Factors Influencing the Dimer to Monomer Transition of an Antibody Single-Chain Fv Fragment†

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ABSTRACT: Antibody single-chain Fv (scFv) fragments are able to form dimers under certain conditions, and the extent of dimerization appears to depend on linker length, antibody sequence, and external factors. We analyzed the factors influencing dimer−monomer equilibrium as well as the rate of interconversion, using the scFv McPC603 as a model system. In this molecule, the stability of the V_H-V_L interaction can be conveniently varied by adjusting the ionic strength (because of its influence on the hydrophobic effect), by pH (presumably because of the presence of titratable groups in the interface), and by the presence or absence of the antigen phosphorylcholine, which can be rapidly removed due to its very fast off-rate. It was found that the monomer is the thermodynamically stable form with linkers of 15 and 25 amino acids length under all conditions tested (35 μM or less). The dimer is initially formed in periplasmic expression, presumably by domain swapping, and can be trapped by all factors which stabilize the V_H-V_L interface, such as the presence of the antigen, high ionic strength, and pH below 7.5. Under all other conditions, it converts to the monomer. Predominantly monomer is obtained during in vitro folding. Monomer is stabilized against dimerization at very high concentrations by the same factors which stabilize the V_H-V_L interaction. These results should be helpful in producing molecules with defined oligomerization states.

Fv fragments are the smallest functional units of antibody molecules that still contain the complete antigen binding site. They are heterodimers, consisting of the variable light (V_L)[1] and the variable heavy (V_H) chain of an antibody. The small size and the ability to produce them in functional form in the periplasm of *E. coli (1)* made them an interesting starting point for protein engineering with potential use in immunodiagnostics and therapy.

However, the Fv fragment has limited stability because of the dissociation of the two domains. This dissociation equilibrium varies from antibody to antibody, since the buried surface area and the quality of the interaction at the V_H-V_L interface vary *(2)*. Furthermore, the domain angle as well as the exact interface composition varies such that both very stable and very unstable heterodimers have been observed. To make Fv fragments generally useful, the introduction of a peptidic linker between V_H and V_L domains has been described to create the so-called single-chain Fv fragment (scFv) *(3−5)*. A variety of linkers with different length and sequence have been used [for reviews and representative examples, see *(6−11)*]. Most popular have been linkers of the sequence $(G_4S)_n$, with much of the early work following Huston and co-workers *(4)*, who used a length of 15 amino acids. Single-chain Fv fragments can be made in the orientation either V_H-linker-V_L or V_L-linker-V_H. Since the distance between the C-terminus of V_L and the N-terminus of V_H is larger than the one between the C-terminus of V_H and the N-terminus of V_L *(6)*, a linker of the same length is more strained in the former than in the latter case.

It was noted by several groups *(12−17)* that scFv fragments may form dimers to varying extent, depending on the linker length and on the particular antibody. The dimerization has subsequently been caused on purpose by shortening the linker to about 5−10 amino acids, thus making it too short for monomeric assembly, giving rise to so-called diabodies *(12, 18)*. Reducing the linker even more or removing it altogether can result in trimeric scFvs or so-called triabodies *(19−21)*.

The solution of the crystal structure of a diabody *(18)* showed that the V_H domain of one chain is paired with the V_L domain of the other chain and vice versa. The triabody has three Fv heads with the polypeptides arranged in a cyclic, head-to-tail fashion *(19)*. These observations can be considered to be manifestations of a phenomenon called 'domain swapping', which is defined as one domain of a monomeric protein being replaced by the same domain from an identical protein chain *(22, 23)*. Domain swapping has been suggested to be caused by the prior history of the protein. In the case of a scFv fragment *(24)*, a predominantly monomeric scFv was found to form a dimer after freezing and thawing. Additionally, the expression conditions can play a role. When CD2 was expressed as a fusion to the dimeric protein

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[1] Abbreviations: BBS, borate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PC, phosphorylcholine; scFv, single-chain Fv fragment; SDS, sodium dodecyl sulfate; V_L, variable light chain of an antibody; V_H, variable heavy chain of an antibody.

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glutathione *S*-transferase, domain swapping was shown by X-ray crystallography to occur, while denaturation and refolding in the absence of the fusion partner converted the dimeric CD2 to the monomer *(25)*.

For application of single-chain Fv fragments, it is necessary that these molecules have a defined oligomerization state. We therefore investigated the factors beyond the linker length, such as pH, ionic strength, protein concentration, and presence or absence of antigen, which might favor particular oligomerization states of a single-chain Fv fragment and influence the rates of interconversion. Such a study of conditions, we reasoned, might give clues also about the structural parameters which favor a particular oligomerization state.

For these studies, we used the phosphorylcholine binding antibody McPC603 *(26−28)* in the form of a single-chain Fv fragment in the orientation V_H-$(G_4S)_3$-V_L as well as a mutant of this fragment which has better in vivo folding properties, but the same thermodynamic stability as the wild-type protein *(29)*. We investigated the oligomerization state of both constructs under a variety of conditions and compared it to the same molecule with a 25-mer linker and to the corresponding Fv fragment. We found a dynamic equilibrium which is influenced by external factors, and we attempt a rationalization based on interface stability. While the monomer is the thermodynamically stable form with a linker of 15 amino acids or longer under all conditions tested, the protein can be trapped in the form of a dimer, if the transition to the monomeric form is prevented.

EXPERIMENTAL PROCEDURES

Plasmid Construction. The expression vectors used were based on the plasmid pASK30 *(30)*: pLisc_SAFH11 expresses the scFv fragment of the antibody McPC603 with three stabilizing mutations in the heavy chain [P40A, S63A, A64D, sequential numbering, *(29)*] and an N-terminal FLAG-peptide *(31)*; pLisc_SAF expresses the wt-MCPC603 scFv fragment without these mutations, but with the same N-terminal short FLAG-peptide; pLisc_SAH11 expresses the scFv with the three mutations in the heavy chain, but without the FLAG-peptide. All constructs are in the V_H-$(G_4S)_3$-V_L orientation, preceded by an OmpA signal sequence. In addition, a $(G_4S)_5$ linker, which was obtained by site-directed mutagenesis and verified by DNA sequencing (C. Freund, unpublished), was cloned into the pLisc_SAFH11 vector using *Xho*I/*Hin*dIII to give the plasmid p*K*A30_fH11-25.

