# Miniantibodies: Use of Amphipathic Helices To Produce Functional, Flexibly Linked Dimeric $F_V$ Fragments with High Avidity in *Escherichia coli*†

Peter Pack and Andreas Plückthun*

*Genzentrum der Universität München, c/o Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, FRG*

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ABSTRACT: We have designed dimeric antibody fragments that assemble in *Escherichia coli*. They are based on single-chain $F_V$ fragments, with a flexible hinge region from mouse IgG3 and an amphiphilic helix fused to the C-terminus of the antibody fragment. The sequence of the helix was taken either from that of a previously reported four-helix bundle design or from a leucine zipper, optionally extended with a short cysteine-containing peptide. The bivalent fragments associate in vivo, either with covalent linkage or with a monomer–dimer equilibrium, and results from ultracentrifugation sedimentation studies and SDS–PAGE are consistent with dimers. All constructs are able to bind to surface-bound antigen under conditions in which only bivalent but not monovalent antibody fragments bind. The covalent bundle helix construct shows binding characteristics nearly identical to those of the much larger whole mouse antibody, resulting in substantially more stable immunoglobulin–antigen complexes than in the case of monovalent fragments. This modular design of natural and engineered protein domains directly leads to a boost of avidity, and it allows the construction of bispecific antibody fragments in functional form in *E. coli*.

Recent advances in the production of recombinant antibody fragments in *Escherichia coli* (Skerra & Plückthun, 1988; Better et al., 1988; Plückthun, 1990, 1991) make it now possible to study antigen–antibody binding interactions by the combined use of protein engineering and structural studies (Glockshuber et al., 1990a,b, 1991; Bhat et al., 1990; McManus & Riechmann, 1991; Steipe et al., 1991). The modification of the recombinant antibody is greatly facilitated by the use of these secretory *E. coli* expression systems, since functional proteins are obtained.

Antibody fragments that have been found most advantageous for antigen-binding studies are the $F_V$ fragment of the antibody (Skerra & Plückthun, 1988), the covalently linked single-chain $F_V$ fragment ($scF_V$) (Glockshuber et al., 1990a; Bird & Walker, 1991), and the $F_{ab}$ fragment (Better et al., 1988; Plückthun & Skerra, 1989). It has been demonstrated that all these fragments are compatible with secretion in *E. coli* and have thermodynamic affinities to monomeric antigen identical to those of the whole antibody, as shown in the case of the phosphocholine-binding antibody McPC603 (Skerra & Plückthun, 1988; Glockshuber et al., 1990a; Skerra et al., 1990). However, all recombinant fragments produced in *E. coli* that have been reported so far are monovalent.

To be able to harvest the gain in "avidity" (an empirical measure of the increased apparent binding constant caused by the entropic effect; Crothers & Metzger, 1972) in bivalent fragments, we investigated ways to engineer small "dimerization domains". We show in this paper that amphiphilic helices are suitable for bringing about this dimerization, that they are compatible with in vivo transport in *E. coli*, and that the dimers assemble in vivo. Therefore, it is possible to secrete bivalent "miniantibodies" based on dimeric single-chain $F_V$ fragments.

These experiments were carried out using the well-characterized phosphocholine-binding antibody McPC603 (Perlmutter et al., 1984), of which the crystal structure is known (Satow et al., 1986).

## Experimental Procedures

*General Techniques.* Recombinant DNA techniques were based on those of Sambrook et al. (1989). Functional expression of the single-chain $F_V$ fragments and the minianti-

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* To whom correspondence should be addressed.

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bodies in *E. coli* JM83 was carried out with vectors similar to pASK-lisc (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). SDS–PAGE was carried out as described by Fling and Gregerson (1986). Concentrations of affinity-purified proteins were measured by $OD_{280}$ using calculated extinction coefficients (Gill & von Hippel, 1989).

*Functional Expression of the Single-Chain $F_V$ Fragments and the Miniantibodies 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 grown another 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). For small-scale preparations, the protein was prepared from the periplasm. The periplasmic extract was prepared by centrifuging the cell culture (10000*g*, 4 min), shaking the cell pellet for 90 min at 4 °C with 0.1 volume of BBS containing 1 mM EDTA, and collecting the supernatant after another centrifugation.

*Ultracentrifugation.* Affinity-purified protein samples were loaded at a concentration of 1 mg/mL in BBS buffer containing 5 mM phosphocholine. The same buffer was placed in the reference cell. A Beckman Model E centrifuge was used at 30 000 and 36 000 rpm. The temperature was 20 °C in the sample compartment. The protein was detected by recording $OD_{280}$. The sedimentation coefficient *s* was determined from the slope in ln *r* vs *t* plots.

