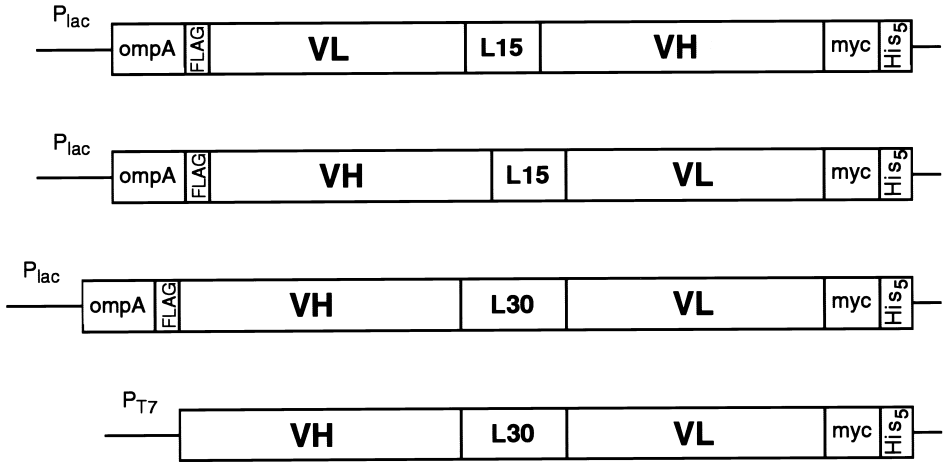

Figure 7. A representation of the different scFv constructs used in this work.

myc tag and His_5 tag sequences are contributed by the vector and are useful for detection and purification purposes. Construct V_H-$(Gly_4Ser)_3$-V_L was prepared by inserting V_H and V_L separately into vector pLiscSEF (Knappik *et al*., 1993), using standard cloning methods. Construct V_H-$(Gly_4Ser)_6$-V_L was produced by elongating the linker in the former construct by oligonucleotide cassette mutagenesis. For cytoplasmic expression, the V_H-$(Gly_4Ser)_6$-V_L gene was amplified by PCR without the signal sequence and inserted into vector pTFT74 (Freund *et al*., 1993), which places expression under control of the T7 promoter. The FLAG sequence was omitted in this construct because it is not useful for detection, due to the additional N-terminal methionine residue. Periplasmic expression was carried out in strain *E. coli* JM83 (λ^-, *ara*, Δ(*lac*, *proAB*), *rpsL*, *thi*, ϕ80, *dlacZ*ΔM15; Yanisch-Perron *et al*., 1985) and for cytoplasmic expression strain *E. coli* BL21DE3 (F^-, *ompT*$^-$, $r_B^- m_B^-$ (λimm21, *lac*I, *lac*UV5, T7 pol, *int*); Studier & Moffatt, 1986) was used.

Small-scale expression

LB media (2 ml) containing 100 µg/ml ampicillin and 0.5% (w/v) glucose were inoculated with a single bacterial colony and incubated overnight at 25°C. Then 10 ml of SB media (20 g/l tryptone, 10 g/l yeast extract, 10 g/l NaCl, 50 mM K_2HPO_4) containing 100 µg/ml ampicillin (in a 100 ml flask) were inoculated with 0.5 ml of the overnight preculture and incubated at 25°C with shaking at 200 rpm. Expression was induced by addition of IPTG to a final concentration of 0.5 mM at an A_{550} = 0.5 and incubation was continued for another six hours. Cultures were centrifuged (5000 ***g***, ten minutes at 4°C) and cell pellets suspended in BBS (50 mM borate, 150 mM NaCl, pH 8), normalizing the cell densities of the samples to an A_{550} = 20. Cell disruption was achieved by French Press lysis, and the resulting crude extracts were centrifuged (15,000 ***g***, ten minutes, 4°C). The supernatant was collected (soluble fraction) and the pellets suspended in BBS (insoluble fraction).

Western blots

Samples of soluble and insoluble fractions (see above) were submitted to SDS-PAGE, and gels were blotted on PVDF membranes. For immunodetection, the monoclonal anti-FLAG antibody M1 (Kodak) (1:10,000 in TBST (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween-20), 60 minutes at room temperature) or the monoclonal anti-myc antibody 9E10 (1:5000 in TBST; Munro & Pelham, 1986) were used, followed by incubation with a polyclonal anti-mouse-peroxidase conjugate (Pierce; 1:5000 in TBST, 60 minutes at room temperature).