Protein Expression. Plasmids were transformed into *E. coli* JM83 (F^- *ara thi* Δ(*lac-pro*AB) *rps*L (*str*^{R}) [*ϕ*80d*lac*Z-ΔM15]) thi). Protein expression was carried out in LB broth in shake flasks, containing 0.1 g/L ampicillin. Overnight cultures were grown at 30 °C. The main culture was inoculated to give an initial OD_{550} of 0.15 (typical dilution of 1:25−1:30) and grown at 26 °C. Cells were induced at an OD_{550} of 0.5−0.6 and harvested after 4 h by centrifugation at 5000*g* for 10 min at 4 °C. Cell pellets were directly used or immediately frozen at −80 °C and stored until use.

Protein Purification. Cell pellets were resuspended in a 5−6-fold volume of BBS buffer (200 mM H_3BO_3, 160 mM NaCl, adjusted to pH 8.0 with NaOH) containing 10 mM EDTA. Cells were disrupted by sonification 4 times in an ice−water bath (duty cycle 50%). The single-chain Fv fragment was purified to homogeneity by phosphorylcholine affinity chromatography as described *(32, 33)* but with a washing step with 1 M NaCl in BBS buffer and elution with 20 mM phosphorylcholine in BBS buffer. The purity was checked by SDS−PAGE, stained with Coomassie Brilliant Blue according to standard methods. The concentration was calculated from the absorbance at 280 nm *(34)*. The mass of the scFv with the 15-mer and 25-mer linker was verified by electrospray mass spectrometry performed after reversed-phase HPLC (LC-MS). The mass obtained deviated less than 2 Da from the calculated mass.

Size-Exclusion Chromatography. The protein size and thus the oligomerization state were determined using a size-exclusion chromatography column (Superose-12 PC3.2/30, Pharmacia, Sweden) on an HPLC system (SMART system, Pharmacia, Sweden) at 20 °C which was calibrated with a molecular mass standard (cytochrome *c*, 12.4 kDa; carbonic anhydrase, 29 kDa; alcohol dehydrogenase, 150 kDa; *β*-amylase, 200 kDa; blue dextran (2000 kDa), void volume) (Sigma, USA). The samples (40−50 *μ*L) were injected using different running buffers, and elution was monitored at 280 and 254 nm. Preparative gel filtration was performed with a Superdex 75 column (HiLoad 16/60 prep grade, Pharmacia, Sweden) at 4 °C in BBS buffer.

RESULTS

The dimerization behavior of the McPC603 single-chain Fv fragment was characterized both in terms of the ratio between monomer and dimer and in terms of the conversion rate under various conditions. We investigated the influence of the antigen and of buffers with different pH and ionic strength. Additionally, we characterized the influence of the linker length and the expression and folding method used to produce the recombinant scFv on the dimerization.

For our characterization, we chose a mutant of the phosphorylcholine binding single-chain Fv fragment McPC603 *(29)* in the form V_H-$(G_4S)_3$-V_L. This mutant carries three mutations in the heavy chain (P40A, S63A, A64D, sequential numbering), which result in better in vivo folding properties. This scFv gives higher yield in functional expression, but leaves the thermodynamic stability in vitro unchanged within experimental error *(29)*. For convenient detection, a shortened FLAG-tag (Asp-Tyr-Lys-Asp) was fused to the N-terminus of the protein *(31)*. Unless specifically mentioned, the experiments were carried out with this protein. We also compared the properties of this scFv with the corresponding wild-type scFv fragment not carrying the mutations, and in the presence or absence of the FLAG-tag and the linker. In all cases, unless specifically mentioned, the antibody fragments were functionally expressed in the periplasm of *E. coli* and purified by phosphorylcholine affinity chromatography.

Influence of the Antigen on the Oligomerization State of the scFv Fragment of McPC603. The influence of antigen binding on the dimerization of the scFv fragment was investigated in BBS buffer (200 mM borate, 160 mM NaCl, pH 8.0), which had been used for affinity purification and previous gel filtration experiments which always showed this scFv fragment to be monomeric under these conditions *(35, 36)*. A Superose-12 gel filtration column was equilibrated with BBS buffer with or without 20 mM phosphorylcholine (PC), and the sample was loaded in BBS buffer

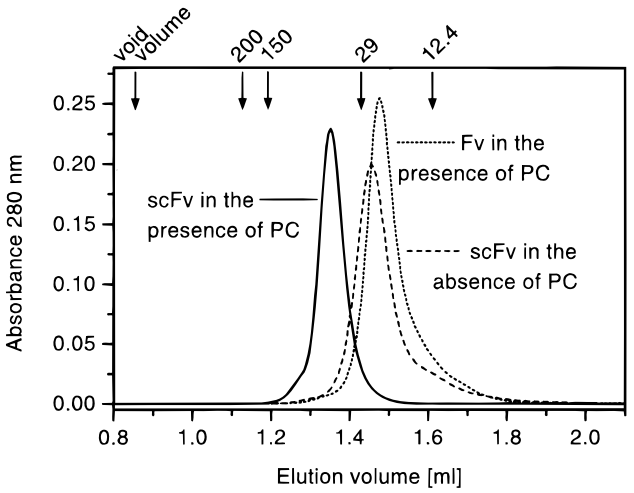

FIGURE 1: Comparison of single-chain Fv fragment with the corresponding Fv fragment in the presence and absence of antigen by Superose-12 gel filtration. All samples were loaded in BBS buffer with 20 mM PC (BBS/PC). ScFv, running buffer BBS/PC (—); scFv, running buffer BBS (- - -); Fv, running buffer BBS/PC (···). The elution volumes of marker proteins are indicated by arrows, the molecular mass is given in kilodaltons (cytochrome *c*, 12.4 kDa; carbonic anhydrase, 29 kDa; alcohol dehydrogenase, 150 kDa; *β*-amylase, 200 kDa; blue dextran (2000 kDa) indicates the void volume).

containing 20 mM PC. As a control, the corresponding Fv fragment was used (Figure 1). All runs show one main peak, but with different elution volumes. In the presence of the antigen in the running buffer, the elution volume of the scFv corresponds to that of a dimer, as judged from a calibration curve of molecular weight standards, whereas in the absence of the antigen, a monomer was found. In contrast, the Fv fragment was always exclusively monomeric, both in the absence and in the presence of the antigen, even at 10-fold higher protein concentrations than used for the scFv.