*Functional ELISA.* The ELISA plates (Nunc, Macrosorp) were coated with 400 μg/mL phosphocholine–BSA in PBS buffer (20 mM phosphate, pH 7.2, 115 mM NaCl). The hapten reagent was prepared from (nitrophenyl)phosphocholine (Sigma), which was reduced and diazotized essentially as described (Chesebro & Metzger, 1972), and reacted by azo coupling to BSA (Sigma) in borate/saline buffer (52.5 mM sodium borate, pH 9, 120 mM NaCl) at 4 °C for 48 h with subsequent dialysis against PBS. After the noncoated plate surface was blocked with 5% skim milk (Nestle) in PBS buffer for at least 2 h, the periplasmic extract or the purified protein was incubated in BBS buffer on the plate for 90 min at room temperature. After thorough washing (three times), remaining functional antibody fragments were detected according to standard procedures (Harlow & Lane, 1988) with rabbit anti-McPC603 serum and anti-rabbit immunoglobulin linked to peroxidase (Sigma) according to Gallati (1979).

## Results

*Molecular Design.* The oligomerization domains were selected for a fairly small molecular weight and for being compatible with transport of the fusion protein through the membrane, which is vital for correct folding of antibody domains in *E. coli*. Also, they should not influence the folding or yield of the antibody domains. We chose to investigate amphiphilic helices.

With amphipathic helices, two basic molecular structures seemed feasible: a four-helix bundle or a coiled-coil helix. The design and formation of helix bundles have been studied extensively (Eisenberg et al., 1986; Ho & DeGrado, 1987; Regan & DeGrado, 1988; Hill et al., 1990), but only circumstantial evidence for the precise molecular association is currently available, as no crystal structure has been reported yet except that of a shortened version of the synthetic helix-forming peptide (Eisenberg et al., 1986). Since the arrangement of the helices is antiparallel in the naturally occurring bundles (Richardson, 1981), the four-helix bundle may be formed from four separate molecules (each contributing one helix), two helix–dimer molecules, or one molecule containing a four-helix construct. Since we were interested in investigating whether dimerization or tetramerization of the fusion protein would take place, the single-chain $F_V$ fragment was extended by one helix of the sequence designed by Eisenberg et al. (1986). This construct is termed scHLX. In an additional construction, scHLX was further extended by a small hydrophilic tail ending in a cysteine (scHLXc; Figure 1A,B). The helices from scHLX and scHLXc will be referred to as "bundle helices". Despite the four-helix prototype, it appears as if the predominant forms of scHLX and scHLXc are dimers (see below).

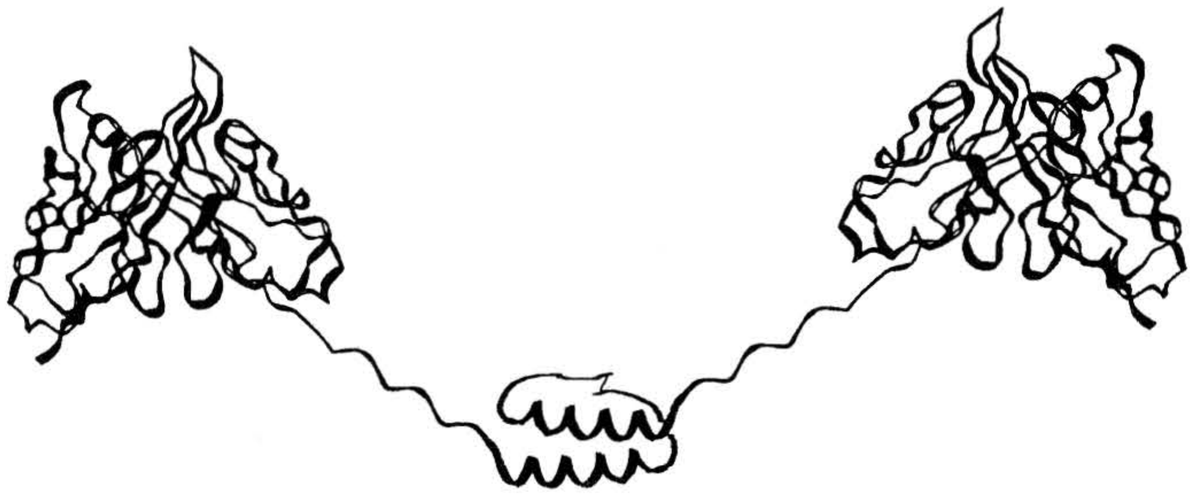
The helices were not fused directly onto the antibody $F_V$ fragment but instead were separated by a hinge peptide. The relatively long "upper hinge" of mouse IgG3 was used, since fluorescence spectra and complement fixation experiments (Dangl et al., 1988; Tan et al., 1990; Burton, 1990) showed a correlation between the length of the upper hinge region and the flexibility of the two $F_{ab}$ fragments with respect to each other in the whole antibody. While the upper hinge regions of mouse IgG2a or IgG2b appear to give rise to slightly higher flexibility than that of mouse IgG3, the upper hinge of IgG3 is more hydrophilic and was therefore considered to be most compatible with membrane transport of the fusion protein into the periplasmic space of *E. coli*, which is necessary for functional expression. This design strategy thus allows the dimerized (or oligomerized) fragments to accommodate surface-bound antigen separated by various distances, just as in a natural antibody.

