

## Articles

The Disulfide Bonds in Antibody Variable Domains: Effects on Stability, Folding in Vitro, and Functional Expression in *Escherichia coli*<sup>†</sup>Rudi Glockshuber,<sup>‡</sup> Thomas Schmidt,<sup>§</sup> and Andreas Plückthun\*

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**ABSTRACT:** The formation of the disulfide bonds in the variable domains  $V_H$  and  $V_L$  of the antibody McPC603 was found to be essential for the stability of all antigen binding fragments investigated. Exposure of the  $F_v$  fragment to reducing conditions in vitro resulted in irreversible denaturation of both  $V_H$  and  $V_L$ . In vitro refolding of the reduced  $F_v$  fragment was only possible when the disulfide bonds were allowed to form under oxidizing conditions. The analysis of a series of mutants of the  $F_v$  fragment, the  $F_{ab}$  fragment and the single-chain  $F_v$  fragment, all secreted into the periplasm of *Escherichia coli*, in which each of the cysteine residues of the variable domains was replaced by a series of other amino acids, showed that functional antigen binding fragments required the presence of both the disulfide bond in  $V_H$  and the one in  $V_L$ . These results were also used to devise an alternative expression system based on the production of insoluble fusion proteins consisting of truncated  $\beta$ -galactosidase and antibody domains, enzymatic cleavage, and refolding and assembly in vitro. This strategy should be useful for providing access to unstable antibody domains and fragments.

**A**ntibodies are designed by nature to bind to a large variety of antigens, yet all use the same chain topology. The ability to manipulate these molecules opens the door to many applications of such engineered antigen binding molecules in basic research, biotechnology, and medicine. We have recently developed the methodology to produce antigen binding fragments of an antibody ( $F_v$ ,  $F_{ab}$ , and single-chain  $F_v$  fragments) in the native state in *Escherichia coli* (Skerra & Plückthun, 1988; Plückthun & Skerra, 1989; Glockshuber et al., 1990a; Plückthun 1990, 1991). This has made site-directed mutagenesis and access to altered fragments very convenient.

As a model system, we used the well-characterized antibody McPC603, an IgA from the mouse that binds phosphocholine. Its three-dimensional structure (Segal et al., 1974; Satow et al., 1986) as well as binding constants of various haptens (Perlmutter et al., 1984) have been determined. We have previously shown that the  $F_v$  fragment, the  $F_{ab}$  fragment, and the single-chain  $F_v$  fragment produced in *E. coli* have the same antigen binding properties as the whole antibody (Skerra & Plückthun, 1988; Glockshuber et al., 1990a; Skerra et al., 1991).

Disulfide bonds are one of the hallmarks of the antibody domain architecture (Alzari et al., 1988; Davies et al., 1990), and their strict conservation has even been used in the alignment of distantly related molecules of the immunoglobulin superfamily (Williams & Barclay, 1988). In each antibody domain, they connect both  $\beta$ -sheets of the  $\beta$ -barrel and are almost completely inaccessible to solvent molecules in the native structure of the antibody domain. The disulfide bonds found in antibodies have conformations typical for this type

of bond [reviewed by Alzari et al. (1988) and Davies et al. (1990)]. It is of great interest to examine their contributions to folding and stability. If the disulfide bonds were unnecessary for stabilization, expression of antibodies in reducing environments would become generally feasible, an exciting prospect for expressing antibodies in the cytoplasm of many types of cells, e.g., as a "molecular Trojan horse". Until now, at least one antibody has been found, in which the disulfide bond in  $V_H$ <sup>1</sup> is absent [caused by the exchange Cys(h96)Tyr (consecutive numbering)], and where it has also been demonstrated that the antibody still binds to the antigen (Rudikoff & Pumphrey, 1986; Victor-Kobrin et al., 1990). Other investigators proposed that functional antibodies assemble in the cytoplasm of mammalian cells (Biocca et al., 1990) and that  $F_{ab}$  fragment can be produced in the cytoplasm of yeast (Carlson, 1988; Tang, 1991). Cabilly (1989) proposed that at very high expression levels and low temperature, some  $F_{ab}$  fragment might assemble in the cytoplasm of *E. coli*, although all cytoplasmic compartments are generally assumed to be reducing (Gilbert, 1990). However, the proportion of correctly assembled molecules and the state of the disulfides before and after workup remained unclear in these experiments. Furthermore, the hypothesis of the existence of transport pathways in eukaryotes not dependent on "classical" signal sequences has been put forward (Muesch et al., 1990).

We therefore chose to study the importance of the disulfide bond for folding and stability in the well-characterized antibody McPC603. We investigated several amino acids substituted for each cysteine residue, the influence of the type of fragment in which it is expressed ( $F_{ab}$ ,  $F_v$ , or single-chain  $F_v$ ), and the localization and stability of the altered proteins in the cell. In the single-chain  $F_v$  fragment used here, the C-terminus of  $V_H$  is connected by the linker sequence (Gly<sub>4</sub>Ser)<sub>3</sub> to the N-terminus of  $V_L$  (Glockshuber et al., 1990a).

Furthermore, we have investigated the question of whether the oxidation of disulfide bonds is mandatory for domain

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<sup>1</sup> Abbreviations:  $V_L$ , variable domain of the light chain;  $V_H$ , variable domain of the heavy chain.

folding and stability. This was done by measuring the sensitivity of the folded molecule to reducing agents under native conditions, and by determining the yield of refolding under reducing and oxidizing conditions.

To gain general access to antibody fragments independently of their folding and binding properties for these physical studies, we also developed an expression system, in which the antibody domains are produced as fusion proteins to  $\beta$ -galactosidase, and can be cleaved and refolded to a completely native  $F_v$  fragment.

#### EXPERIMENTAL PROCEDURES

**Recombinant DNA Techniques.** Recombinant DNA techniques were based on Sambrook et al. (1989). The plasmid pLZPWB1 (Wetekam et al., unpublished results) was kindly provided by Dr. Wetekam, Hoechst AG, Frankfurt. It was constructed from the pUR series of plasmids (Rüther & Müller-Hill, 1983) and is similar to the pUC family of plasmids, but instead of the  $\alpha$ -fragment of the *lacZ* gene it contains a version of the *lacZ* gene encoding amino acids 1–46 fused to 600–1007, followed by a polylinker. Functional expression of the native and the mutated  $F_v$ ,  $F_{ab}$ , and single-chain  $F_v$  fragments in *E. coli* JM83 (Vieira & Messing, 1982) was carried out with vectors similar to pASK30 (Skerra et al., 1991). Site-directed mutagenesis was directly performed in these vectors according to Kunkel et al. (1987) and Geisselsoder et al. (1987) using the helper phage M13K07 (Vieira & Messing, 1987).

**Functional Expression of the  $F_v$ ,  $F_{ab}$ , and Single-Chain  $F_v$  Fragments in *E. coli*.** In preparative expression experiments, cells were grown in LB medium at room temperature. At an  $OD_{550}$  of 0.5, IPTG was added to a final concentration of 1 mM, and the cells were further grown for 3 h before harvest. The cells were then suspended in BBS buffer [200 mM borate/NaOH (pH 8.0)/160 mM NaCl; 1:100 of the culture volume], disrupted in a French pressure cell (18 000 psi), and purified by phosphocholine affinity chromatography as described previously (Glockshuber et al., 1990a).