Since PC is commercially obtained as a calcium salt, two further control experiments were carried out to confirm that the observed dimerization was indeed due to the antigen and not to the bivalent calcium ion. By incubating PC with a chelating ion-exchange resin (Chelex-100, BioRad), the calcium ion of PC was substituted by sodium ions. The same gel filtration experiments were performed with Ca^{2+}-free PC (data not shown), and identical results to those of Figure 1 were obtained. Furthermore, in BBS buffer with and without 20 mM $CaCl_2$, a monomer was found (Figure 2C, data discussed in the next section).

Thus, a dimer is found for the scFv fragment only in the presence of antigen, but not in its absence, while the Fv fragment always remains monomeric. The dimer converts to the monomer when the column is run in the absence of antigen (Figure 1). This behavior is independent of the presence or absence of Ca^{2+}. Clearly, the dimerization requires the presence of the linker, and molecules of a defined size are obtained. A nonspecific aggregation of Fv or scFv fragment is not observed. Since pure monomeric species are observed in BBS buffer, the formation of the monomer must be fast.

Dependence of the Dimer to Monomer Conversion Rate on Buffer Conditions. After observing that the dimeric form can be converted to the monomeric form, we then characterized the influence of different buffers on the rate of the dimer

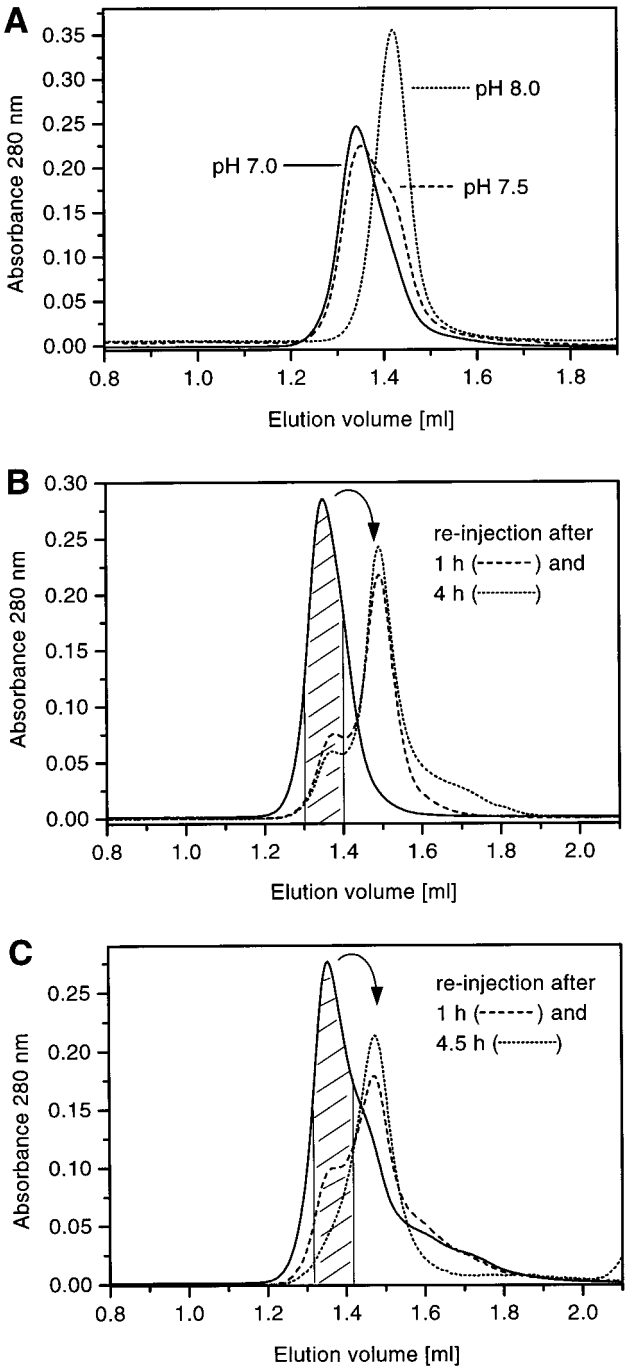

FIGURE 2: Elution profiles of Superose-12 gel filtration column runs. All samples were loaded in BBS buffer, pH 8.0, with 20 mM PC (BBS/PC). (A) Influence of pH on the dimer to monomer equilibration of scFv. Running buffer 20 mM Tris, 150 mM NaCl, pH 7.0 (—), pH 7.5 (- - -), pH 8.0 (···). (B) Investigation of the equilibrium state of scFv in the absence of antigen. Running buffer 20 mM Tris, 150 mM NaCl, pH 7.0. The peak (—) was collected (hatched area), and half was re-injected 1 h (- - -), the second half 4 h (···) after the first run. (C) Same as (B) but running buffer BBS, pH 8.0, with 20 mM $CaCl_2$. First injection (—), re-injection of the collected peak (hatched area) after 1 h (- - -) and 4.5 h (···).

to monomer conversion, when the antigen is removed during gel filtration. For these experiments, the scFv was loaded in its dimeric form (in BBS, 20 mM PC, pH 8.0), and the conversion to the monomeric form was monitored by running gel filtration columns equilibrated with the respective buffers of different pH and ionic strength in the absence of the antigen (Figure 2A, Figure 3). Thus, the antigen present in