Protein purification

Periplasmic expression

LB media (20 ml containing 100 µg/ml ampicillin, 0.5% glucose) were inoculated with a single bacterial colony and incubated overnight at 25°C. One liter of SB media (100 µg/ml ampicillin) was inoculated with the preculture and incubated at 25°C (5 l flask, 180 rpm). Expression was induced at an A_{550} = 0.5 by addition of IPTG to a final concentration of 0.5 mM. Incubation was continued for about five to six hours until the culture reached stationary phase. Cells were collected by centrifugation (8000 ***g***, ten minutes at 4°C), suspended in 25 ml of MHA buffer (33 mM Mes, 33 mM Hepes, 33 mM sodium acetate, pH 7.5) and disrupted by French Press lysis. The crude extract was centrifuged (48,000 ***g***, 60 minutes at 4°C), the supernatant passed through a 0.2 µm filter and directly applied to IMAC chromatography (see below).

Cytoplasmic expression

One liter of SB media (100 µg/ml ampicillin) was inoculated with the preculture and incubated at 37°C (5 l flask, 180 rpm). Expression was induced at an A_{550} = 1.5 to 2 by addition of IPTG to a final concentration of 0.5 mM. Incubation was continued for about four hours. After centrifugation (8000 ***g***, ten minutes, 4°C), cells were suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 2 mM $MgCl_2$ and disrupted by French Press lysis. Inclusion body protein was isolated following a standard protocol (Buchner & Rudolph, 1991).

The inclusion body protein pellet from 1 l of bacterial culture was solubilized at room temperature in 50 ml of solubilization buffer (0.2 M Tris-HCl (pH 8 at room temperature), 6 M guanidinium hydrochloride (Gdn-HCl), 10 mM EDTA), containing 50 mM DTT. The

resulting solution was centrifuged (48,000 *g*, ten minutes at 4°C) and the supernatant dialyzed extensively against solubilization buffer to remove DTT. A portion (10 ml) of this solution was diluted in 1 l of refolding buffer (0.2 M Tris-HCl (pH 9.0 at 5°C), 0.8 M arginine, 2 mM EDTA, 0.5 mM ϵ-amino-caproic acid, 0.5 mM benzamidinium·HCl), incubated for two to ten days at 4°C and then applied to antigen affinity chromatography (see below). The formation of disulfide bonds was catalyzed either by the presence of reduced and oxidized glutathione in the refolding buffer (standard molar ratio of GSH to GSSG was 0.2:1.0, but other ratios were tested), or simply by air oxidation, catalyzed by 5 μM $CuSO_4$.

IMAC chromatography

The crude extract from periplasmic expression (see above) was loaded onto an imino-diacetate (IDA) column (column volume 1.66 ml, flow-rate 10 ml/minute), equilibrated with Mes-Hepes-acetate (MHA) buffer, on a BIOCAD Workstation (Perseptive Biosystems). After washing with ten column volumes of MHA buffer containing 500 mM NaCl, ten containing 10 mM NaCl, ten containing 150 mM NaCl and 2 mM imidazole, and five containing 150 mM NaCl and 10 mM imidazole, bound scFv protein was eluted in three column volumes of MHA buffer containing 150 mM NaCl and 100 mM imidazole.

Antigen affinity chromatography

To prepare the affinity matrix, levan from *Erwinia herbicola* (Sigma) was hydrolyzed under mild conditions (acetate buffer (pH 4), 60°C, 60 minutes) and then precipitated by adding ethanol (50% (v/v) final concentration). The pellet was solubilized in 0.2 M NaOH, 0.2 M KOH (pH 12.5) and coupled to epoxy-activated Sepharose 6B (Pharmacia) following the supplier's instructions. Refolding mixtures or scFv protein containing fractions from IMAC chromatography were directly loaded onto the affinity column (column volume 12 ml, equilibrated with borate-buffered saline (BBS), flow-rate 1 ml/minute). After washing with BBS, bound scFv protein was specifically eluted with acid-hydrolyzed antigen (0.2% (w/v) in BBS). Acid-hydrolyzed antigen solution was prepared by incubating bacterial levan for 60 minutes at pH 2 and 70°C, following neutralization with BBS. The scFv eluates were dialyzed against BBS (membrane cutoff 10,000 Da) to remove antigen, and concentrated. Yields were calculated from the A_{280} values, using calculated extinction coefficients (Gill & von Hippel, 1989).

Protein characterization

Gel permeation chromatography

Samples of purified and concentrated scFv proteins were analyzed on a Superose 12 or a Superdex 75 column, equilibrated with BBS, on a SMART-system (Pharmacia). The sample volumes were usually 20 μl (containing about 2 to 5 μg of protein) and the flow-rate was 50 μl/minute. Lysozyme (14 kDa), carbonic anhydrase (31 kDa), bovine serum albumin (66 kDa), aldolase (150 kDa) and catalase (230 kDa) were used as molecular mass standards.