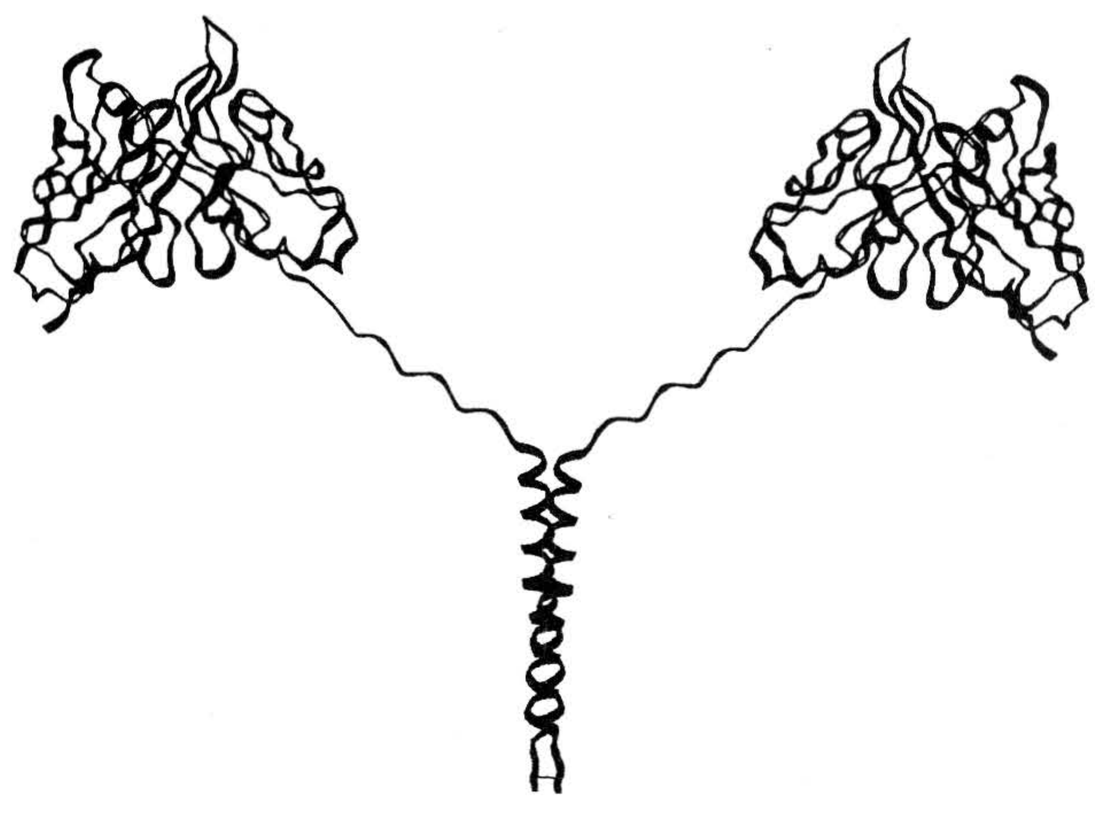
In the second design, we took advantage of recent progress in the molecular understanding of coiled coils. The sequence chosen was that of a "leucine zipper" (Landschulz et al., 1988; O'Shea et al., 1989), a dimerization device used in eukaryotic transcription factors such as GCN4 from yeast. Recent biochemical studies with synthetic peptides (O'Shea et al., 1989) and the solved crystal structure (O'Shea et al., 1991) show that the leucine zipper is a parallel coiled-coil helix. The leucine zipper has also previously been attached to other proteins (Hu et al., 1990; Blondel & Bedouelle, 1991). Again, the zipper helix was fused alone (scZIP) or fused with a cysteine-containing extension (scZIPc) to the $scF_V$ protein via the hinge peptide (Figure 1).

*Protein Characterization.* All resulting fusion proteins could be purified by hapten affinity chromatography, as expected for correctly folded antibody fragments of normal functionality. The amounts isolated were similar to those of the $scF_V$ fragment. This shows that none of the helix peptides interfere with folding, secretion, or antigen binding of the $scF_V$ fragment. Therefore, all constructions are suitable for use in *E. coli* expression systems.

Proteins of the expected sizes were observed on SDS–PAGE under reducing conditions (data not shown). Under nonreducing conditions, covalent dimers are seen both for the bundle helix with the cysteine-carrying tail and for the leucine zipper with the cysteine-carrying tail (Figure 2).