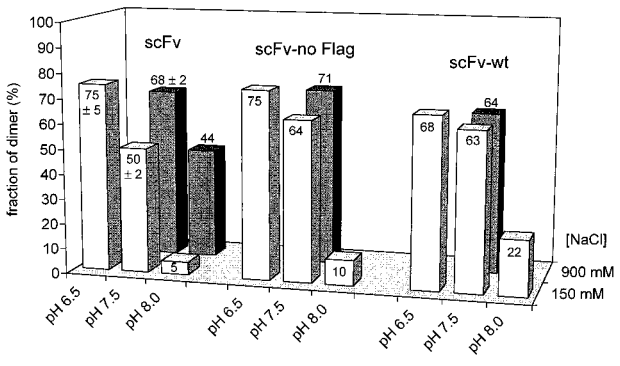

FIGURE 3: Influence of the triple V_H mutation (P40A, S63A, A64D) and the FLAG-peptide on the equilibration rate, as a function of pH and ionic strength. The percentage of remaining dimer was calculated from Superose-12 gel filtration profiles (as shown in Figure 2A for the scFv with FLAG-peptide) obtained in 20 mM Tris, 150 mM NaCl, at pH 7.0, 7.5, and 8.0 in the absence of PC. The fraction of remaining dimer was calculated by fitting the first part of the measured curve, which corresponds to the dimeric fraction remaining after the column run, with a Gaussian function and relating its area to the total area of the measured curves. The white columns show the pH variation at 150 mM NaCl, the gray columns at 900 mM NaCl. The numbers give the percentage of dimer. For the scFv with FLAG-peptide in three cases, multiple measurements were performed, and the mean value with its error is given. *ScFv*, protein with the triple mutation P40A, S63A, A64D; *scFv-no FLAG*, the same protein without the N-terminal FLAG; *scFv-wt*, protein with FLAG, but without the triple mutation.

the sample is separated from the scFv on the column.

The variation of pH between 8.0 and 7.0 (20 mM Tris, 150 mM NaCl) had a dramatic influence on the conversion rate of dimer to monomer. The conversion was fastest at pH 8.0, when a single peak was immediately observed with an elution volume corresponding to a monomer, indicating that under these conditions the conversion rate is fast in comparison with the time for the gel filtration run (~50 min) (Figure 2A). The same observation was made when using BBS buffer, pH 8.0, as a running buffer (Figure 1), indicating that this is a pH effect and not due to any other compounds in the buffer. At pH 7.5, two peaks were found, merging into each other, thus indicating that the conversion rate was of the same order of magnitude as the time needed for a gel filtration run (~50 min). In contrast, at pH 7.0, the main peak still corresponded to a dimer with only a shoulder pointing toward lower molecular weight. The same measurement was carried out at pH 6.5 in Bis-Tris-propane buffer (data not shown), and no difference was seen from the data obtained at pH 7.0. To verify that the observed peaks of Figure 2A monitored the rate of dimer to monomer conversion and not the equilibrium state at a given pH, the center fraction of the peak of the sample run at pH 7.0 was collected, and half of it was re-injected after two different time periods (Figure 2B). The first re-injection, directly after the first run, already showed largely monomeric species, with only a small amount of dimer, which was even smaller at the second re-injection, done 4 h after the first run. This result clearly shows that the equilibrium is on the side of the monomer also at pH 7.0, and that the pH has a strong influence on the equilibration rate.

The characterization of the influence of the ionic strength was carried out in a similar way, using 150 and 900 mM NaCl in the running buffer at pH 7.5 and 8.0. As in the experiment before, dimeric scFv (in BBS, 20 mM PC) was loaded onto the gel filtration column. The result was comparable to that of the pH variations (Figure 3). At any given pH, at high ionic strength (900 mM NaCl) the fraction of dimer was higher than at lower ionic strength (150 mM NaCl), revealing that the conversion rate is accelerated by decreasing the ionic strength. Since the ionic strength depends strongly on the valency of the ions, the influence of $CaCl_2$ on the dimer to monomer conversion rate was also investigated. BBS buffer was used with the addition of 20 mM $CaCl_2$, because in BBS buffer alone the dimer to monomer conversion was very fast (see above). The addition of 20 mM $CaCl_2$ to the BBS buffer decreased the rate of the dimer to monomer conversion significantly (Figure 2C). The elution volume of the major peak corresponded to a dimer, but the increased width of the peak and the appearance of the shoulder showed that dynamic changes are occurring during the run. To define the equilibrium state, the center fraction of the peak was re-injected directly after the first run and once again 4.5 h later. The first re-injection already showed mainly a monomer, but still about 25% dimer, which could be further reduced after 4.5 h. As seen in the case of the pH variation, the equilibrium in the absence of the antigen is on the side of the monomer, and the ionic strength influences only the equilibration rate.

Comparison of the H11 Mutant with the Wild-Type Protein. In the experiments described so far, the triple mutant of the scFv of the antibody McPC603 was used, in which three amino acids in the heavy chain (P40A, S63A, A64D) have been exchanged, since the expression properties of this variant are significantly improved *(29)*. Since the amino acid at position 40 of the heavy chain makes contacts to the light chain, it might thus influence the monomer−dimer equilibration. To investigate whether these mutations and the FLAG-peptide fused to the N-terminus have any influence, the wt protein with the FLAG-peptide (scFv-wt) and the mutant with and without the FLAG-peptide (scFv-noFlag) were analyzed in the same way and compared. Figure 3 shows the summary of all data where the fraction of dimer obtained after chromatography is compared. The measurements were highly reproducible, as indicated by the standard error from some repeated measurements (Figure 3). However, the quantitative evaluation of the measured curves is difficult as the curves do not consist of only two stable species, dimer and monomer, but show a dynamic behavior, and thus the peak is asymmetric and its width is increased (as seen in Figure 2A, pH 7.5 curve). Therefore, the first part of the measured curves, which corresponds to the fraction of dimer still present after the time of one column run, was fitted with a Gaussian function. The percentage of dimer was calculated by relating the area of this curve fit to the integral of the whole curve, including dimer and monomer, which was present before or was formed during the gel filtration run.

The data show that lowering the pH and increasing the salt concentration from 150 mM to 900 mM NaCl resulted in the same behavior for all scFv fragments investigated within experimental error (Figure 3). It appears that the conversion rate at a given condition might be slightly reduced when the FLAG-peptide is missing (scFv-noFlag) and for the wild-type (scFv-wt) in comparison to the scFv. However, for this study it is important to note that all three species initially form dimers after purification from the periplasm

in the presence of antigen, and that the dimer to monomer transition is influenced for all three of them by salt concentration and pH variation in the same way.