**Expression of Fusion Proteins and Purification from Inclusion Bodies.** Cultures of *E. coli* W3110 (Bachmann, 1972) harboring either the plasmid pLZPWB1/Xa/VL or the plasmid pLZPWB1/Xa/VH were grown at 37 °C in 1 L of LB medium to an  $OD_{550}$  of 1.0 and induced with IPTG (final concentration 1 mM). The cells were grown for further 16 h, harvested, and suspended in 100 mL of 50 mM sodium phosphate, pH 7.0. They were disrupted in a French pressure cell (18 000 psi), and the lysates were centrifuged through 400 mL of 50 mM sodium phosphate, pH 7.0, containing 40% (w/v) sucrose (17000g, 1 h, 4 °C). The supernatants were discarded. The insoluble fusion proteins were suspended in 3 mL of 10 mM Tris-HCl, pH 8.3, and solubilized by dropwise addition to 50 mL of 8 M urea/10 mM Tris-HCl, pH 8.3. The mixture was again centrifuged (35000g, 30 min, 4 °C), and the supernatants were dialyzed twice against 5 L of 10 mM Tris-HCl, pH 8.3, at 4 °C. Typically, 75 mg of the purified, soluble fusion proteins was obtained from 1 L of *E. coli* at concentrations of about 1 mg/mL.

**Cleavage with Factor Xa.** The blood clotting factor X was purified to homogeneity from bovine blood and activated to factor Xa with Russell's viper venom protease according to Fujikawa et al. (1972a,b). The fusion proteins were cleaved with factor Xa (Nagai & Thøgersen, 1984, 1987) at an enzyme:substrate ratio of 1:75 in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 1 mM  $CaCl_2$  at 4 °C, at a substrate concentration of 0.5 mg/mL for 24–48 h. From 1 mg of the corresponding fusion protein, 100  $\mu$ g of correctly cleaved  $V_H$

and  $V_L$  was generally obtained.

**Reconstitution and Purification of the  $F_v$  Fragment.** The cleavage mixtures containing about equimolar amounts of  $V_L$  and  $V_H$  were combined. After addition of urea and 2-mercaptoethanol to a final concentration of 8 M and 1 mM, respectively, the solution was incubated for 1 h at 37 °C to reduce incorrectly formed disulfide bonds. It was subsequently dialyzed extensively against 4  $\times$  1 L of 8 M urea, 150 mM NaCl, and 50 mM Tris-HCl, pH 8.0, at 4 °C for 2 days to allow the formation of the intramolecular disulfide bonds in  $V_H$  and  $V_L$  by air-oxidation. The urea concentration was then reduced to 0.5 M by the addition of cold 150 mM NaCl/50 mM Tris-HCl, pH 8.0, containing 1 mM phosphocholine. After incubation for another 24 h at 4 °C, the solution was concentrated by ultrafiltration (Amicon YM10), dialyzed extensively against 150 mM NaCl/50 mM Tris-HCl, pH 8.0, and applied to a phosphocholine affinity column (Chesebro & Metzger, 1972). The native  $F_v$  fragment was eluted with 5 mM phosphocholine, 150 mM NaCl, and 50 mM Tris-HCl, pH 8.0.

**Quantitative Disulfide Analysis.** The formation of the intramolecular disulfides in  $V_L$  and  $V_H$  was analyzed according to Inglis et al. (1976). A solution of 10  $\mu$ g of the purified, refolded  $F_v$  fragment in 100  $\mu$ L of 8 M urea, 150 mM NaCl, and 50 mM Tris-HCl, pH 8.0, was mixed with 10.5  $\mu$ L of 4-vinylpyridine (final concentration 1 M) and incubated for 90 min at 20 °C under an argon atmosphere. An identical sample of the  $F_v$  fragment was completely reduced with 1 M 2-mercaptoethanol (30 min, 37 °C) before the addition of 4-vinylpyridine. The reactions were quenched by adding 50  $\mu$ L of formic acid. After addition of 900  $\mu$ L of  $H_2O$ , the samples were dialyzed extensively against  $H_2O$ , dried in vacuo, and hydrolyzed in 200  $\mu$ L of 6 M HCl for 24 h at 110 °C. The samples were dried again, and the amino acids were separated on a DC6A cation-exchange column (Durrum Chemical Corp.) using an automatic E6000 amino acid analyzer (Biotronic GmbH). The eluted amino acids were detected with ninhydrin.

**Protein Sequencing.** The sequencing of the  $NH_2$ -terminal amino acids in  $V_H$  and  $V_L$  was performed according to Eckerskorn et al. (1988) after separation of the variable domains on a 14% SDS-PAGE (Fling & Gregerson, 1986) and transfer onto a glass matrix.

**Separation of  $V_L$  and  $V_H$ .** The variable domains of the  $F_v$  fragment were separated by cation-exchange chromatography in the presence of 8 M urea and refolded subsequently as described previously (Glockshuber et al., 1990b).

**Protein Concentration.** The concentrations of the purified antibody fragments were determined by their absorption at 205 nm assuming an extinction coefficient  $\epsilon_{205}^{1mg/mL}$  of 31 (Scopes, 1982). From these values, the extinction coefficients at 280 nm were calculated:  $F_v$ ,  $\epsilon_{280}^{1mg/mL} = 1.95$ ;  $V_L$ ,  $\epsilon_{280}^{1mg/mL} = 0.89$ ;  $V_H$ ,  $\epsilon_{280}^{1mg/mL} = 2.92$ . The yields of  $V_L$  and  $V_H$  after cleavage with factor Xa and the yields of the reconstituted  $F_v$  fragment were estimated from SDS-polyacrylamide gels stained with Coomassie Brilliant Blue by comparison with simultaneously applied samples of known concentration of the purified  $F_v$  fragment.

**Fluorescence Measurements.** Fluorescence measurements were performed at 20 °C using a Shimadzu RF-5000 fluorescence spectrometer. Hapten binding was analyzed by protein fluorescence from titrations of the protein with phosphocholine as described previously (Glockshuber et al., 1990a). The investigation of the quantitative reassociation of separately refolded  $V_L$  and  $V_H$  was based on the fact that

the specific fluorescence of the  $F_v$  fragment increases with association and hapten binding (Glockshuber et al., 1990a). To achieve a complete association of  $V_L$  and  $V_H$ , the experiment was performed in the presence of excess phosphocholine. A solution of  $V_H$  in BBS was added stepwise to a solution of  $V_L$  in BBS containing phosphocholine (5 mM). Thirty minutes after each addition of  $V_H$ , the fluorescence at 328 nm (excitation at 280 nm) was recorded, averaged, and corrected for the volume increase.

**Kinetics of Denaturation.** The denaturation of the  $F_v$  fragment with 2-mercaptoethanol was performed at 37 °C at protein concentrations of 7.5  $\mu$ M in BBS containing 5 mM phosphocholine, where the native  $F_v$  fragment is fully stable. After different times of incubation (0–5 h) in the presence of 100 mM 2-mercaptoethanol, or after 1 h in the presence of 0–175 mM 2-mercaptoethanol, samples of 50  $\mu$ L were removed, put on ice (30 s), and centrifuged (1 min, room temperature). Thirty microliters of each supernatant was mixed with 10  $\mu$ L of a BSA solution (5 mg/mL in BBS) and applied onto a 14% SDS-PAGE. The amount of soluble protein was determined densitometrically after the gel was stained with Coomassie Brilliant Blue using the BSA band as an internal standard. The pseudo-first-order kinetics obtained had correlation coefficients of 0.99. The apparent first-order rate constants were used to calculate the second-order rate constants  $k_2$ .