To test the functionality of the fusion proteins and to quantitate the gain in avidity by the dimerization, the fragments were tested on ELISA plates (Figure 3) coated with phosphocholine-derivatized BSA. An enormous gain in binding, and thus sensitivity, is observed for all miniantibody constructs, compared to the monomeric $scF_V$ fragment. This





| construct | Fv-fragment | hinge | amphipatic helix | *cys-tail* |
|---|---|---|---|---|
| *scFv* | **VH-linker-VL** | | | |
| *scHLX* | **VH-linker-VL**- | PKPSTPPGSS- | GELEELLKHLKELLKGEF | |
| *scHLXc* | **VH-linker-VL**- | PKPSTPPGSS- | GELEELLKHLKELLKGPR | *KANSRNC* |
| *scZIP* | **VH-linker-VL**- | PKPSTPPGSS- | RMKQLEDKVEELLSKNYHLENEVARLKKLVGER | |
| *scZIPc* | **VH-linker-VL**- | PKPSTPPGSS- | RMKQLEDKVEELLSKNYHLENEVARLKKLVGER | *GGCGG* |

FIGURE 1: (A, top left) Molecular model of the dimeric scHLXc miniantibody construct. The antibody $F_V$ fragment of McPC603 was taken from the X-ray coordinates of Satow et al. (1986). The hinge region was modeled according to a polyproline-II helix with $\phi = -78°$ and $\psi = 149°$, consistent with the limited structural information available about hinge regions (Marquart et al., 1980; Kessler et al., 1991). A standard $\alpha$-helix with $\phi = -57°$ and $\psi = -47°$ was used for the amphiphilic helix. The Cys-tail peptide is presumably disordered, and the structure drawn should be taken only as a guide to the topology. There is currently no unambiguous evidence for an antiparallel arrangement of the helices. (B, top right) Molecular model of the scZIPc miniantibody construct. The parameters are identical with those in (A), except that a parallel coiled coil with about a quarter turn of the superhelix was assumed for the leucine zipper part (O'Shea et al., 1991). (C, bottom) Sequences of the miniantibody constructs. The name of the construct is given on the left. All constructs are derived from the single-chain $F_V$ fragment of the McPC603 as described by Glockshuber et al. (1990a). This is immediately followed by the sequences of the hinge and the amphipathic helix as indicated. The cysteine tail present in the constructs with the suffix *c* is emphasized in italics.

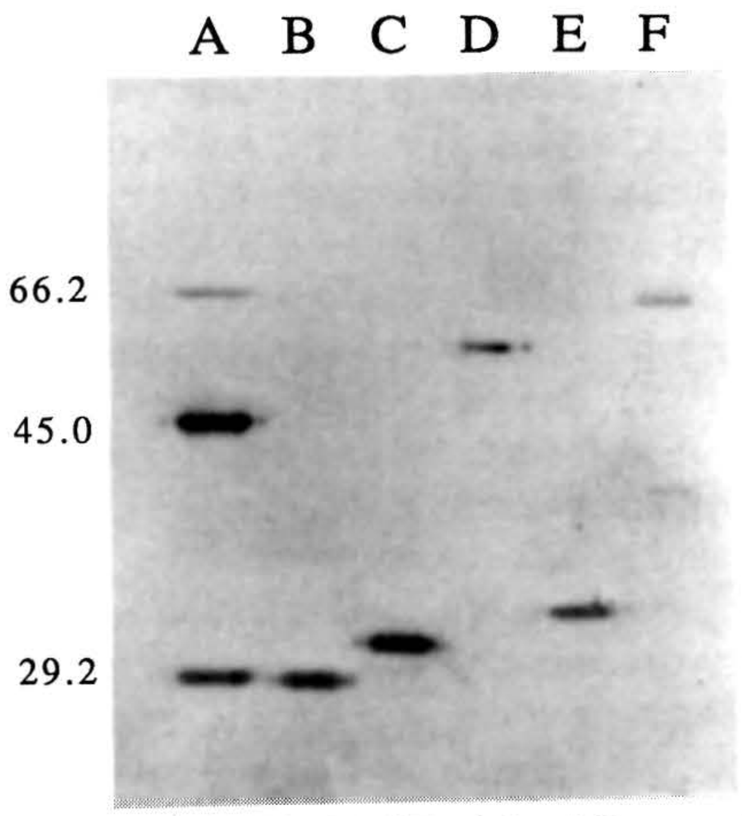



FIGURE 2: Silver-stained SDS–PAGE of the different miniantibody constructs under nonreducing conditions: (A) protein MW standard; (B) single-chain $F_V$ fragment; (C) scHLX; (D) scHLXc; (E) scZIP; (F) scZIPc.

is consistent with the simultaneous binding of two or even more binding sites to the same surface.