Reversibility of the Dimerization. In the above experiments, the conversion of dimeric scFv to the monomeric form was characterized. We next investigated whether the monomer to dimer conversion can be observed when adding the antigen to the monomeric form. To first remove the antigen from the protein, a preparative gel filtration (Superdex 75) was performed with a purified sample of scFv in BBS buffer. An aliquot from the sample applied to the preparative gel filtration column was kept for comparison. The gel-filtrated sample was split into two parts. The first part of this antigen-free sample was used to investigate the dimerization state by analytical gel filtration. For this purpose, it was important to keep the actual amount of dimer and monomer unaltered without further change on the analytical column, and, therefore, the column was equilibrated with a buffer known to ensure slow dimer−monomer conversion, and PBS buffer (50 mM sodium phosphate, 150 mM NaCl, pH 6.5) was chosen. The antigen-free sample was found to be almost completely monomeric (data not shown). The second part of the antigen-free sample at a protein concentration of about 60 μg/mL (2 μM) was supplemented with its antigen PC to a final concentration of 20 mM, and after different incubation times at 4 °C at pH 8.0, the resulting molecular weight was monitored by analytical gel filtration in BBS buffer, pH 8.0, supplemented with 20 mM PC. The gel filtration runs showed no change after adding PC, and even after 12 days incubation, the sample remained monomeric in the presence of PC (Figure 4A). In contrast, the aliquot kept always in the presence of PC, diluted to a comparable concentration, showed a single peak corresponding to the dimer (Figure 4A). These results make clear that once the antigen is removed, the molecule becomes monomeric at these concentrations, even after addition of PC. We therefore conclude that the antigen is necessary to maintain the dimeric state, but is not sufficient to achieve dimerization. Most likely, the antigen does not shift the equilibrium and change the oligomerization state, but simply traps the current state of the molecule, which is dimeric after periplasmic expression, but monomeric after removal of the antigen.

Concentration Dependence of the Dimer. To test the hypothesis of a trapped dimer further, a dilution series of freshly purified sample (1 mg/mL, 1:10, 1:50, 1:100, 1:500 dilution) in BBS with 20 mM PC, pH 8.0, was prepared and incubated over 2 days, and the fraction of dimer was measured by gel filtration, using BBS buffer supplemented with 20 mM PC (Figure 4B).

The results showed that the dimer was stable, as no change was observed at any concentration investigated. This strengthens the hypothesis of a trapped dimer, because if the reaction had been at equilibrium, the proportion of dimer to monomer should depend on the concentration. The fact that even after several days only dimeric molecules were seen in gel filtration requires that this trapped species has a long half-life, meaning that the conversion to the thermodynamically favored monomeric species is blocked by a high activation barrier. To prove this point, a 1-year-old sample, stored in BBS with 20 mM PC, pH 8.0, at 4 °C, was analyzed and compared with a freshly prepared one (data not shown). The 1-year-old sample still gave a reasonable elution profile

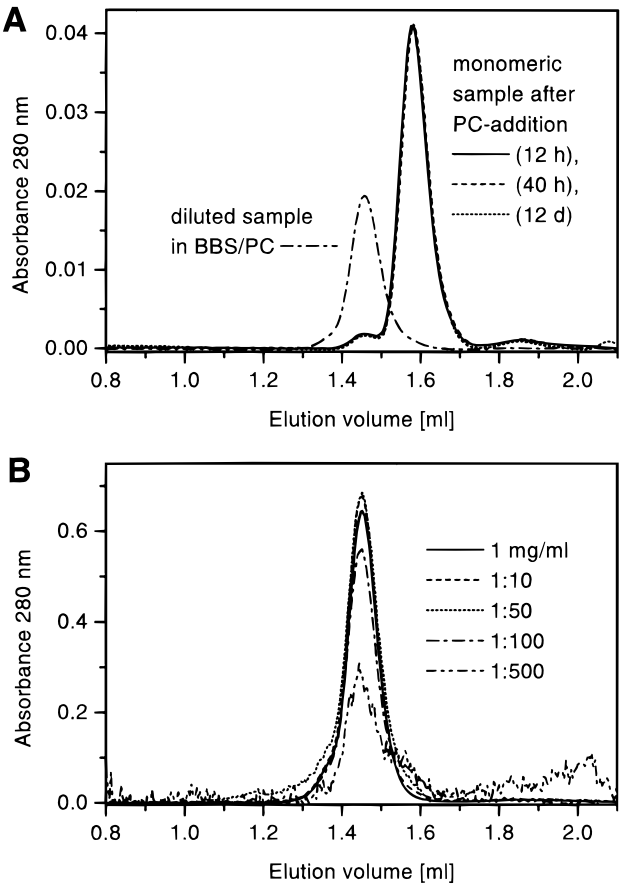

FIGURE 4: Elution profiles of Superose-12 gel filtration column runs. The running buffer was in all cases BBS, pH 8.0, with 20 mM PC. (A) A PC-free sample of scFv was incubated with PC for 12 h (—), 40 h (- - -), and 12 days (•••). For comparison, a PC-containing sample was diluted with the running buffer to a similar concentration (— • —). (B) Concentration dependence of scFv in its dimeric form. A PC-containing sample of 1 mg/mL (—) was diluted 1:10 (- - -), 1:50 (•••), 1:100 (— • —), and 1:500 (— •• —). Shown are the normalized elution profiles, multiplied by the dilution factor.

with mostly dimeric species with only a small shoulder pointing to lower molecular weight, showing that the dimer is indeed very stable over time. Furthermore, even after lyophilization in the presence of antigen, monomer remained in excess (*37*), as tested with a further sample (data not shown).