**Cell Fractionation.** After inducing an *E. coli* culture (20 mL) harboring the desired plasmid at an  $OD_{550}$  of 0.5 with 1 mM IPTG, and continued growth for 3 h, 10 mL of the cells was centrifuged (10 min, 4000g, 4 °C). The cells were taken up in 1 mL of BBS containing 1 mM EDTA (Marvin et al., 1989). The suspension was transferred to microfuge tubes and shaken for 30 min at 4 °C. The spheroplasts were centrifuged (5 min, 10000g, 4 °C), and the supernatant was saved as the periplasmic fraction. The spheroplasts were resuspended in BBS containing 1 mM EDTA and passed twice through a French press homogenizer at 18000 psi. The lysate was centrifuged (30 min, 13000g, 4 °C). The supernatant was saved as the soluble spheroplast fraction. The insoluble portion was resuspended in 1 mL of BBS. To all fractions was added 250  $\mu$ L of SDS-PAGE loading buffer, and the samples were heated to 100 °C for 5 min.

If the whole cell protein was to be separated only into a soluble and an insoluble portion, then the bacteria were taken up in 1 mL of BBS and passed twice through a French press homogenizer at 18000 psi. All subsequent steps were as above.

**Trypsin Accessibility.** The procedure was essentially analogous to the one described by Minsky et al. (1986). The periplasmic fraction was prepared with 100 mM Tris-HCl, pH 8.0, containing 300 mM sucrose and 1 mM EDTA. The spheroplasts were centrifuged, and the supernatant was saved as the periplasmic fraction. The spheroplasts were taken up in 1 mL of buffer (100 mM Tris-HCl, pH 8.0, 300 mM sucrose, and 10 mM  $MgCl_2$ ), and trypsin was added to a final concentration of 0.1 mg/mL. The incubation was carried out for 3 h at 15 °C and stopped by the addition of trypsin inhibitor to a final concentration of 0.2 mg/mL. The further workup of the samples was as above.

Controls included samples in which no trypsin was added as well as samples in which the spheroplasts were passed through a French press homogenizer before the addition of trypsin.

The quality of the cell fractionation was validated by measuring  $\beta$ -lactamase activity (Sigal et al., 1984). Usually, greater than 95% of the total  $\beta$ -lactamase activity was found

in the periplasmic fraction. Additionally, some controls were fractionated using lysozyme (Witholt et al., 1976), but the results were found comparable to the EDTA procedure described above.

**Immunoblotting.** Samples separated on SDS-polyacrylamide gels (12.5% for  $F_{ab}$  and sc $F_v$  fragments, 15% for  $F_v$  fragments; Fling & Gregerson, 1986) were transferred onto nitrocellulose membranes and incubated with rabbit anti-McPC603 antibodies. The recombinant antibody chains were detected according to Blake et al. (1984) using alkaline phosphatase conjugated swine anti-rabbit antibodies.

**Nomenclature.** The amino acids are numbered as in the crystal structure (entry 2MCP in the Brookhaven Protein Data Base; Satow et al., 1986). Mutations are denoted as, e.g., C(h98)V/C(122)L, which describes a double mutant protein (the mutations being separated by the slash) carrying the changes Cys  $\rightarrow$  Val in the heavy chain (denoted by h) at position 98 and Cys  $\rightarrow$  Leu at position 22 in the light chain (denoted by l).

## RESULTS

To investigate the influence of the disulfide bonds, which are essential components of almost all immunoglobulin domains (Alzari et al., 1988; Davies et al., 1990), on folding and stability, two approaches were taken. First, the stability of the  $F_v$  fragment in the presence of reducing agents was examined, and its folding under reducing and oxidizing conditions was compared. Second, the stability of mutants, in which one or several of the cysteine residues were altered, was studied in *E. coli*. We will use the word "in vivo" to refer to *E. coli* expression experiments.

**Unfolding in Vitro.** It was first demonstrated that the disulfide bonds were quantitatively present in the recombinant secreted  $F_v$  fragment (Skerra & Plückthun, 1988; Plückthun & Skerra, 1989). After derivatization with 4-vinylpyridine in 8 M urea, the reaction product with cysteine, 4-pyridylethylcysteine, was not found in amino acid analysis. In contrast, we observed the expected 4 molar equiv when the  $F_v$  fragment was first treated with 1 M 2-mercaptoethanol in urea before the addition of 4-vinylpyridine (data not shown, exactly analogous to Figure 4).

The  $F_v$  fragment was then subjected to reducing conditions at 37 °C in BBS buffer containing 5 mM phosphocholine. Both  $V_H$  and also  $V_L$  precipitated with first-order kinetics in the presence of 100 mM 2-mercaptoethanol, with  $V_H$  precipitating at a faster rate (Figure 1). If the results of a 1-h incubation with varying 2-mercaptoethanol concentrations were analyzed, a first-order dependence of the precipitation rate on 2-mercaptoethanol concentration could also be demonstrated. These experiments show that the  $F_v$  fragment is unstable to reducing agents and suggest that the reduction of the disulfide bond is rate-determining in the overall precipitation reaction. The apparent second-order rate constant for the reduction with 2-mercaptoethanol is about  $(1.6 \pm 0.4) \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$  for  $V_L$  and about  $(2.85 \pm 0.5) \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$  for  $V_H$ .

The temperature dependence of this reaction was then measured. Using 100 mM 2-mercaptoethanol and an incubation time of 60 min, transition temperatures for the irreversible precipitation of  $V_H$  and  $V_L$  could be determined. In the absence of hapten, this temperature is about 25 °C for both  $V_L$  and  $V_H$ ; in the presence of the hapten phosphocholine, the transition temperature is increased by about 10 °C for both chains (data not shown).

These results are consistent with a model of the irreversible denaturation of the variable domains, in which the reduction

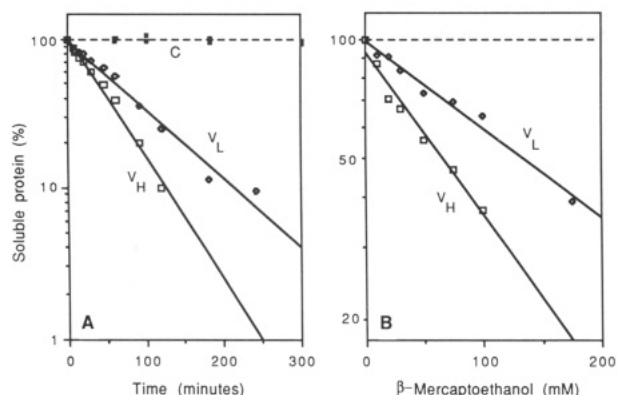


FIGURE 1: Kinetics of the irreversible denaturation of  $V_H$  and  $V_L$  by 2-mercaptoethanol. The purified  $F_v$  fragment (7.5  $\mu$ M in BBS in the presence of 5 mM phosphocholine) was incubated with 2-mercaptoethanol at 37 °C. The amount of soluble protein after the incubation was determined densitometrically after SDS-polyacrylamide gel electrophoresis of the soluble part of the samples (see Experimental Procedures). (A) Kinetics of precipitation of  $V_H$  and  $V_L$  at a concentration of 100 mM 2-mercaptoethanol; (B) precipitation of  $V_H$  and  $V_L$  after a 1-h incubation at different concentrations of 2-mercaptoethanol (0–175 mM). C denotes a control incubation in the absence of 2-mercaptoethanol.

of the intramolecular disulfide bonds in  $V_H$  and  $V_L$  can only occur in an at least partially unfolded state. This is plausible, because all four cysteine residues of the variable domain have an accessibility of zero to a probe of 1.4-Å radius in the native state. In the absence of a reducing agent, this partially unfolded state appears to be in equilibrium with the native state, and only the reduction reaction commits the partial denaturation to an irreversible precipitation pathway.