The avidity of the fusion protein scHLXc was comparable to that of the natural antibody McPC603, which could be detected with antigen-coated ELISA, while the monomeric $scF_V$ fragment could not (Figure 3B). The thermodynamic affinity of the natural antibody to soluble phosphocholine is about $1.6 \times 10^5$ $M^{-1}$ and is thus relatively weak (Metzger et al., 1971), and this is apparently not sufficient for a monomeric fragment–hapten complex to survive the repeated washing steps of a functional ELISA (Kemeny & Challacombe, 1988).

Figure 3 also shows some difference in binding among the various constructs. The fragment with a bundle helix not containing the covalent dimerization anchor (scHLX) did not perform as well as the cysteine-containing counterpart, probably because the equilibrium is more on the side of the monomer. This shows that the covalent linking of the dimer is beneficial for the bundle-helix construct but that assembly takes place in the absence of covalent linking as well. The noncovalent leucine zipper (scZIP) behaves very similarly to the bundle helix (scHLX), suggesting comparable binding properties. Interestingly, however, the covalent linking of the leucine zipper (scZIPc) does not improve the binding to the ELISA plate but rather makes it somewhat poorer, although the data on the purified protein are consistent with at least the major portion of the protein being present as a covalently linked dimer (Figure 2). The faster migrating band in lane F may contain incorrect disulfide bonds in one of the arms, since this band disappears upon reduction. At identical molar protein concentrations, the single-helix construct with the cysteine tail is clearly binding best to the hapten-coated surface. This covalent dimer is apparently able to accommodate best to the surface-bound antigen to bind with both arms simultaneously. This must be the most crucial feature, because the $scF_V$ fragment is detected at least 2 orders of magnitude more poorly.

Functional binding of the miniantibodies to the ELISA plate was demonstrated by showing that the binding can be inhibited with soluble phosphocholine. Below $10^{-11}$ mol of protein per well, binding was almost completely inhibited by 1 mM

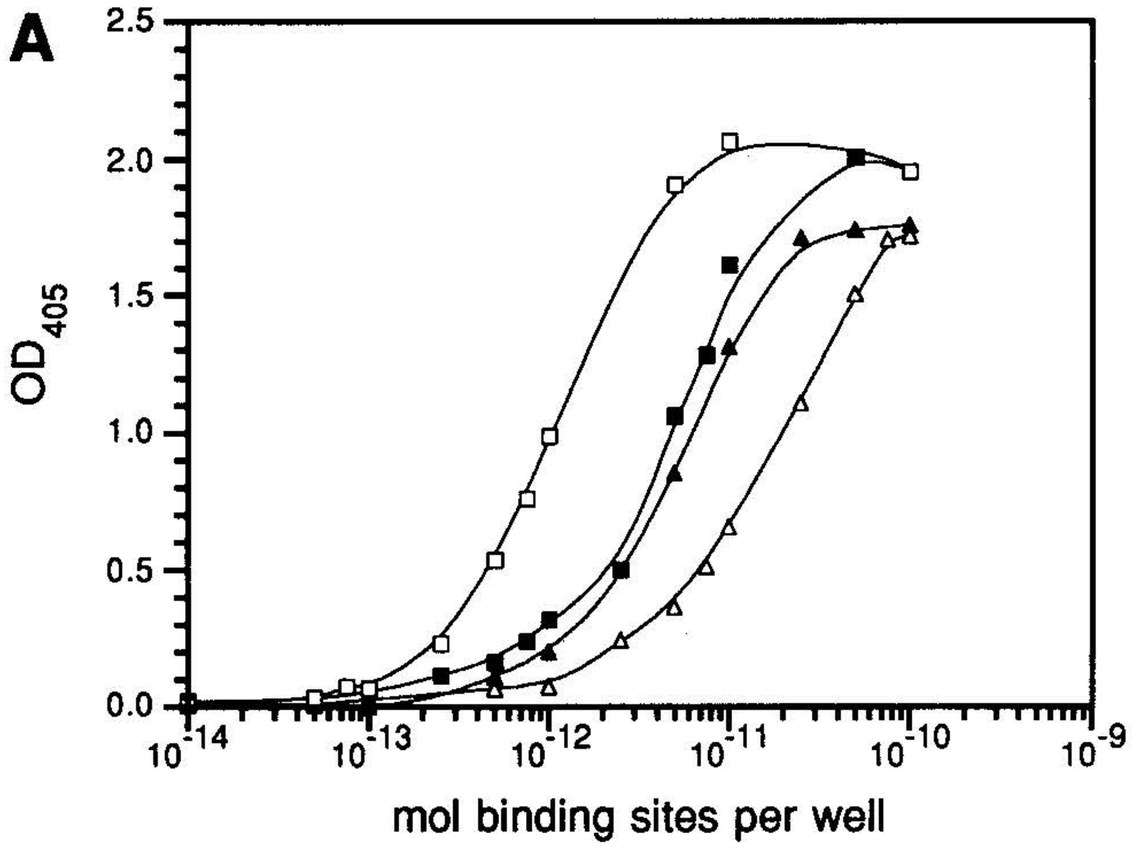



FIGURE 3: Functional ELISA of the various miniantibodies. The concentrations of the affinity-purified proteins, measured by $OD_{280}$, refer to the number of binding sites per well. The ELISA plates were coated with phosphocholine–BSA, and the purified phosphocholine-specific miniantibody proteins were bound and detected by an anti-McPC603 antiserum. Symbols: (A) (□) scHLXc, (■), scHLX, (▲) scZIP, and (△) scZIPc; (B) (◆) McPC603 whole antibody, (□) scHLXc, and (○) single-chain $F_V$ fragment.