Comparison of Different Expression Methods. We further investigated whether the amount of dimer may depend on the folding history of the protein, and thus on the expression method. As described above, the single-chain Fv fragment is completely dimeric, when functionally expressed in the periplasm of *E. coli* and purified by PC-affinity chromatography by elution with antigen (Figure 1). It makes no difference whether the cells had been frozen for storage or used directly in purification. In contrast, the same single-chain Fv fragment, when expressed in inclusion bodies and refolded in vitro, leads to predominantly monomeric protein with only a small amount of usually 10−20% dimer (data not shown). Thus, dimerization on the affinity column can be excluded, as the protein is purified using the same affinity purification scheme in both cases. Therefore, the amount of dimer observed after affinity purification depends strongly on the expression method. The differences between periplasmic folding and in vitro refolding include the protein

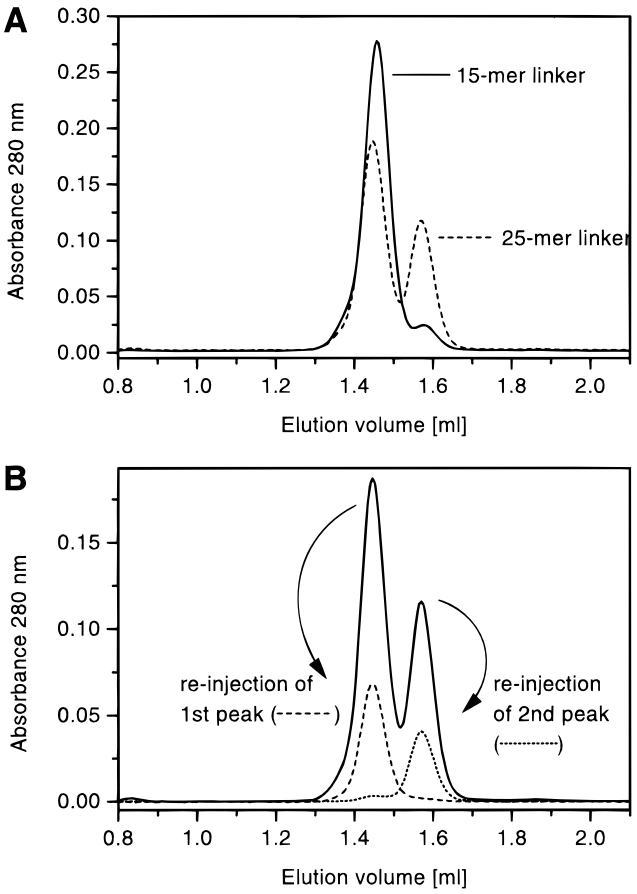

FIGURE 5: Elution profiles of Superose-12 gel filtration column runs. The sample and running buffer was BBS buffer with 20 mM PC, pH 8.0. (A) Comparison of scFv with 15-mer (—) and 25-mer linker (- - -). (B) Investigation of the trapped form in the presence of the antigen PC for the scFv with a 25-mer linker. Re-injection of the dimeric fraction (- - -) and monomeric fraction (•••) of the two peaks obtained for the scFv with a 25-mer linker (—).

concentration, the presence and nature of disulfide-forming factors, and also potentially the presence of other noncovalent chaperones during folding. The concentration of single-chain Fv fragment in the periplasm may be roughly estimated to be at least 500 μM or 13 mg/mL, if one assumes only 5 mg of functional protein in a 2 L culture of OD_{550} 2.0, treats the cell as a capped cylinder with 1 μm in diameter and 2 μm in length, and assumes that the periplasmic space is about 10% of the total cell volume *(38)*. In contrast, the single-chain Fv fragment concentration in the standard refolding mixture is only about 1 μM (27 μg/mL) *(37)*. Thus, one striking difference between both methods is the concentration difference during folding of the protein, which is over 500-fold. Of course, cellular factors might influence dimer formation as well, but we assume the high concentration during periplasmic folding plays a critical role in dimer formation, which then gets trapped in the presence of the antigen.

Influence of Linker Length. As seen in Figure 1, the linker is absolutely necessary for dimerization, as the respective Fv fragment, which has no linker, remains monomeric. To obtain further insight into the properties of the dimers, the influence of the antigen on the same scFv molecule, but with a longer linker with 5 repeats instead of 3 of the $(G_4S)_n$ motif, was compared by size-exclusion chromatography under the same conditions (Figure 5A). In the single-chain construct with the 25-mer linker, expressed in the periplasm, the dimeric fraction was reduced to about 60% in the presence of antigen.

These results indicate that the percentage of dimer is higher with shorter linkers, which is obvious for very short linkers, where only dimers can be formed for steric reasons *(12, 15, 17)*. However, as shown before, the single-chain Fv fragment with the 15-mer linker quantitatively forms monomers in the absence of antigen, which makes steric hindrance unlikely as an explanation. Furthermore, the molecule with the 25-mer linker indicates that elongation of linkers might not be sufficient to obtain solely monomers in periplasmic expression.

In a further experiment, the stability of dimeric and monomeric fractions obtained for the scFv with the 25-mer linker in the presence of the antigen was analyzed. A gel filtration run was performed in BBS buffer in the presence of the antigen and fractionated. The fractions of the dimeric and of the monomeric species, respectively, were re-injected (Figure 5B). No change was observed; the dimeric fraction remained dimeric, and the monomeric one remained monomeric. This strengthens the assumption made above that the dimer gets trapped in the presence of the antigen.

DISCUSSION

The phenomenon of dimerization or even higher oligomerization of single-chain antibody Fv fragments has been reported previously *(12−14, 16, 24, 39−41)* and has been investigated in the context of the linker length *(15, 17, 42)*. However, using the same linker length, different antibodies were found to give different distributions *(14, 39, 41)*. While the structures of both a diabody *(18)* and a triabody *(19)* have been solved, the factors influencing the monomer−dimer equilibrium have remained unclear.

Here, we have investigated this problem by using a model system which could easily be manipulated. We could identify additional factors influencing the dimerization of a single-chain Fv fragment and can thus propose a general explanation. Using the model antibody McPC603, we could conveniently change the dimerization state by adding and removing antigen, since its on- and off-rates are very fast *(43)* and therefore do not delay the interconversion rate. First, the expression method was shown to have a profound effect. Only dimeric single-chain Fv fragment was found after functional periplasmic expression, whereas after expression in inclusion bodies and refolding in the presence of antigen, the fraction of dimer was usually less than 20%. Second, the presence of the antigen clearly influences the oligomerization state. Dimeric scFv quantitatively converts to its monomeric form when removing the antigen. In contrast, when adding antigen to the monomeric form, it remains unchanged. Similarly, when adding it to the dimeric form, the dimer remains stable. Third, ionic strength and pH were shown to influence the rate of the dimer to monomer conversion.