**Folding in Vitro.** The refolding of the domains of the  $F_v$  fragment is of immediate interest in developing production methods alternative to secretion systems. While native secretion systems are of unparalleled convenience [reviewed in Plückthun (1990, 1991)], in special cases such as, e.g., the production of unstable variants or specifically labeled proteins, in vitro refolding may become necessary.

For this purpose, expression vectors containing fusions between a truncated form of  $\beta$ -galactosidase (encoding amino acids 1–46 fused to amino acids 600–1007) and the antibody variable domains containing a linker encoding the cleavage sequence for the blood clotting protease factor Xa (Figure 2A,B) were constructed. A similar approach was used for the expression of a  $V_L$  domain fused to the cII gene of  $\lambda$  phage (Baldwin & Schultz, 1989).

The cytoplasmic expression of the fusion proteins (in separate experiments for  $V_L$  and  $V_H$ ) lead to insoluble inclusion bodies, which were solubilized and cleaved with factor Xa as described under Experimental Procedures. The refolding of the  $F_v$  fragment from the combined factor Xa cleavage mixtures was performed in two steps according to Hochman et al. (1976). First, the intracellular disulfides in  $V_H$  and  $V_L$  were allowed to form in the presence of 8 M urea by air-oxidation. This procedure appears to be especially suitable for single antibody domains, since they contain only two cysteine residues, and no wrong intramolecular disulfides can form. Additionally, the denaturing conditions should strongly favor the correct intramolecular disulfides over incorrect intermolecular linkages. In a second step, the urea concentration was decreased to native conditions by dilution.

This sequential refolding procedure appeared to be necessary for obtaining native  $F_v$  fragments in high yields and suggests an early formation of disulfide bonds in the folding process (Hochman et al., 1976). Using this strategy, the refolded  $F_v$

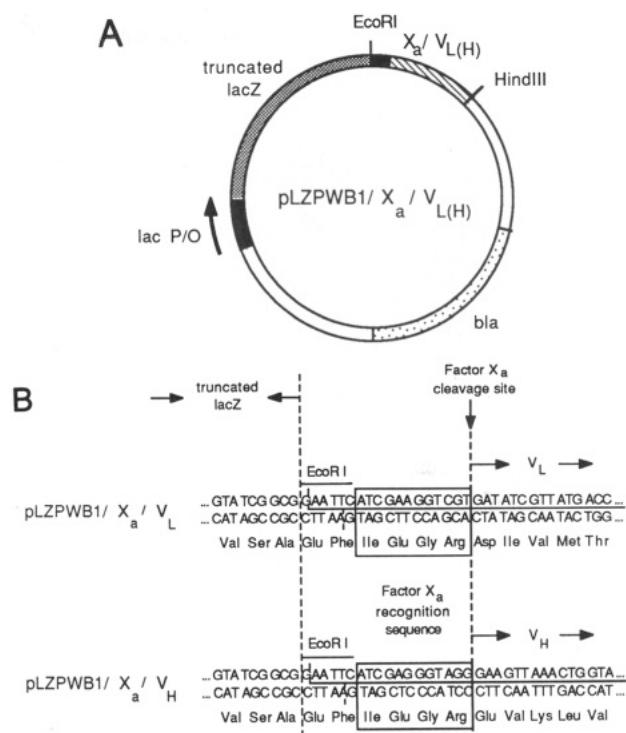


FIGURE 2: (A) Plasmids pLZPBW1/X<sub>a</sub>/V<sub>L</sub> and pLZPBW1/X<sub>a</sub>/V<sub>H</sub> for the cytoplasmic expression of  $V_L$  and  $V_H$  as fusions with a truncated  $\beta$ -galactosidase. The plasmid pLZPBW1 (Wetkum et al., unpublished results) contains the origin of replication and the ampicillin resistance gene (*bla*) from the plasmid pBR322 taken from the pUR series of plasmids (Rüther & Müller-Hill, 1983). The truncated *lacZ* gene is under the control of the wild-type *lac* promoter-operator sequence (*lac p/o*) and is inducible by isopropyl  $\beta$ -D-thiogalactoside (IPTG). It encodes the amino acids 1–46 and 600–1007 of the *lacZ* gene followed by a multiple cloning site, where the synthetic genes of  $V_H$  and  $V_L$  were inserted via the *EcoRI* and *HindIII* restriction sites. The vector is thus similar to the pUC vectors except for the version of the *lacZ* gene, and it does not contain the region 454–683 of pUC19, and it carries the *ori* and the *bla* genes derived from pBR322. (B) Precise fusion of the genes of  $V_L$  and  $V_H$  to the truncated  $\beta$ -galactosidase gene. The factor Xa recognition sequence was introduced into the genes of  $V_L$  and  $V_H$  (Plückthun et al., 1987) by replacing the NH<sub>2</sub>-terminal methionine codons using synthetic cassettes ranging from the *EcoRI* to the *PstI* site in  $V_L$  and from the *EcoRI* to the *HinI* site in  $V_H$ .

fragment could be purified to homogeneity with phosphocholine affinity chromatography (Figure 3). As in the unfolding experiments with 2-mercaptoethanol described above, the presence of the disulfides appeared to be a prerequisite for the stability of  $V_H$  and  $V_L$ : Refolding under identical but reducing conditions did not give rise to functional  $F_v$  fragments able to bind to the phosphocholine affinity column.

An early formation of disulfide bonds in the overall folding process of reduced  $V_H$  and  $V_L$  is also suggested by the fact that the folding reaction was found to be fully reversible when oxidized domains were used. The  $F_v$  fragment, expressed by the periplasmic route, was denatured with 8 M urea and the chains were separated as described previously (Glockshuber et al., 1990b). Separated  $V_L$  and  $V_H$  chains were each renatured independently by dialysis against BBS buffer, and the assembly to functional  $F_v$  fragment was followed fluorometrically. After the addition of 1 molar equiv of  $V_H$  to  $V_L$  in the presence of phosphocholine, a break in the spectral titration plot is apparent, suggesting that the initially added  $V_H$  combines with  $V_L$  to form  $F_v$  fragment up to 1 mol/mol, and the excess  $V_H$  cannot combine (Figure 5). It should be noted that native  $V_L$  has almost no intrinsic fluorescence in the native state (Figure 5). This experiment also showed that the as-

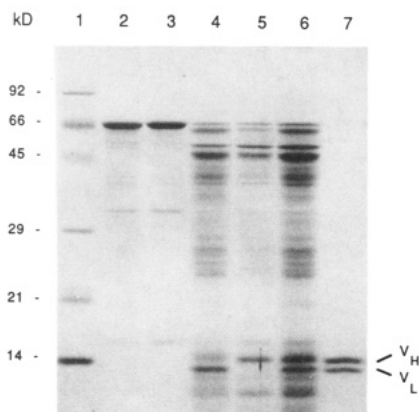


FIGURE 3: Cytoplasmic expression and refolding of the  $F_v$  fragment of McPC603. A 14% SDS-polyacrylamide gel (Fling & Gregerson, 1986) stained with Coomassie Brilliant Blue is shown. The insoluble cytoplasmic fusion proteins of  $V_H$  and  $V_L$  expressed in *E. coli* W3110 were enriched and solubilized. After cleavage with factor Xa,  $V_L$  and  $V_H$  were refolded to the functional  $F_v$  fragment from the combined cleavage mixtures after oxidation of the intramolecular disulfides in urea. The native  $F_v$  fragment was subsequently purified to homogeneity by phosphocholine affinity chromatography (see Experimental Procedures). (Lane 1) Molecular size marker; (lanes 2 and 3) enriched and solubilized  $\beta$ -galactosidase fusion proteins of  $V_L$  and  $V_H$ , respectively; (lanes 4 and 5) fusion proteins after cleavage with factor Xa; (lane 6) combined cleavage mixtures; (lane 7) affinity-purified, refolded  $F_v$  fragment.