phosphocholine. Above $10^{-11}$ mol of protein per well, however, the inhibition was incomplete and may signify nonfunctional adsorption of the fragments onto the well surface. We believe, therefore, that the binding observed for the monomeric $scF_V$ fragment at such concentrations is largely nonspecific.

To test this conclusion more quantitatively, and to demonstrate the practical utility of these fragments, we titrated the ELISA incubation mixture with various amounts of phosphocholine directly in a periplasmic extract of *E. coli* expressing scHLXc (Figure 4). Half-maximal inhibition of BSA–PC binding is already observed at about 5 $\mu$M soluble phosphocholine, and this is in the range expected for the association constant of $1.3 \times 10^5$ $M^{-1}$ measured for the $scF_V$ fragment of McPC603 (Glockshuber et al., 1990a).

For further characterization of the oligomerization state of the various constructs, we determined the sedimentation velocity of the purified proteins by analytical ultracentrifugation (Table I). The proteins with a dimerization domain all had a significantly higher sedimentation coefficient than the monovalent $scF_V$ fragment. Only in the case of the noncovalent single-bundle-helix protein (scHLX) and the noncovalent leucine zipper protein (scZIP) was a small fraction of faster sedimenting material visible, which might constitute higher order oligomers (perhaps a tetramer in the case of scHLX). The data in Table I for the major fraction are consistent with

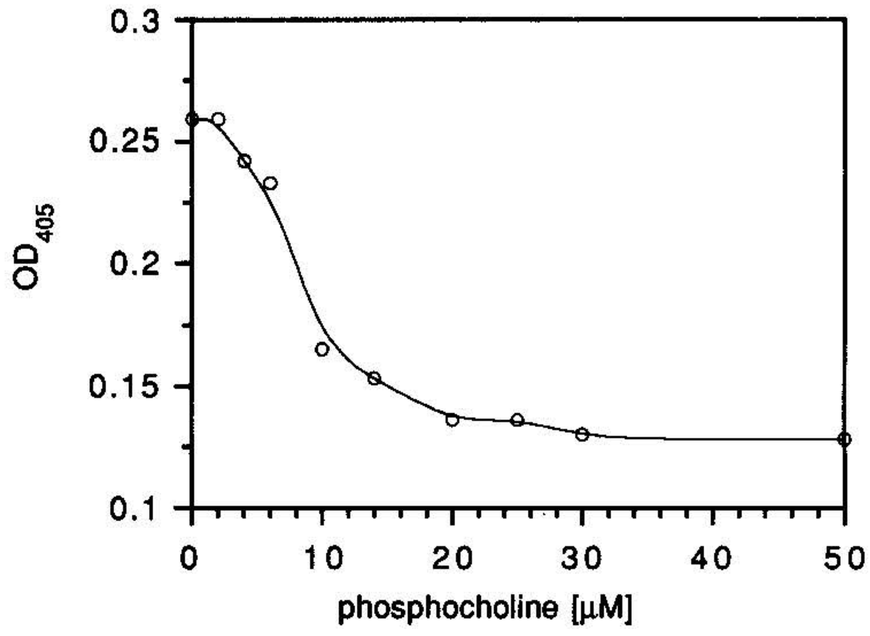



FIGURE 4: Inhibition of binding of the miniantibody scHLXc out of the periplasmic extract to the ELISA plate by soluble phosphocholine. The ELISA plate was coated with phosphocholine–BSA, and the binding phosphocholine-specific miniantibody proteins were assayed with an anti-McPC603 antiserum after extensive washing.