To explain our findings, we propose the following model of a trapped dimeric form (Figure 6). The model requires four forms of the scFv, a closed monomer and dimer and in addition two corresponding open forms (open monomer, open dimer), in which the interface between V_H and V_L is

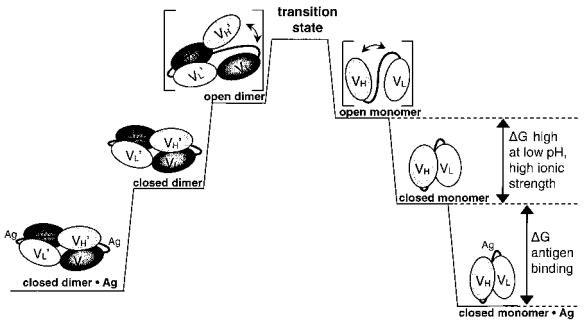

FIGURE 6: Model to explain the occurrence of dimers and monomers under the various conditions. The model describes the monomeric and dimeric species in two different forms, an open and a closed form. The closed form represents the stable molecule with an intact V_H-V_L interface, which is further favored in the presence of antigen. In the open form, the interaction between V_H and V_L is weakened, and the two domains can separate to change the oligomerization state. The closed monomeric form is shown with a lower free energy with respect to the closed dimeric form, which represents the experimental conditions investigated (35 μM or less). Increasing the concentration would shift the equilibrium toward the dimeric form, according to the law of mass action.

destabilized. The dimeric form is a product of domain swapping *(22, 23)* and presumably forms a diabody (see below). Figure 7 shows the model of this proposed McPC603 diabody. The closed forms are favored when the antigen is present, because it stabilizes the molecule by simultaneously binding to V_H and V_L *(27)*. In contrast, the open forms are intermediates which are only populated in the absence of the antigen. The energy barrier between closed monomer and closed dimer depends on the presence of antigen, on the ionic strength, and on the pH. The law of mass action requires that the relative energy of monomer and dimer be dependent on the absolute concentration (see below).

The dimer exists most likely in the form of a domain-swapped diabody, which is supported by the following experiments. First, the gel filtration runs revealed only dimers, monomers, or a mixture of both but never higher aggregates. Therefore, the dimer must have a defined structure. Second, unspecific aggregates should also be found for the Fv fragment, which are not observed, whereas a diabody requires a linker between V_H and V_L. Third, the dimeric species was found directly after affinity purification and must therefore be able to bind its antigen, which also indicates a defined state. Fourth, NMR results (Freund et al., in preparation) with different portions of dimer and monomer showed no significant difference in chemical shifts, thus requiring that both structures are very similar.

In the absence of antigen, the monomeric form is favored under all conditions investigated and represents the equilibrium state as demonstrated in Figure 2B,C. As the interface between V_H and V_L is not very stable in the absence of bound antigen—for the Fv fragment of McPC603, the V_H-V_L dissociation constant was estimated to be 10^{-6} M *(5)*—the open forms should be present to a significant extent. The open forms can react either in an intramolecular reaction to a monomer or in an intermolecular reaction to a dimer, respectively. The law of mass action requires that the equilibrium constant between monomer and dimer depends on the effective concentration of the domains in the scFv molecule, and the position of the equilibrium depends also on the absolute concentration. Even though we can only roughly estimate the local concentrations, these must be significantly higher than the maximal concentration of 35 μM used in the experiments described here, and thus the observed equilibrium was on the side of the monomer.

In the presence of antigen, the closed monomeric and dimeric forms are favored and get trapped, which means that the current state is frozen and the equilibrium cannot be reached on the experimental time scale. This trapping is best seen by the fact that the dimeric form remains dimeric when diluted in the presence of antigen, and the monomeric form remains monomeric after addition of antigen. The fact that the dimer is trapped only in the presence of the antigen is consistent with this model, since the presence of the antigen stabilizes the V_H-V_L interaction, thus increasing the activation energy needed to separate both domains, which is an essential intermediate to form a monomer. The same holds true for the monomer: in the presence of the antigen, the closed form is favored, and thus the concentration of the open form is so low that the formation of a diabody is kinetically forbidden, although it might be thermodynamically favored at very high concentrations.

The differences in dimeric fraction resulting from the expression method can also be best explained by a trapped dimer. During periplasmic expression, the dimeric form is favored, probably due to the higher concentration during folding in the periplasm, which was estimated to be at least 500-fold higher than for in vitro refolding. In addition, some other unknown factors in the periplasm might promote dimer formation. Most likely, the affinity purification in the presence of antigen and the elution with antigen trap the current state of the molecule, which is dimeric when expressed in the periplasm, but predominantly monomeric after refolding in dilute solutions. Thus, we can exclude that the affinity purification leads to dimerization; however, we still lack an explanation why the dimeric form after periplasmic expression is retained during the cell disruption. Possibly, the high ionic strength and the presence of other cell-specific proteins stabilize the antibody fragment, thus increasing the activation barrier to the open form. In gel filtration experiments, we demonstrated that the observed dimer after periplasmic expression is *not* the equilibrium state in the absence of antigen, which strengthens the proposal that this dimer is a trapped species. This assumption is further confirmed by the fact that even the single-chain Fv fragment with a 25-mer linker is found to contain a dimeric fraction under these conditions.