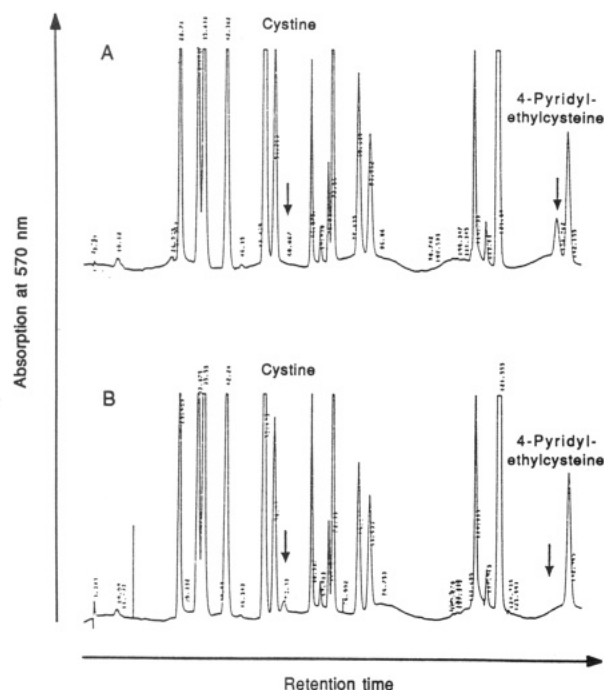


FIGURE 4: Quantitative formation of the intramolecular disulfide bridges in  $V_L$  and  $V_H$ . Ten micrograms of the purified, refolded  $F_v$  fragment was treated with 4-vinylpyridine in 8 M urea before (B) and after (A) reduction with 2-mercaptoethanol and hydrolyzed with 6 M HCl as described under Experimental Procedures. The samples were applied onto a DC6A cation-exchange column (Durrum Chemical Corp.). The eluted amino acids were stained with ninhydrin and detected at 570 nm.

sociation of  $V_H$  with  $V_L$  is tighter than that of any putative  $V_L$  dimers in the presence of the hapten, as there is no obvious deviation from linearity in Figure 5, and the break occurs at approximately 1 mol/mol. This break occurs since the  $F_v$  fragment has an increased molar fluorescence when phosphocholine is bound (Glockshuber et al., 1990a). This experiment thus distinguishes the fluorescence of the functional fragment from the sum of the subunit contributions.

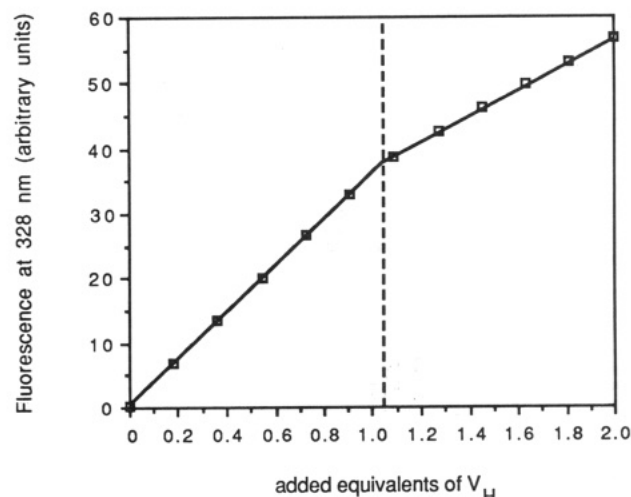


FIGURE 5: Quantitative association of  $V_L$  and  $V_H$  to the functional  $F_v$  fragment. The variable domains of the purified  $F_v$  fragment were separated as described previously (Glockshuber et al., 1990). Separated  $V_L$  and  $V_H$  chains were renatured by dialysis as described under Experimental Procedures. The association of  $V_H$  and  $V_L$  was detected fluorometrically at 25 °C. A solution of refolded  $V_H$  (2.60  $\mu$ M) in BBS was added stepwise (portions of 133  $\mu$ L) to 2 mL of a solution of refolded  $V_L$  (1.01  $\mu$ M) in BBS containing 5 mM phosphocholine. Thirty minutes after each addition of  $V_H$ , the fluorescence was recorded at 328 nm (excitation wavelength 280 nm) and corrected for the volume increase.

In conclusion, the domain association of  $V_H$  and  $V_L$  appears to be reversible, as long as the intramolecular disulfide bonds are present. The disulfide bond is clearly correlated with stability in vitro, both in the irreversible denaturation as well as in the refolding reaction.

**In Vivo Properties of Antibody Fragments with Mutations in Cysteine Residues.** The in vitro results were then correlated with in vivo expression experiments, in which one or more of the cysteine residues were replaced by other residues. The secretory expression system was used.

In a first set of experiments, the two cysteines of either chain of the  $F_v$  fragment were replaced by alanine residues. It was found that the chain containing the mutations was completely degraded (Figure 6), giving rise to no functional  $F_v$  fragment. Interestingly, the partitioning of the remaining chain between soluble and insoluble forms seemed to be identical with that of the wild-type  $F_v$  fragment, and therefore independent of the mutation in the other domain. This suggests that this partitioning is at least partially independent of the presence of the other domain. The reported cases of antibodies naturally lacking disulfide bonds (Kabat et al., 1987) all seemed to contain Cys/Tyr exchanges. The genetic reason is probably that this exchange can occur with a single base change and that all other single base changes that a cysteine codon may undergo are creating codons unsuitable for a buried residue or result in stop codons. Each of the four cysteine residues was therefore, in separate experiments, converted to a tyrosine residue. However, the result was unchanged: only the *unmutated* chain was visible on a Western blot (Figure 7), and in no case could any functional  $F_v$  fragment be isolated by affinity chromatography, while the wild-type control behaved normally.

It was then of interest to investigate whether different results are obtained for the same Cys  $\rightarrow$  Tyr mutations in the context of the  $F_{ab}$  fragment (Figure 8). Again, in no case was the functional  $F_{ab}$  fragment obtained for any of the mutants, as assayed by phosphocholine chromatography. This was also confirmed for an  $F_{ab}$  fragment, in which the mutations C-



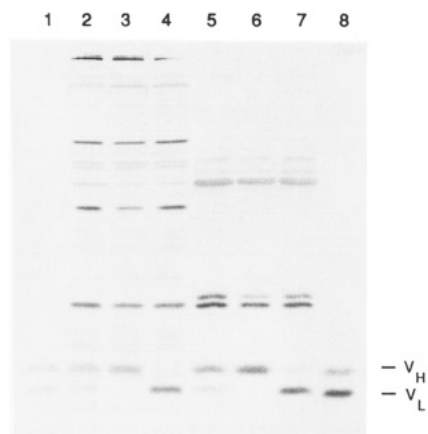


FIGURE 6: Coexpression and cosecretion of  $V_L$  and  $V_H$  into the periplasm of *E. coli* after deletion of the intramolecular disulfides. The expression of the native  $F_v$  fragment and the mutated  $F_v$  fragments C(123)A/C(194)A and C(h22)A/C(h98)A in *E. coli* JM83 was performed as described under Experimental Procedures using pASK30. After cell lysis, equivalent amounts of the soluble and insoluble fractions of the lysates were subjected to a reducing 14% SDS-PAGE. The expressed variable chains were detected by immunoblotting. The samples in lanes 2–4 correspond to the soluble parts of the cellular protein and the samples in lanes 5–7 to the insoluble parts. (Lanes 1 and 8) Purified  $F_v$  fragment; (lanes 2 and 5) native  $F_v$  fragment; (lanes 3 and 6) mutant C(123)A/C(194)A; (lanes 4 and 7) mutant C(h22)A/C(h98)A.