Table I: Ultracentrifugation Studies of Different Miniantibody Constructs

| construct | MW | $s_{20} \times 10^{13}$ | $\left(\frac{MW_{dimer}}{MW_{mono}}\right)^{0.66}$ | $\left(\frac{MW_{dimer}}{MW_{mono}}\right)^{0.5}$ | $\frac{S_{exp}}{S_{scFv}}$ |
|---|---|---|---|---|---|
| $scF_V$ | 27 403 | 2.74 | | | |
| scHLX | 30 447[a] | 3.20 | 1.70[b] | 1.49[c] | 1.17[d] |
| scHLXc | 31 198 | 3.99 | 1.73 | 1.51 | 1.45 |
| scZIP | 32 300 | 3.51 | 1.73 | 1.53 | 1.28 |
| scZIPc | 32 632 | 3.62 | 1.78 | 1.54 | 1.32 |

[a] The MW of the monomer is shown in all cases. [b] $s_1/s_2 = (MW_1/MW_2)^{0.66}$ is a relation found for globular particles of different MW (see text). The dimer MW (twice the value in the table) is compared to the MW of $scF_V$. [c] $s_1/s_2 = (MW_1/MW_2)^{0.5}$ + const is a relation found for rods of different MW (see text). The dimer MW (twice the value in the table) is compared to the MW of $scF_V$. [d] Ratio of the experimental $s$ value to the $s$ value of $scF_V$.

dimeric species. The measured sedimentation coefficients are composites of the molecular weight and shape and, in the case of noncovalent dimers, the dissociation constants. However, the expected molecular shape of these dimers is anything but spherical, but will probably largely resemble a dumbbell with a flexible joining region. For DNA (a more or less flexible rod), the sedimentation coefficient was found empirically to be approximately equal to $s = a + b \times MW^{0.5}$ ($a$ and $b$ being empirical constants, MW being the molecular weight), whereas for globular proteins, $s$ is roughly proportional to $MW^{0.66}$ (Freifelder, 1982). In view of these uncertainties, we can only deduce that the sedimentation coefficients are consistent with the molecules being dimeric. A comparison of the $s$ values of the two bundle-helix constructs with and without covalent linking (scHLX and scHLXc) suggests a rapid equilibrium of the noncovalent dimer with the monomeric form. In contrast, the $s$ values of the noncovalent leucine zipper (scZIP) and the covalent leucine zipper (scZIPc) are much more similar to each other, suggesting that the equilibrium of the noncovalent zipper is more on the side of the dimer than that of the noncovalent bundle helices. This is consistent with previous measurements of the free peptides (Ho & DeGrado, 1987; O'Shea et al., 1989). In summary, the sedimentation data are consistent with all species largely being present as dimers and the noncovalent variants being in a fast equilibrium with the monomeric species and only a small fraction being tetramers or higher order aggregates.

## Discussion

In this study, we have shown that dimeric $scF_V$ fragments could be produced that assemble in vivo. The strategies chosen, namely, using amphiphilic helices as dimerization domains, were compatible with expression, transport, in vivo folding,

and antigen binding of the $scF_V$ fragment. Furthermore, the dimerization seems to occur spontaneously, including the disulfide-bond formation in those fragments, which contain properly placed cysteine residues.

With this molecular design, one can now take advantage of one of the conserved features of naturally occurring antibodies with far-reaching potential: their bivalence. There are two immediate applications of this design. First, one may now study antibodies with low thermodynamic affinities. These are not, as is occasionally surmised, "poor" antibodies. Instead, many of these are directed against polymeric structures, such as polysaccharides. The well-studied anti-phosphocholine antibodies (Perlmutter et al., 1984) are directed against surface polysaccharides carrying phosphocholine moieties (Potter, 1971). Bivalent binding to PC–protein conjugates and PC–polysaccharides must clearly be inferred from the observation that binding of the whole antibody is still observed under such experimental conditions, where monomeric fragments do not bind. Thus, nature does seem to take advantage of this gain in entropy. The thermodynamic binding affinity of mono- and divalent antibody molecules has been shown to be identical when measured with soluble hapten, as is to be expected (Metzger et al., 1971; Skerra et al., 1990). Especially with the scHLX construct, we can now almost quantitatively mimic the entropic effect of bivalency with small dimeric $F_V$ fragments that assemble by themselves in *E. coli*. This boost of avidity can be employed to quantify the functional antibody fragments with very low affinity, down to amounts of $2 \times 10^{-13}$ mol of protein per well in a simple colorimetric assay, directly out of periplasmic extracts.

However, this gain in avidity is of course not limited to low starting affinities. Rather, the gain is expected to be proportional to the thermodynamic affinity of the monovalent fragment (Crothers & Metzger, 1972). Provided there are no steric constraints to bivalent binding, an already high starting affinity of an antibody fragment may thus be converted to an even higher functional avidity. This effect may turn out to be vital for applications in tumor diagnostics and therapy. This strategy will be possible whenever the surface geometry of the antigen allows it and whenever the natural antibodies show this effect. Now, however, this effect can be achieved with a very much smaller molecule than an antibody or even an $(F_{ab}')_2$ fragment. The molecular weight of the miniantibodies is hardly larger than that of a monovalent $F_{ab}$ fragment.