The conversion from the dimeric to the monomeric form, and thus the energy barrier, depends not only on the presence of the antigen but also on the ionic strength and the pH of the buffer, as these factors also have an influence on the stability of the interface between V_H and V_L. We found in the case of the antibody McPC603 that the conversion to the monomeric species is faster at physiological conditions than at higher salt concentrations. This can be rationalized by higher salt concentrations stabilizing the interface by increasing the strength of hydrophobic interactions. These conditions disfavor the open form which is necessary for the conversion to the monomer. The influence of the pH is more difficult to rationalize and might vary for different molecules. We have not yet been able to pinpoint the titratable groups responsible for the pH-dependent stability

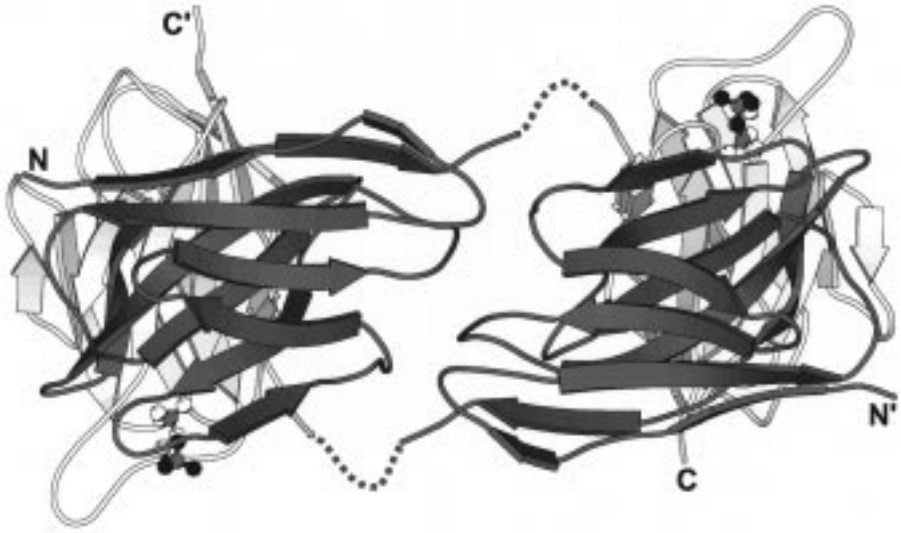

FIGURE 7: Structural model of the diabody form of the single-chain Fv fragment McPC603. V_H is drawn in dark gray, and V_L in light gray; the antigen is shown as balls-and-sticks, and the linker is indicated by a dotted line. The model was built in INSIGHT II using the structures of a diabody *(18)* (PDB file 1LMK) and of the Fab fragment of the antibody McPC603 *(27)* (PDB file 2MCP). The secondary structure elements were assigned according to Kabsch and Sander *(51)*, and the representation was made with MOLSCRIPT *(52)*.

of the interface. The interface becomes destabilized in this antibody above pH 7, which suggests that at least one titratable protonated group is needed for stability. While one might propose that either AspL97 or GluH35 is still protonated at neutral pH and the protonated form is needed for stability, pK_a calculations (A. Caflisch, unpublished data) do not indicate a value that high for either of them. Other candidates are LysL36 and HisL98, but they are not located directly in the interface and thus do not seem to be prime suspects. The pH dependence of PC binding has been experimentally investigated in the range of pH 4 to pH 9 *(44)*, and a drop in affinity was noted below pH 6, which also does not explain the observed behavior, as the ability to bind the antigen and thus also the antigen-mediated interface stability decrease with pH *below* 6, while we are searching for an explanation why it decreases *above* pH 7. We do not suggest that the observed pH-dependent behavior is a general feature of scFv fragments. Instead we propose that this is peculiar to McPC603, because of the nature of titratable groups in the interface, which allowed us to conveniently vary the stability in a reversible manner. However, it is likely that the salt dependence is a very general phenomenon, as all V_H-V_L interfaces have substantial hydrophobic components *(2, 45)*.

Earlier studies with the McPC603 single-chain Fv fragment were—although we were not aware of this phenomenon—always carried out with the monomeric form, since for functional analysis the antigen was always removed by extensive dialysis, and gel filtration runs were performed in BBS buffer in the absence of the antigen, where the scFv is converted fast enough into its monomeric form *(35−37)*. On the other hand, we are now able to explain the short T_2 relaxation times of NMR spectra of the scFv obtained in the presence of the antigen *(46)* with the presence of some dimeric fraction, which are further stabilized at the millimolar concentrations used for NMR (Freund et al., in preparation). It should be stressed, however, that the chemical shifts of Fv and monomeric and dimeric scFv are virtually identical. Furthermore, all kinetic folding experiments were carried out under conditions where only monomers are obtained.

An important conclusion from our studies is that single-chain Fv fragments might be obtained in a dimeric form directly after periplasmic expression, but can convert to the monomeric form under certain physiological conditions, which will depend on the V_H-V_L interface energy. Furthermore, by stabilizing the interface, one should be able to maintain the monomeric form even at high concentrations as used in NMR, crystallography, and lyophilization. While these experiments have all been carried out with only one scFv fragment and closely related variants, this model system, because of its convenient adjustment of V_H-V_L energy by ionic strength, pH, and presence of antigen, allows some general predictions. We have also preliminary data for other single-chain Fv fragments (S. Jung, unpublished results), which support the assumption of a general phenomenon.

In principle, the monomeric form is the thermodynamically stable state of a scFv fragment in dilute solutions, even with a 15-mer linker, at least in the V_H-linker-V_L orientation. A longer linker makes the dimer even less favored. However, if the protein is produced in an expression system, where it folds at concentrations of at least 0.5 mM, an estimate for the *E. coli* periplasm, the dimer may form initially. In the case of McPC603, the V_H-V_L interface of the Fv fragment is not very stable, witnessed by the dissociation constant of 10^{-6} M *(5)* for V_H and V_L. A range of dissociation constants has been observed for Fv fragments from antibodies, and they can be as low as 10^{-7} and 10^{-9} M *(47−50)*. Thus, in cases where the interface is intrinsically more stable than the one of McPC603, the reequilibration of dimer to monomer may be incomplete or hardly occurring, even in the absence of antigen. Thus, different scFv fragments may be trapped in the dimeric state to different degrees, explaining the observation that different scFv molecules give rise to widely varying monomer-to-dimer ratios, even when they have the same linker *(13, 14)*. In conclusion, to obtain defined monomeric fragments, it is advisable to use longer linkers than 15-mer, especially in the V_L-linker-V_H orientation. Furthermore, conditions of rapid equilibration or, in extreme cases, folding at low concentrations need to be considered. Fortunately, the monomeric scFv appears to be

the thermodynamically favored form. The domain swapping phenomenon of such proteins *(22, 23)* is a factor which needs to be understood and fully controlled for successful protein design.

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