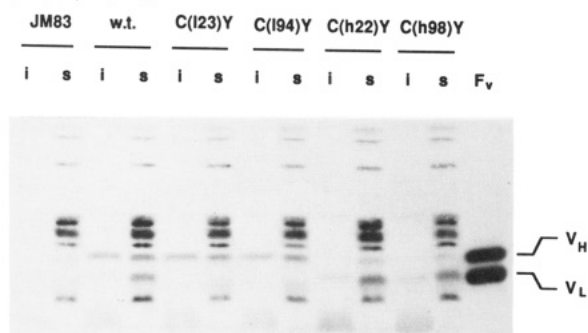


FIGURE 7: Effect of deleting a disulfide bond in the  $F_v$  fragment by substituting each cysteine residue individually by tyrosine. The soluble and insoluble fractions are denoted by s and i, respectively. JM83, the host strain without a plasmid; w.t., the wild-type  $F_v$  fragment of McPC603 expressed with the plasmid pASK30 in *E. coli* JM83; C(123)Y, C(194)Y, C(h22)Y, and C(h98)Y are single point mutants of the  $F_v$  fragment in pASK30.

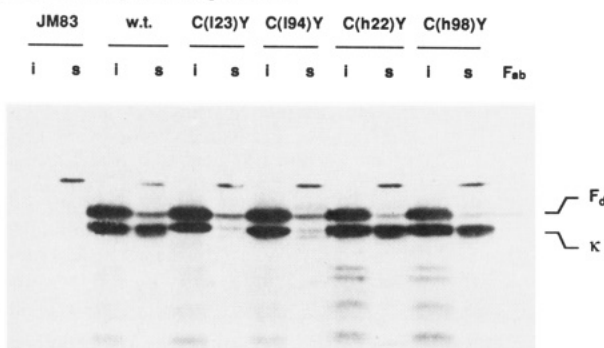


FIGURE 8: Effect of deleting a disulfide bond in the  $F_{ab}$  fragment by substituting each cysteine residue of the variable domains individually by tyrosine. The soluble and insoluble fractions are denoted by s and i, respectively. JM83, the host strain without a plasmid; w.t., the wild-type  $F_{ab}$  fragment of McPC603, expressed with the plasmid pASK29 in *E. coli* JM83; C(123)Y, C(194)Y, C(h22)Y, and C(h98)Y are single point mutants of the  $F_{ab}$  fragment in pASK29. pASK29 (Skerra & Plückthun, 1991) is identical to pASK30 (Skerra et al., 1991) but contains in addition the constant domains  $C_L$  and  $C_H1$ .

(h22)A and C(h98)A were introduced, and which did not give rise to functional  $F_{ab}$  fragment either (Skerra & Plückthun,

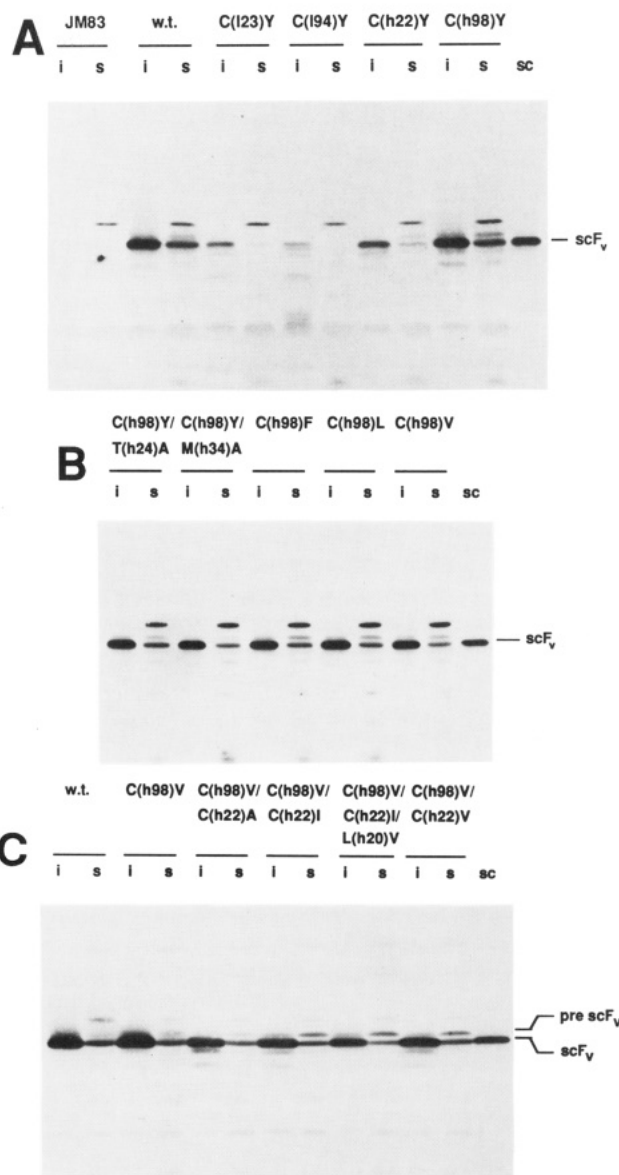


FIGURE 9: Effect of mutations in the single-chain  $F_v$  fragment. The soluble and insoluble portions are denoted by s and i, respectively. The expression was carried out in JM83 with derivatives of the plasmid pASK-lisc (Skerra et al., 1991). The mutants are denoted as explained under Experimental Procedures. (A) Single Cys  $\rightarrow$  Tyr changes: C(123)Y, C(194)Y, C(h22)Y, and C(h98)Y. (B) Single and double mutants missing the h22-h98 disulfide bond in scF<sub>v</sub>: C(h98)Y/T(h24)A, C(h98)Y/M(h34)A, C(h98)F, C(h98)L, and C(h98)V. (C) w.t. (the wild-type single-chain  $F_v$  fragment encoded by pASK-lisc); the single, double, and triple mutants are C(h98)V, C(h98)V/C(h22)A, C(h98)V/C(h22)I, C(h98)V/C(h22)I/L(h20)V, and C(h98)V/C(h22)V. sc denotes the purified single-chain  $F_v$  fragment, and the precursor and the mature forms are denoted by pre-scF<sub>v</sub> and scF<sub>v</sub>, respectively.

1991). However, Western blot analysis showed weaker effects of the mutations on the solubility of the chains in the case of the  $F_{ab}$  fragment than found for the  $F_v$  fragment, and the amount of the insoluble portion of the  $F_{ab}$  fragment seemed to be independent of the presence of the mutations (Figure 8). While the mutated light chain is almost completely absent in the soluble fraction, the amount of soluble mutated heavy chain is reduced by only about 50%.

Finally, the effect of the mutations was examined in the context of a secreted single-chain  $F_v$  fragment. The mutant protein C(194)Y was sensitive to proteolysis and could only be faintly detected in the insoluble fraction. The mutations C(123)Y and C(h22)Y take intermediate positions in that faint

bands of soluble protein were visible, while C(h98)Y behaved like wild type on Western blots (Figure 9A). This is the same exchange as in the antibody ABPC48 (Rudikoff & Pumphrey, 1986). Yet, in the case of McPC603, neither this mutation nor any of the others gave rise to single-chain F<sub>v</sub> fragment binding to the affinity column.