Ellman's assay

An aliquot (100 μl) of protein solution (about 60 to 80 μg) was mixed with 500 μl of assay buffer (0.3 M Tris-HCl (pH 8), 1 mM EDTA, 6 M GdnHCl) and 3 μl of dithio-bis(nitrobenzoic acid) stock solution (10 mM in 0.3 M Tris-HCl (pH 8), final concentration 50 μM). After 15 minutes incubation at room temperature, the A_{405} value was measured.

Antigen binding ELISA

ELISA plates (Nunc) were coated with a solution of levan from *E. herbicola* (Sigma) (10 μg/ml in TBS (25 mM Tris-HCl (pH 7.5), 150 mM NaCl)) overnight at 4°C and blocked with 4% low-fat milk. Samples of ABPC48 and ABPC48-cys scFv protein (200 ng in 100 μl of TBS) were premixed with different amounts of solubilized bacterial levan and incubated on levan-coated ELISA plates for 60 minutes at room temperature. Bound scFv protein was detected using the monoclonal anti myc-tag antibody 9E10 and a polyclonal anti-mouse/peroxidase conjugate (Pierce).

Electrospray mass spectrometry

Data were collected on a Sciex API III$^+$ mass spectrometer. The ion spray voltage was 5000 V, the dwell time 0.7 ms and the scan step size 0.15 amu. The samples (10 pmol/μl scFv protein in 10 mM ammonium acetate) were injected into the ion source at a flow-rate of 7 μl/minute.

Equilibrium denaturation

Sample preparation

Stock solutions of urea and GdnHCl (about 9 M and 7 M) were prepared in BBS and exact concentrations were determined from refractive indices. ScFv protein/denaturant-mixtures (2 ml) contained a final protein concentration of 5 μg/ml and denaturant concentrations varying from 0 to 4 M (GdnHCl) or 0 to 8 M (urea). Pipetting was monitored on an analytical balance to minimize concentration errors. After overnight incubation at 10 °C, the fluorescence emission of the samples was recorded.

Fluorescence spectroscopy

Emission spectra of the equilibrated samples were recorded from 320 to 370 nm, at an excitation wavelength of 280 nm, with a Shimadzu fluorescence spectrometer (model RF-5000). A 3 ml cuvette was used, which allowed stirring of the sample during measurement, and the temperature was kept constant at 20°C, using a temperature-controlled waterbath connected to the cuvette holder. For every sample, five spectra were recorded and averaged.

Data evaluation

With increasing concentrations of denaturant, the maxima of the recorded emission spectra shifted from about 336 to 348 nm. The maxima of the averaged spectra were determined by fitting a Gaussian function, and the unfolded fraction of scFv protein, depending on denaturant concentration, was calculated according to Pace (1990).

Model building

For model building, the InsightII (Homology module) and Discover program packages (Version 95/3.0, MSI) were used. Molecular dynamics and energy minimization were performed using the cff91 forcefield of the Discover program and a distance-dependent dielectric constant. Charges were assigned for a protonation state corresponding to an assumed pH of 7.4. The models of the V_H domain were predominantly based on the X-ray structure of the galactan-binding antibody J539 (Brookhaven Protein Data Base entry pdb2fbj, 1.95 Å resolution), which showed 86.3% sequence identity with the V_H-domain of ABPC48 (90.8% identity if CDR3 is omitted from the comparison). All substitutions leading to the model of the disulfide-containing mutant of ABPC48 could be accommodated without problems. After substitution of the disulfide bridge by a cysteine-tyrosine pair (classical model of the ABPC48 V_H-domain), bad steric interactions resulted that could be relieved by manual rotamer search for the substituted residues and for clashing side-chains, followed by energy minimization. To generate the alternative model, the conformation of residues H21 to H23 were altered manually in such a way that Cys22 faced outward. The model was annealed by a short molecular dynamics run at 300 K. For the first 20 ps, the C^{α} positions of all beta-strands except strand b were tethered to their initial positions, allowing the altered strand and the loop regions to relax. For a further 50 ps, these restraints were dropped. Every 5 ps, a sample structure was extracted and energy-minimized through 250 cycles of conjugate-gradient minimization.

Acknowledgements

We thank Dr P. Gehrig for mass spectrometry measurements.

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Edited by I. A. Wilson

(Received 23 July 1996; received in revised form 14 October 1996; accepted 15 October 1996)