The second direction opened by these new strategies is a very facile access to bispecific antibodies in *E. coli*. Further engineering of the dimerization handles (P. Pack, F. Müller, and A. Plückthun, manuscript in preparation) will allow the selective combination of only bispecific fragments in vivo, at the expense of the homodimers. Although bispecific antibodies have attracted much attention, for example, in the field of tumor research [summarized in Sedlacek et al. (1988) and Jung and Müller-Eberhard (1988)], there is currently no method to exclusively generate heterobifunctional antibodies. Two important rationales for bispecific antibodies are (i) a greater selectivity for tumor homing by incorporating the reactivity to two tumor markers into the same antibody and (ii) the recruitment of T-cells by combining an anti-T-cell reactivity with a reactivity directed against a tumor marker. However, large antibodies are unsatisfactory in their penetrating powers and their time window of selectivity, whereas $F_V$ fragments show great promise in this area (Colcher et al., 1990). Therefore, the current approaches discussed here show how biotechnological production in *E. coli*, bivalence, and small size can be combined. Regardless of unsolved questions concerning immunogenicity of the newly designed proteins, our miniantibodies may serve as a first model for medically relevant applications. Monomers may pass independently through tissues at low concentrations, find themselves at the target cells, and create highly effective dimers because of the increased local concentration of the dimerization domains at the target site.

A detailed comparison of the bundle approach with the leucine zipper approach is hampered by the fact that, except for that of a shortened peptide, the structure of the four-helix bundle, from which this sequence was taken, is not known (Eisenberg et al., 1986). It should especially be noted that structures other than four-helix bundles have been considered (Hill et al., 1990) for both the one-helix peptides and the two-helix peptides. The overall association constant of the one-helix peptides to a tetramer in solution ($4.2 \times 10^{13}$ $M^3$) is only modest (Ho & DeGrado, 1987), and the dimer formation constant is not known. We favor a model for explaining the present data, in which a transient association of sufficient lifetime is the prerequisite for the formation of the disulfide bond at the end of the helix. The stability of the helical secondary structure may then be increased by the high local concentration forced upon the helix dimer by the covalent link. We have currently no direct evidence for an antiparallel association of the bundle helices, as anticipated in the design by Eisenberg et al. (1986), although the charge complementarity should strongly favor this arrangement. Clearly, the models in Figure 1A,B are only working hypotheses. In our design, the hydrophilic anchor of the scHLXc construct should be long enough to connect the ends of *either* antiparallel or parallel helices with a disulfide bond. This linker may sterically prevent tetramer formation in scHLX, as there is no indication of faster moving species in the scHLXc protein in the ultracentrifugation studies.

The leucine zipper, on the other hand, has probably a considerably higher association constant than the helix bundle and exists as a parallel dimer at micromolar concentrations (O'Shea et al., 1989, 1991). Interestingly, the performance of the constructs containing the leucine zippers in recognizing a surface-bound antigen in an ELISA format is actually somewhat poorer than that of the covalent bundle-helix construct. The fraction of incorrectly folded scZIPc, which migrates faster than the covalent dimer on nonreducing SDS–PAGE, might be partially responsible for the observed decrease of binding in the functional ELISA in comparison with the other constructs. While we have purified all molecules by affinity chromatography, we have no direct evidence that *both* domains are correctly folded in every individual molecule, and some of the differences seen in Figure 3A might be due to this effect. Another possible explanation would be that the tight association in the leucine zipper restrains the molecule too much to accommodate to the surface-bound antigen. The more weakly associating bundle-helix dimer, once covalently bound, might actually reversibly open and close the helix association and thus span a much wider distance in space. In addition, if the bundle helix is really dimerizing in an antiparallel orientation, a wider gap than in the parallel scZIP is already bridged by the helix itself (Figure 1).

The most effective miniantibody construct presented here is the fragment with the bundle helix followed by a hydrophilic anchor with a cysteine (scHLXc). This construct shows almost the same sensitivity in the functional ELISA as the whole antibody McPC603, a bivalent mouse IgA. Small remaining differences in these measurements may be due to uncertainties

of the exact protein concentration (Gill & von Hippel, 1989) and possibly some difference in signal intensity at equal molarity in the ELISA, since the whole antibody has a larger surface to be recognized by the polyclonal anti-McPC603 serum. Therefore, the miniantibody scHLXc probably approaches the same avidity as its natural prototype, the whole antibody.

In conclusion, we have demonstrated the feasibility of minimizing the size of a bivalent immunoglobulin, where both arms are able to interact flexibly and simultaneously with antigens. While there are numerous ways to chemically link $F_{ab}$ fragments to $(F_{ab}')_2$ and, perhaps analogously, $scF_V$ fragments, our fragments assemble by themselves to the functional bivalent structure in the periplasm of *E. coli*. The noncovalent association of two single-chain $F_V$ fragments with the means of hinge regions and amphiphilic helices alone leads to a considerable gain in binding to polymeric antigens. This effect can be increased further by covalent posttranslational linkage with a disulfide bridge. This modular design offers a very practical access to functional homobivalent or heterobivalent miniantibodies.

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