A sequence comparison between the antibody ABPC48 (not containing the disulfide in V<sub>H</sub>) and McPC603 in conjunction with the three-dimensional structure of the latter revealed a remarkable conservation in the environment of the hydrophobic core around the disulfide bond. The packing of the hydrophobic core has been shown to be important for protein stability (Yutani et al., 1987; Matsumura et al., 1988; Das et al., 1989; Kellis et al., 1988, 1989; Lim & Sauer, 1989; Sandberg & Terwillinger, 1989; Karpusas et al., 1989; Ganter & Plückthun, 1990), and therefore the effect of substitutions in the interior of the protein was evaluated. One difference between the two proteins is the nature of residue h24, which is alanine in ABPC48 and threonine in McPC603. We do not know what orientation the new tyrosine residue at H98 would assume, and therefore the substitution T(h24)A was carried out to generate a similar environment as in ABPC48. At position h34, both McPC603 and ABPC48 contain methionine, but alanine was introduced as another mutation to accommodate alternative conformations of Tyr(h98). Finally, the cysteine residue h98 was also substituted with various other hydrophobic residues. However, in none of the new single and double mutants of the single-chain F<sub>v</sub> fragment, C(h98)Y/T(h24)A, C(h98)Y/M(34h)A, C(h98)F, C(h98)L, and C(h98)V, antibody protein binding to the affinity column was isolated, while Western blots were very similar to wild-type single-chain F<sub>v</sub> fragment (Figure 9A,B). This result was not changed, either, by the additional substitution of the remaining CysH22 to Ala, Ile, or Val (Figure 9C). Interestingly, the latter mutants show a similar expression level as wild type, while the mutation C(h22)Y by itself leads to a very low expression level. Apparently, Tyr(h22) cannot be accommodated in the hydrophobic core in the presence of Cys(h98), and the result is a protein easily degraded.

Interestingly, the mutations C(h98)V/C(h22)V, C(h98)V/C(h22)I, and C(h98)V/C(h22)I/L(h20)V lead to the accumulation of detectable precursor protein. This is not the case for C(h98)V/C(h22)A, suggesting that a larger hydrophobic residue than Ala or Cys at position h22 is less compatible with protein transport than wild type.

In conclusion, only the wild-type arrangement of the disulfide bonds lead to protein purifiable by antigen affinity chromatography, in the context of either the F<sub>v</sub> fragment, the single-chain F<sub>v</sub> fragment, or the F<sub>ab</sub> fragment.

**The Mutated Single-Chain F<sub>v</sub> Fragments Are Transported but Precipitate.** The localization of the mutated single-chain F<sub>v</sub> fragments was investigated using cell fractionation and protease accessibility studies. Only for the wild-type single-chain F<sub>v</sub> fragment was soluble protein found in the periplasmic fraction. In the other mutants examined (Figure 10), the soluble protein cofractionated with the spheroplast fraction. This strongly suggests that these proteins are largely insoluble and precipitate in the periplasm, being resolubilized during the separation of the soluble and insoluble spheroplast fraction.

To confirm this interpretation, it had to be shown that these proteins are actually accessible to a protease added to the outside of the spheroplasts. It was demonstrated that trypsin can digest both the soluble and also the insoluble portion of mature protein sedimenting with the spheroplasts (Figure 11). In contrast, the precursor protein, presumably on the inside

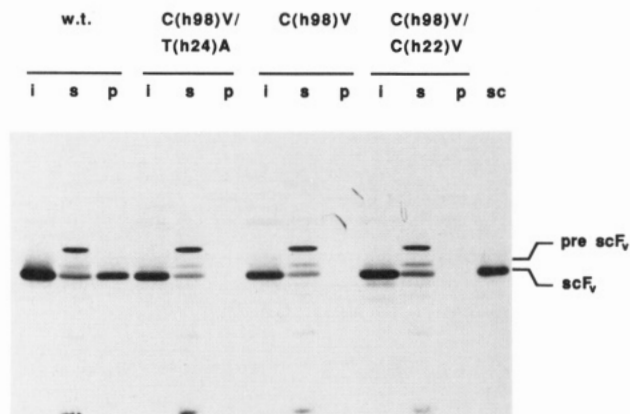


FIGURE 10: Cell fractionation of mutant single-chain F<sub>v</sub> fragments. The insoluble portion of the total cell protein is denoted i, the soluble portion of the spheroplasts is s, and the soluble periplasmic fraction is denoted p. The single-chain F<sub>v</sub> fragment was expressed in JM83 harboring pASK-lisc. w.t. denotes the wild-type single-chain F<sub>v</sub> fragment, and C(h98)Y/T(h24)A, C(h98)V, and C(h98)V/C(h22)V are double and single mutants of the single-chain F<sub>v</sub> fragment. scF<sub>v</sub> denotes purified single-chain F<sub>v</sub> fragment. The precursor is denoted as pre-scF<sub>v</sub> and the mature form scF<sub>v</sub>.

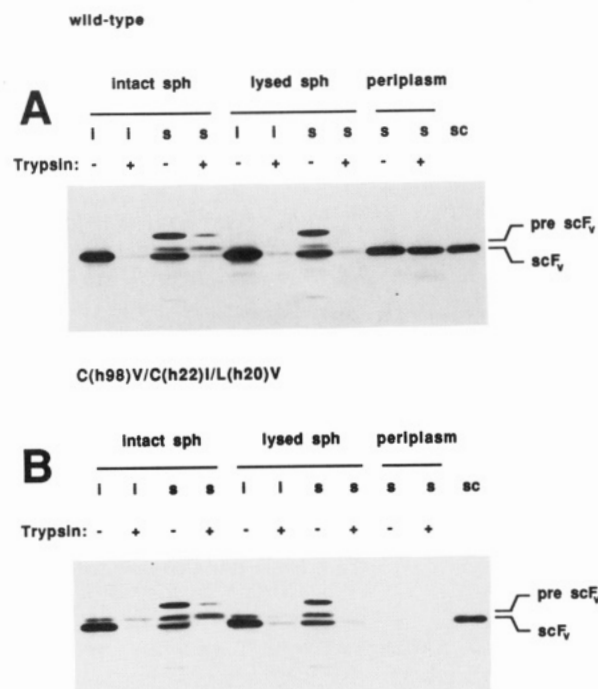


FIGURE 11: Trypsin accessibility experiment of (A) the wild-type single-chain F<sub>v</sub> fragment and (B) the triple mutant C(h98)V/C(h22)I/L(h20)V. Spheroplasts were incubated with trypsin for 3 h at 15 °C, and after the reaction was stopped, they were passed through a French press cell afterward (lanes denoted intact sph). In the lanes denoted lysed sph, the spheroplasts were lysed first by passage through a French press cell and then incubated with trypsin. The soluble periplasmic fraction was also tested (periplasm). The insoluble portion is denoted i, the soluble portion is s, and trypsin addition is indicated with + and -. scF<sub>v</sub> denotes purified single-chain F<sub>v</sub> fragment.

of the spheroplast, is inert to added protease. Also, the periplasmic, folded wild-type protein is only marginally affected by added protease.

We deduce from these experiments that the inactive mutant proteins are transported to the periplasm where they largely precipitate. A portion is resolubilized by French press lysis before the separation of soluble and insoluble total protein, or insoluble and soluble spheroplast fractions, respectively. Only the wild-type single-chain F<sub>v</sub> fragment fractionates into the periplasmic fraction, from which it can be isolated in functional form.

The *in vivo* experiments therefore reflect the results *in vitro*. The absence of the disulfide bonds in  $V_H$  or  $V_L$  does not lead to proteins able to bind to an antigen affinity column, no matter whether in the context of an  $F_v$  fragment, a single-chain  $F_v$  fragment, or an  $F_{ab}$  fragment. This suggests incomplete or incorrect folding of the domain missing the disulfide bond. The solubility of the fragments carrying the same mutations is somewhat different for  $F_v$ , single-chain  $F_v$ , and  $F_{ab}$  fragments *in vivo*, as is their proteolytic sensitivity, probably because of the influence of the additional domains. However, the folding of the variable domains seems to be equally affected for the same mutations in all fragments since no functional protein can be isolated. We deduce that the disulfide bonds in the variable domains are crucial for correct folding and are thus required for antigen binding activity of the investigated fragments of the antibody McPC603.

#### DISCUSSION

Both *in vitro* and *in vivo* results showed that both disulfide bonds of the  $F_v$  fragment (one in  $V_H$  and one in  $V_L$ ) are necessary for the folding and the stability of the  $F_v$  fragment, of the  $F_{ab}$ , and of the single-chain  $F_v$  fragment, at least in the investigated case of the antibody McPC603.

Disulfide bonds have been added to a variety of proteins [reviewed by Wetzel (1987)], and this has resulted in substantial increases in protein stability in certain cases (Matsumura et al., 1989). On the other hand, disulfides have also been removed from proteins without great loss in stability, e.g., in the case of  $\beta$ -lactamase (Schultz et al., 1987; Laminet & Plückthun, 1989). For this protein, several substitutes for the cysteine residues have already been tested and were found to be functional. The folding and stability of this particular protein do not seem to depend on the presence of the disulfide bond for stability *in vitro* or *in vivo*. It can even be expressed in the cytoplasm in reduced, but folded form (Kadonaga et al., 1984; Plückthun & Knowles, 1987). These two cases,  $\beta$ -lactamase and antibody domains, may illustrate two opposite extreme cases of the importance of disulfide bonds *in vivo*.

The  $\beta$ -barrel architecture, which makes up the antibody domain, is apparently not intrinsically labile, nor dependent on disulfide bonds. Several unrelated  $\beta$ -barrel proteins have been studied crystallographically, and only the antibody domains contain the central disulfide bond (Getzoff et al., 1989; Baker, 1988; Guss & Freeman, 1983; Richardson et al., 1976). Yet the topologically distantly related superoxide dismutase has been found to be one of the most stable proteins known (Getzoff et al., 1989; Richardson et al., 1976). The PapD protein, a molecular chaperone of pili assembly in *E. coli*, consists of two domains with an immunoglobulin fold, but neither contains the typical intradomain disulfide bond (Holmgren & Bränden, 1989).

Disulfide bonds of antibody domains are remarkably well conserved. Only in a few rare cases have antibodies been found, in which a cysteine residue has been exchanged. One antibody, ABPC48, has been studied in more detail, and its functionality has been demonstrated (Rudikoff & Pumphrey, 1986). This observation stands in contrast to the results reported here as it was not possible to achieve correctly folded  $F_v$ , single-chain  $F_v$ , or  $F_{ab}$  fragments in the antibody McPC603 if any of the cysteine residues of the variable domains were substituted.

These results were obtained with a variety of different mutants, using a variety of substitutions for the cysteine residues and additional changes in the hydrophobic core. The results did not depend on the size of the antigen binding fragment expressed, suggesting that it is an intrinsic effect of

the variable domain folding. Despite the fact that the hydrophobic core of a protein contributes strongly to its stability [see, e.g., Yutani et al. (1987), Matsumura et al. (1988), Das et al. (1989), Kellis et al. (1988, 1989), Lim and Sauer (1989), Sandberg and Terwillinger (1989), Karpusas et al. (1989), and Ganter and Plückthun (1990)], the mutations tested had only a marginal effect on stability of the antibody fragments *in vivo*. Only mutants carrying a large hydrophobic residue at position h22 show some evidence of accumulated precursor. *In vivo*, the mutants lacking either disulfide bond of the variable domain show various degrees of proteolytic degradation and/or mostly precipitate in the periplasm. The disulfide bond must therefore contribute strongly to the stability or be involved in early folding steps *in vivo*.

The *in vitro* studies showed that precipitation strictly correlates with the reduction of the native molecule and that no refolding is possible under reducing conditions. However, a satisfactory refolding yield is obtained when the formation of the disulfide bonds is carried out in the presence of molecular oxygen and strong denaturant, consistent with a very early formation of the disulfide bonds at least during *in vitro* folding.

Unfortunately, neither the *in vivo* nor the *in vitro* experiments can rigorously distinguish whether the stability deficiency is a thermodynamic or a kinetic one. In the thermodynamic hypothesis, the disulfide bond would be needed to shift the equilibrium to the native state, in the simplest case by lowering the entropy of the unfolded state. In this case, the disulfide-missing antibody ABPC48 would have to be intrinsically more stable, e.g., by more favorable interactions of its shorter hypervariable loops within the native structure, or the details of packing of the hydrophobic core.

In the kinetic hypothesis, we would have to postulate that an early disulfide formation is required for the folding of  $V_H$  and  $V_L$ . It may be possible that certain molecular chaperones not present in *E. coli* open new pathways for folding of  $V_H$  and  $V_L$  in higher cells, circumventing an early disulfide intermediate. In this case, most antibodies would behave like McPC603 in *E. coli*, but would tolerate, like ABPC48, the removal of disulfide bonds when expressed in eukaryotes. The true difference would then be one of the different environments for *in vivo* folding in eukaryotes and prokaryotes and independent of their intrinsic structures.

Rather little is known about the formation of disulfide bonds *in vivo* [summarized by Gilbert (1990)]. The involvement of an enzymatic activity, protein-disulfide isomerase (PDI), has been demonstrated in eukaryotic cells (Freedman & Hillson, 1980; Bulleid & Freedman, 1988; Lyles & Gilbert, 1991a,b). Recently, an enzyme with the same activity has been discovered in the periplasm of *E. coli* (Bardwell et al., 1991; Kamitani et al., 1992), but its reaction mechanism and substrate specificity remain to be investigated. The involvement of other folding modulators (molecular chaperones) (Fischer & Schmid, 1990) must also be taken into account, and needs to be investigated further. If a folding modulator is required for allowing the folding of antibody domains not containing the central disulfide bond to proceed to the native state, it must open up a pathway *not* depending on the formation of this disulfide bond. Further experiments with eukaryotic expression and coexpression of folding modulators can now be used to address this question.

We have also shown how the *in vitro* results can be applied to a rational approach to *in vitro* refolding and thus to alternative expression strategies that may be useful, e.g., for unstable fragments or other one-domain members of the immunoglobulin superfamily. The approach via inclusion bodies



can also be of interest when an enrichment of the desired protein would be difficult to achieve by other methods. The refolding strategy will have to be modified for antibody constructs with more than one domain, however, since the statistic oxidation of cysteine residues in 8 M urea would strongly favor wrong intramolecular disulfides. In such a case, disulfide shuffling systems are needed (Buchner & Rudolph, 1991).

This expression strategy via fusion proteins and in vitro refolding avoids some of the problems of the direct cytoplasmic expression of antibody domains. Certain variants may not only be proteolytically unstable but may also give rise to unstable mRNAs and poor translation initiation due to the sequence at the beginning of the coding region. Protein sequencing showed that the processing by factor Xa took place at the desired position and thus an identical N-terminus is obtained as in the mouse. The F<sub>v</sub> fragment assembled by this route was also shown to be fully functional. Therefore, this strategy offers an alternative route to F<sub>v</sub> fragments or similar molecules from *E. coli*.

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